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CYTOKINE GENE EXPRESSION AND CELLULAR IMMUNE RESPONSE IN DOGS WITH LEISHMANIOSIS BEFORE AND UNDER THE TWO FIRST-LINE TREATMENT PROTOCOLS: NEW INSIGHTS INTO THE ANIMAL DISEASE

MARCOS ANDRÉ FERREIRA SANTOS

Orientadoras: Professora Doutora Isabel Maria Soares Pereira da Fonseca de Sampaio

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Ciências Biológicas e Biomédicas

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2020

É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE/TRABALHO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

To science and the search of knowledge,

and to you my brave Ana

ACKNOWLEDGMENTS

While a PhD is generally a “solitary” endeavor and mine was definitely not the exception to the rule, several people played a crucial role during this work and to whom I would like to thank.

First of all, to Professor Isabel Pereira da Fonseca, my supervisor in the past few years since my arrival at the faculty, thank you so much for all the support and help in the most difficult times and all the trust in my capabilities, even when I lacked confidence in myself to achieve the goals. I was truly fortunate to have a supervisor that understood all the struggles, my mindset and my way of thinking and helped to overcome all hurdles because “when the going gets tough, the tough get going”.

To Professor Gabriela Santos-Gomes, my co-supervisor from the IHMT, for all her knowledge and scientific experience, which were essential for understanding this field of immunology with all its nuances and for bringing out the most relevant results of the work.

To Professor Graça Alexandre-Pires for her eternal sympathy and friendship, as well as for all the support for the collaboration protocol in the part of work on Flow Cytometry.

To all the members of the Parasitology and Parasitic Disease Laboratory, past and present, the good times and friendships that were created will stay in my heart forever. And to Dr. Lídia Gomes a special thanks, for the analysis of the IFIs and smears that are part of this study and for all her work.

To all the veterinarians and staff of the Teaching Hospital, Pathology, Clinical Research and Microbiology and Immunology sectors of the Faculty of Veterinary Medicine, University of Lisbon (FMV-ULisboa), that helped in the diagnosis of dogs with canine leishmaniosis, collection of samples and laboratorial analysis.

To the team from the Unit of Imaging and Cytometry at Instituto Gulbenkian de Ciência (IGC), in particular MSc Cláudia Andrade, MSc Cláudia Bispo and PhD Rui Gardner for their support in the design of the flow cytometry experiments and fluorochrome panel. And also, to drivers Rui and Fernando for their company during the travels to IGC and back.

To MSc Telmo Pina Nunes for all the statistical counseling and support.

To all the dogs that participated in the study and their respective tutors who volunteered and trusted in our work, their help was essential, since without them this study would have been impossible to do.

To all those who directly or indirectly contributed to the realization of this work.

To the Portuguese Foundation for Science and Technology (FCT), for funding my PhD scholarship SFRH/BD/73386/2010.

To FMV and CIISA for accepting me and allowing me to use the facilities to do my work.

To my close friends António, Mariana, João Pedro and Teresa for their companionship and pleasant evenings in each other's company.

To my family, grandmother Maria, uncles Sérgio and Anabela, my cousins André and Miguel, Miguel's son Martinho and wife Gabriela, and to their newborn daughter Maria, for their support and for being there when others are not.

To my wife's family, Ana Maria, Inês, Maria Antónia and Pedro, for accepting me as another member, I am truly honored to be part of this family.

And finally, but not least, I want to thank Ana Valente, my best friend in the whole world, the girl I fell in love with and the woman I dedicate my life to. She is the reason I am able to present this work; her irredeemable support and admiration is what drives me forward. The sense of duty that I have for science is only supplanted by the love I have for my wife, and it is for this reason that this thesis is dedicated to Ana, and to you I say, "I would rather share one lifetime with you than face all the ages of this world alone".

FINANCIAL SUPPORT

The present work was funded by:

- PhD fellowship SFRH/BD/101467/2014, from the Portuguese Foundation for Science and Technology (FCT);

- Project PTDC/CVT/118566/2010 - “Regulação da resposta imunológica em cães em várias fases clínicas de leishmaniose e submetidos a diferentes protocolos terapêuticos” (Regulation of the immune response in dogs in various clinical stages of leishmaniosis and submitted to different therapeutic protocols), from the Portuguese Foundation for Science and Technology (FCT);

- Project UIDP/CVT/00276/2020, from the Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), Faculdade de Medicina Veterinária, Universidade de Lisboa;

- Projects UID/Multi/04413/2013 - Contract for the acquisition of training services under the GHTM - Global Health and Tropical Medicine (GHTM), and PTDC/CVT-CVT/28908/2017 - “EXOTRYPANO - Achieving new frontiers through trypanosomatid exosomes (TEx)” at Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, and funded by the Portuguese Foundation for Science and Technology (FCT).

Expressão génica de citocinas e resposta imune celular em cães com leishmaniose antes e sob os dois protocolos de tratamento de primeira linha: novas informações sobre a doença animal

RESUMO

A leishmaniose canina (LCan) causada por *Leishmania infantum* é uma doença visceral zoonótica de interesse mundial. Os fármacos utilizados para o tratamento melhoram o estado clínico do animal, embora, muitas das vezes, os parasitas não sejam totalmente eliminados. O presente trabalho teve como objetivo avaliar a resposta imunitária do cão com leishmaniose antes e durante o tratamento com fármacos de primeira linha, através da análise do perfil de citocinas e subconjuntos de células T $CD4^+$ e $CD8^+$ no sangue periférico, linfonodo e medula óssea. Dois grupos de seis cães diagnosticados com LCan foram tratados com antimoniato de meglumina ou miltefosina em associação com alopurinol. Em simultâneo, outro grupo de dez cães clinicamente saudáveis foi usado como grupo controlo. Aquando do diagnóstico e durante os três meses consecutivos de tratamento, foram registados os sinais clínicos, parâmetros hematológicos e bioquímicos, resultados de urianálise e títulos de anticorpos anti-*Leishmania* obtidos por IFAT. Células mononucleares do sangue periférico, linfonodo e medula óssea foram recolhidas para avaliação da expressão génica de IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α, TGF-β e IFN-γ por qPCR. Em paralelo, estas células foram também analisadas imunofenotipicamente por citometria de fluxo, com anticorpos monoclonais de superfície anti-CD45, CD3, CD4, CD8, CD25 e anticorpo mononuclear intracelular anti-factor nuclear FoxP3. Ambos os protocolos de tratamento promoveram a remissão dos sinais clínicos, a normalização dos parâmetros hematológicos, bioquímicos e dos valores de urianálise. Cães doentes mostraram um aumento generalizado da expressão génica de IFN-γ e diminuição de IL-2, IL-4 e TGF-β. A expressão de IL-12, TNF-α, IL-5 e IL-10 apresentou variações entre os grupos de cães e o tecido analisado. A LCan levou também a um aumento generalizado da percentagem de células T $CD8^+$ em todos os tecidos. No sangue verificou-se ainda diminuição de células T $CD4^+$ e aumento de células T $CD4^+CD25^+FoxP3^+$ e $CD8^+CD25^+FoxP3^+$, com estas últimas aumentando também na medula. As células $CD4^+CD25^-FoxP3^-$ mostraram diminuição acentuada no sangue e medula óssea. Durante o tratamento, foi observada uma tendência para a normalização da expressão génica de citocinas e subconjuntos celulares. No entanto, níveis elevados da expressão génica de IFN-γ foram observados em todos os tecidos. Por sua vez, os tratamentos causaram um aumento da percentagem de células T $CD4^+CD25^+FoxP3^+$ e diminuição de células T $CD8^+CD25^-FoxP3^-$, levando à normalização os valores de células T $CD4^+$ e $CD8^+$ em todos os tecidos. Adicionalmente, o efeito do tratamento na expressão génica de citocinas, que não se encontravam alteradas aquando da infeção, é indicador de que estas terapêuticas combinadas afetam diretamente a produção de citocinas. Ambas as terapêuticas combinadas são eficazes na remissão dos sinais clínicos e parecem influenciar a resposta imunitária do cão, sustentando um ambiente imunológico pró-inflamatório e promovendo a normalização de subconjuntos de linfócitos T.

Estes resultados indicam que *L. infantum* poderá ser capaz de manipular elementos do sistema imunológico do cão para impedir a diferenciação de uma resposta protetora eficaz, evitando o rápido desenvolvimento de patologia grave, enquanto assegura a sobrevivência do parasita, garantindo a possibilidade de vários ciclos de transmissão. Aliado a estes resultados, estudos realizados em colaboração pelo grupo de trabalho sobre o papel dos neutrófilos, hepatócitos e células de Kupffer na LCan, assim como a avaliação do tratamento na leishmaniose felina, permitiram agregar mais conhecimentos na área da leishmaniose animal.

Palavras-chave: Leishmaniose animal; Leishmaniose canina; Citocinas; Linfócitos; fármacos anti-*Leishmania*.

Cytokine gene expression and cellular immune response in dogs with leishmaniosis before and under the two first-line treatment protocols: new insights into the animal disease

ABSTRACT

Canine leishmaniosis (CanL) caused by *Leishmania infantum* is a zoonotic visceral disease of worldwide concern. The drugs used for its treatment improve the animal's clinical condition, although, in most cases, the parasites are not completely destroyed. The current study aimed to evaluate the immune response of the dog with leishmaniosis before and during treatment with first-line drugs, by analyzing the profile of cytokines and subsets of $CD4^+$ and $CD8^+$ T-cells in peripheral blood, lymph node and bone marrow. Two groups of six dogs diagnosed with CanL were treated with either miltefosine or meglumine antimoniate in combination with allopurinol. Simultaneously, another group of ten clinically healthy dogs was used as a control group. Upon diagnosis and during the following three months of treatment, clinical signs, hematological and biochemical parameters, urinalysis results and anti-*Leishmania* antibody titers using IFAT were recorded. Furthermore, peripheral blood, popliteal lymph node and bone marrow mononuclear cells were collected to evaluate the gene expression of IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α, TGF-β and IFN-γ by qPCR. In parallel, these cells were also immunophenotypically analyzed be flow cytometry, using surface monoclonal antibodies anti-CD45, CD3, CD4, CD8, CD25 and intracellular monoclonal antibody anti-nuclear factor FoxP3. Both treatment protocols promoted the remission of clinical signs, normalization of hematological and biochemical parameters and urinalysis values. Sick dogs showed a generalized increase in IFN-γ gene expression and a decrease of IL-2, IL-4, and TGF-β. The expression of IL-12, TNF-α, IL-5, and IL-10 showed variations between groups of dogs and the tissue analyzed. CanL also resulted in an overall increase in the percentage of $CD8^+$ T-cells in all tissues. In the peripheral blood there was also a decrease in $CD4^+$ T-cells and an increase of $CD4^+CD25^+FoxP3^+$ and $CD8^+CD25^+FoxP3^+$ T-cells, with the latter also increasing on the bone marrow. $CD4^+CD25^-FoxP3^-$ T-cells showed a marked decrease in blood and bone marrow. During treatment, a trend towards normalization of cytokine gene expression and T-cell subsets was observed. However, high levels of IFN-γ gene expression were still observed in all tissues. In turn, the treatments caused an increase in the percentage of $CD4^+CD25^+FoxP3^+$ and a decrease in $CD8^+CD25^-FoxP3^-$ T-cells, leading to normalization of $CD4^+$ and $CD8^+$ T cells in all tissues. Furthermore, the effect of treatment on gene expression of cytokines that were not significantly altered by infection indicates that these combined treatment protocols directly affect cytokine production. Both combined treatments are effective in remitting clinical sings and appear to influence the dog's immune response, sustaining a pro-inflammatory immune environment while promoting the normalization of T-cell subsets.
These findings indicate that *L. infantum* may be able to manipulate elements of the dog's immune system to avoid differentiating an efficient protective response, preventing the rapid development of severe pathology while ensuring the parasite's survival and securing the possibility of several transmission cycles. Allied to these results, other studies carried out in collaboration with the working group on the role of neutrophils, hepatocytes and Kupffer cells in CanL, as well as the evaluation of treatment in feline leishmaniosis, have allowed to enhance the knowledge in the area of animal leishmaniosis.

Keywords: Animal leishmaniosis; Canine leishmaniosis; Cytokines; Lymphocytes; anti-*Leishmania* treatment.

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List of abbreviations

ABCD	Advisory Board on Cat Diseases
AHCC	Active hexose correlated compound
ALT	Alanine aminotransferase
Ap	Allopurinol
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BCG	Bacillus Calmette-Guérin
BCR	B cell antigen receptor
BHI	Brain-heart infusion agar medium
BID	*bis in die* – twice a day
bp	Base pair
BUN	Blood urea nitrogen
CanL	Canine leishmaniosis
CBC	Complete Blood Count
CD	Cluster of differentiation
cDNA	Complementary DNA
CG	Control Group
CIISA	Centro de Investigação Interdisciplinar em Sanidade Animal (Centre for Interdisciplinary Research in Animal Health)
CLWG	Canine Leishmaniasis Working Group
CPDA-1	Citrate phosphate dextrose adenine
CL	Cutaneous Leishmaniosis
CVBD	Canine vector-borne disease
D.	*Dirofilaria*
DAT	Direct agglutination test
DCL	Diffuse Cutaneous Leishmaniosis
DGAV	Direção-Geral de Alimentação e Veterinária
DGS	Direcção-Geral de Saúde
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate

DP	Double-positive
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia* - for example
ELISA	Enzyme linked immunosorbent assay
EMTM	Evans' modified Tobie's medium
ESCCAP	European Scientific Counsel Companion Animal Parasites
et al.	*et alii* - and others
FBS	Fetal bovine serum
FCT	Fundação para a Ciência e Tecnologia (Foundation for Science and Technology)
Fig.	Figure
FML	Fucose-mannose ligand
FMO	Fluorescence minus one
FMV-UL	Faculdade de Medicina Veterinária, Universidade de Lisboa (Faculty of Veterinary Medicine, University of Lisbon)
FoxP3	Forkhead box Protein 3
Frw	Forward primer
g	Gram
g	Relative centrifugal force
GPI	Glycosyl phosphatidyl inositol
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
ICT	Immunochromatographic-based dipstick tests
i.e.	*id est* - that is
IL	Interleukin
IFAT	Indirect Fluorescent Antibody Test
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IHMT	Instituto de Higiene e Medicina Tropical (Institute of Hygiene and Tropical Medicine)
iNOS	Inducible nitric oxide synthase
ITS1	Internal transcribed spacer 1
KC	Kupffer cell

L.	*Leishmania*
LCL	Localized cutaneous leishmaniosis
LiESP	*L. infantum* excreted/secreted proteins
LPG	Lipophosphoglycan
Lu.	*Lutzomyia*
MØ	Macrophage
MALDI-TOF MS	Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCL	Mucocutaneous Leishmaniosis
MCV	Mean corpuscular volume
Megl+Al	Meglumine antimoniate and Allopurinol
MG+A	Meglumine antimoniate and Allopurinol
MHC	Major histocompatibility complex
Milt+Al	Miltefosine and Allopurinol
MLEE	Multi-locus enzyme electrophoresis
MLMT	Multi-locus microsatellite typing
MLST	Multi-locus sequence typing
MPV	Mean platelet volume
mRNA	Messenger RNA
MT+A	Miltefosine and Allopurinol
mtDNA	Mitochondrial DNA
NaCl	Sodium Chloride
NE	Neutrophil Elastase
NETs	Neutrophil extracellular traps
NKT	Natural Killer T cell
NNN	Novy, McNeil and Nicolle growth medium
NO	Nitric oxide
NW	New World
OIE	Office International des Epizooties (World Organization for Animal Health)

OW	Old World
p	*p* value
P.	*Phlebotomus*
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-buffered saline
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polimerase Chain Reaction
PDW	Platelet distribution width
PKDL	Post-Kala-azar Dermal Leishmaniosis
P-MAPA	Protein aggregate magnesium-ammonium phospholinoleate-palmitoleate anhydride
PMN	Polymorphonuclear Neutrophils
PNLVERAZ	Plano Nacional de Luta e Vigilância Epidemiológica da Raiva Animal e outras Zoonoses
PRRs	Pattern-recognition receptors
PSG	Promastigote secretory gel
QA-21	*Quillaja saponaria* saponin fraction
qPCR	Real-time PCR
RDW	Red cell distribution width
Rev	Reverse primer
RFLP	Restriction Fragment Length Polymorphism
rK39	Recombinant protein K39
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
S.	*Sergentomyia*
SAG	Sodium antimony gluconate
SID	*semel in die* – once a day
SPLA	Soluble promastigote *Leishmania* antigens
syn.	Synonym

TCR	T cell antigen receptor
TGF-β	Transforming growth factor beta
Th1	Type-1 T-Helper
Th2	Type-2 T-Helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
Tp	Timepoint
Treg	Regulatory T-cells
UK	United Kingdom
UPC	Urine Protein Creatinine ratio
USA	United States of America
VL	Visceral Leishmaniosis
vs	*versus*
v/v	Volume *vs* volume
WHO	World Health Organization
ZVL	Zoonotic Visceral Leishmaniosis

List of publications and presentations

Scientific Articles:

Santos, M.F., Alexandre-Pires, G., Pereira, M.A., Gomes, L., Rodrigues, A.V., Basso, A., Reisinho, A., Meireles, J., Santos-Gomes, G., Fonseca, I.P. 2020. Immunophenotyping of peripheral blood, lymph node and bone marrow T lymphocytes during canine leishmaniosis and the impact of antileishmanial chemotherapy. Frontiers in Veterinary Science. IF - 2.029; Q1.

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Santos, M. "Meglumine antimoniate and miltefosine treatment protocols tend to restore cytokine normal levels in canine leishmaniosis". Course Unit "Seminários de Investigação" by the Interdisciplinary Animal Health Research Center (CIISA), FMV-ULisboa, January 4th 2018.

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CHAPTER I:
INTRODUCTION

1. THE PROTOZOAN PARASITE *LEISHMANIA*

Leishmania parasites are obligatory intracellular protozoa that are dependent on the phlebotomine sand-fly for their transmission to mammalian hosts (Novo et al. 2016), whether human or other animal hosts, such as the domestic dog, to complete the parasite's life cycle. The inoculation of parasites by the sand-fly can result in a state of host infection, which, depending on the parasite species, its virulence and the host's immune response, can further develop into leishmaniosis (Goto and Lindoso 2004), a disease with specific clinical signs.

1.1. *Leishmania* genus

The genus *Leishmania* belongs to the order Trypanosomatida (syn. Kinetoplastida) where parasites of the genus *Trypanosoma* are also found, and comprises about 53 species of five subgenera: *Leishmania*, *Viannia*, *Sauroleishmania*, *Mundinia* (former *L. enrietti* complex) and *Paraleishmania* (Akhoundi et al. 2016; Espinosa et al. 2016). Of these, 31 are mammalian parasites and 20 are considered zoonotic, which include *Leishmania infantum* (syn. *L. chagasi* in the American continent) (Maroli et al. 2013), the etiologic agent of canine leishmaniosis (Gramiccia and Gradoni 2005) (Table. 1).

Leishmaniosis was first reported in 1903 by Lieutenant General Sir William Boog Leishman (1865-1926). This Scottish pathologist, during his stay in the service of the British Army in India, discovered ovoid bodies, similar to those of trypanosomatids, in smears collected *postmortem* from a soldier's spleen in the city of Dum Dum, near Calcutta (Leishman 1903; Steverding 2017). Several weeks later, Charles Donovan (1863-1951), an Irish physician, reported similar ovoid bodies, this time in spleen samples taken during life and at autopsy of Indian natives (Donovan 1903; Steverding 2017). But it was through the hands of Ronald Ross (1857-1932), a British physician, commissioned by the Indian government to investigate the kala-azar disease, that in an article published in November of 1903, he proposed these ovoid bodies as a new protozoan organism, associating them to the clinical symptoms found, thus considering this the causative agent of kala-azar (Ross 1903a; Altamirano-Enciso et al. 2003; Steverding 2017). Ronald Ross then, in a follow-up article, suggested that this novel protozoan should belong to a new genus and proposed the name *Leishmania donovani* for the species in honor of the two previous authors (Ross 1903b; Allison 1993; Steverding 2017). Five years later, the French bacteriologist Charles Jules Henry Nicolle (1866-1936) succeeded in isolating *Leishmania* parasites from a Tunisian child and suggested the specific name *L. infantum* (from "infant") for the etiological agent of Mediterranean Visceral Leishmaniosis, a different species from *L. donovani*, the causative agent of Indian kala-azar (Nicolle 1908; Akhoundi et al. 2016; Steverding 2017).

Table 1. Main zoonotic species of *Leishmania*, geographical distribution, clinical disease and epidemiological role of domestic dogs

Subgenus	Species	Old/New World	Clinical Disease	Domestic dog	Distribution
Leishmania	*L. aethiopica*	OW	LCL, DCL	-	East Africa (Ethiopia, Kenya)
	L. amazonensis	NW	LCL, DCL, MCL	Host	South America (Bolivia, Brazil, Venezuela)
	L. donovani	OW	VL, PKDL	Host	Central Africa, South Asia, Middle East, India, China
	L. infantum	OW, NW	LCL, VL	Reservoir	North Africa, Mediterranean countries, Southeast Europe, Middle East, Central Asia, North, Central and South America (Bolivia, Brazil, Mexico, Venezuela)
	L. major	OW	LCL	Host	North and Central Africa, Middle East, Central Asia
	L. mexicana	NW	LCL, DCL	Host	USA, Ecuador, Peru, Venezuela
	L. tropica	OW	LCL, VL	Host	North and Central Africa, Middle East, Central Asia, India
	L. venezuelensis	NW	LCL	-	Northern South America, Venezuela
	L. waltoni	NW	DCL	-	Dominican Republic
Viannia	*L. braziliensis*	NW	LCL, MCL	Host	Western Amazon Basin, South America (Bolivia, Brazil, Guatemala, Peru, Venezuela)
	L. guyanensis	NW	LCL, MCL	Host	Northern South America (Bolivia, Brazil, French Guiana, Suriname)
	L. lainsoni	NW	LCL	-	Bolivia, Brazil, Peru
	L. lindenbergi	NW	LCL	-	Brazil
	L. naiffi	NW	LCL	-	Brazil, French Guiana
	L. panamensis	NW	LCL, MCL	Host	Central and South America (Brazil, Columbia, Panama, Venezuela)
	L. peruviana	NW	LCL, MCL	Host	Peru, Bolivia
	L. shawi	NW	LCL	-	Brazil
Mundinia	*L. martiniquensis*	OW, NW	LCL, VL	-	Martinique, Thailand
Paraleishmania	*L. colombiensis*	NW	LCL, VL	Host	Colombia
	L. siamensis	OW, NW	LCL, VL	-	Central Europe, Thailand, USA

DCL - diffuse cutaneous leishmaniosis; LCL - localized cutaneous leishmaniosis; MCL - mucocutaneous leishmaniosis; NW - New World; OW - Old World; PKDL - post-kala-azar dermal leishmaniosis; VL - visceral leishmaniosis. Table adapted from information on Shaw et al. (2015), Akhoundi et al. (2016) and Steverding (2017).

In the same year, Nicolle and Comte (1908) isolate *L. infantum* from dogs, becoming the first reference of the domestic dog (*Canis lupus familiaris*) as a reservoir of *Leishmania* parasites (Akhoundi et al. 2016; Steverding 2017). But it was in 1922 that the Brazilian physician Henrique de Beaurepaire Rohan Aragão (1879-1956) reproduced in a dog the clinical signs of leishmaniosis by injecting it with infected sand flies, highlighting their role as vectors of *Leishmania* (Aragão 1927; Akhoundi et al. 2016; Steverding 2017).

Leishmania spp., like many protozoan parasites, has a digenetic life cycle, involving both a vertebrate host and an insect vector, in this case a sand-fly (Sunter and Gull 2017). These parasites have two distinct morphological forms, the promastigote, an extracellular form that differentiates in the sand-fly gut, and the amastigote, the intracellular form present in the mammalian host (Fig. 1). While *Leishmania* species present asexual reproduction, several cases of sexual reproduction giving rise to hybrids have been reported, such as between *L. braziliensis* and *L. peruviana* (Dujardin et al. 1995; Kato et al. 2016), *L. braziliensis* and *L. guyanensis* (Delgado et al. 1997; Bañuls et al. 1999), *L. braziliensis* and *L. panamensis* (Belli

et al. 1994), *L. infantum* and *L. major* (Ravel et al. 2006), *L. donovani* and *L. aethiopica* (Odiwuor et al. 2011), to name a few. These hybrids, in turn, have shown high plasticity, suggesting that they may acquire genetic characteristics that allow for greater growth capacity and less susceptibility to reactive oxygen species (ROS), providing mechanisms to avoid host cells (Cortes et al. 2018).

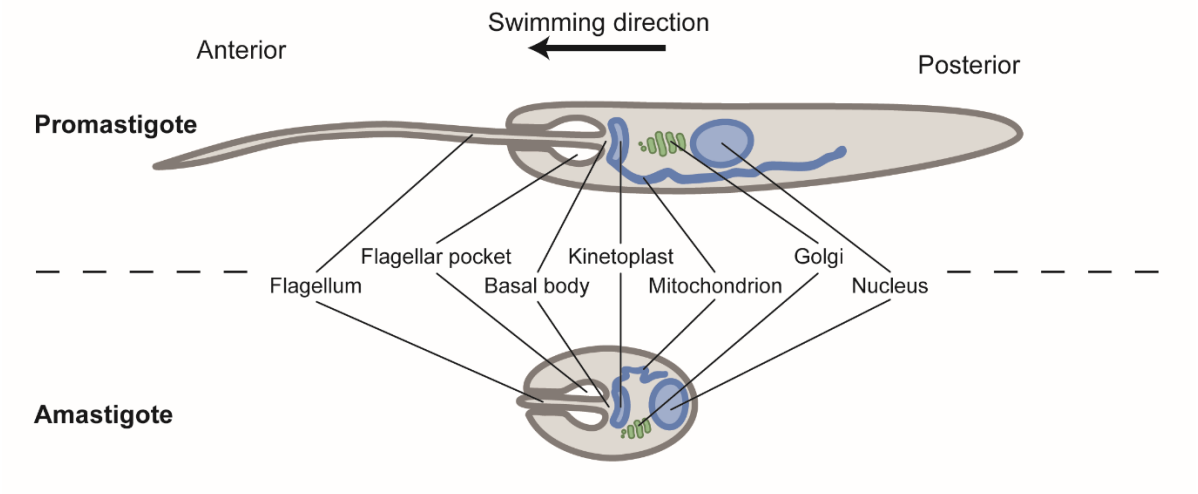

Figure 1. Schematic representation of *Leishmania* morphological forms
Promastigote and amastigote forms aligned along the anterior posterior axis with key structures indicated. Adapted from Sunter and Gull (2017).

In both cell morphologies the nucleus is posteriorly positioned in relation to the other main structures, together with single copy organelles, such as the mitochondria and the Golgi apparatus (Fig.1) (Rudzinska et al. 1964; Sunter and Gull 2017). Anterior to the nucleus is the kinetoplast, a mass of concatenated mitochondrial deoxyribonucleic acid (mtDNA), which is directly connected to the basal body from which the flagellum extends. The promastigote morphology is defined by a fusiform cell body with a long motile flagellum that extends out of the flagellar pocket, which provides propulsive force and appears to be responsible for facilitating the traverse through the sand-fly's digestive tract (Cuvillier et al. 2003; Sunter and Gull 2017). The amastigote form is ovoid and smaller than the promastigote, and is generally regarded as non-flagellated, when in fact it presents a short and immotile flagellum, which barely emerges from the flagellar pocket and is potentially more focused on sensory functions (Gluenz et al. 2010; Sunter and Gull 2017). Despite this, both forms retain the same basic cellular layout, with the kinetoplast anterior to the nucleus and a flagellum extending from the basal body.

1.2. Vectors and vertebrate hosts of *Leishmania* spp.

Leishmania species have a heteroxenous life cycle, requiring at least two types of hosts (Akhoundi et al. 2016; Alemayehu and Alemayehu 2017), a biting insect and a vertebrate. A myriad of vertebrate hosts of parasites of the genus *Leishmania* have been reported, including rock hyraxes, rodents, mongooses, opossums, dogs, cats, foxes, jackals, wolves, bats, armadillos, anteaters, coatis, sloths, porcupines, kinkajous, raccoons, red squirrels, marsupials, primates and humans, among others (Roque and Jansen 2014; Alemayehu and Alemayehu 2017). Of these, the domestic dog is the most important reservoir of *L. infantum*, mainly due to its close relationship with humans (Roque and Jansen 2014). Another carnivorous species closely linked to humans is the cat, which in recent years has gained more relevance to *Leishmania* epidemiology (Pennisi et al. 2013), with multiple reported cases of infection and of clinical manifestations of feline leishmaniosis (Maroli et al. 2007; Martín-Sánchez et al. 2007; Maia et al. 2010; Basso et al. 2016).

1.2.1. Life cycle and routes of transmission

Canine leishmaniosis (CanL) is considered a Canine Vector-Borne Disease (CVBD), being the main route of transmission via the bite of *Leishmania*-infected sand flies (Ready 2013). In addition, the dog is recognized as the main reservoir of *L. infantum* (Lainson et al. 1987). Female phlebotomine sand flies are small hematophagous insects of the order Diptera, family Psychodidae, subfamily Phlebotominae (Maroli et al. 2013; Ready 2013). There are over 800 species of sand flies (Maroli et al. 2013; Akhoundi et al. 2016) and, although the subdivision of this subfamily is still debated by the scientific community, the current classification recognizes six genera (Maroli et al. 2013; Akhoundi et al. 2016):

- Three genera from the Old World comprising the genus *Phlebotomus* with 13 subgenera, genus *Sergentomyia* with 10 subgenera and genus *Chinius* with four species.
- Three genera from the New World comprising the genus *Lutzomyia* with 26 subgenera, genus *Brumptomyia* with 24 species and genus *Warileya* with six species.

Of these, only two genera (*Phlebotomus* and *Lutzomyia*) are of medical importance, being vectors of *Leishmania* (Killick-Kendrick 2002; Dostálová and Volf 2012). In Europe, the predominant vector of *L. infantum* belongs to the genus *Phlebotomus* (mainly *Phlebotomus perniciosus*), and in Latin America the genus *Lutzomyia* (mostly *Lutzomyia longipalpis*) (Maroli et al. 2013). Both male and female sand flies are phytophagous, feeding on sources of sugar such as plant sap or honeydew from aphids (Cameron et al. 1995), but only females feed on

blood, which provides the nutrients required for the production of eggs (Killick-Kendrick 2002). The increased contact between vectors and mammalian hosts, due to frequent blood meals, increases the opportunity of *Leishmania* transmission (Killick-Kendrick 2002) and, since infected sand flies need to probe several times when feeding, the efficiency of transmission increases even further (Killick-Kendrick et al. 1977; Rogers and Bates 2007).

In the phlebotomine vector, *Leishmania*'s life cycle begins after the female sand-fly ingests amastigote-infected macrophages and neutrophils from an infected mammalian host (Fig. 2). The uptake of *Leishmania*-infected macrophages is enhanced by the cutting action of the sand-fly's saw-like mouthparts into the skin, which they agitate to produce a small wound into which the blood flows from superficial capillaries, hence their status as pool feeders (telmophages) (Lane 1993). It is the skin damage that leads to increased recruitment of infected macrophages and other phagocytes to the wound site (Bates 2007). The passage from the mammalian host to the sand-fly's abdominal midgut together with the subsequent decrease in temperature and increase in pH triggers the differentiation of the amastigote, that becomes a procyclic promastigote, which is the first stage within the phlebotomine vector (Bates and Rogers 2005; Dostálová and Volf 2012).

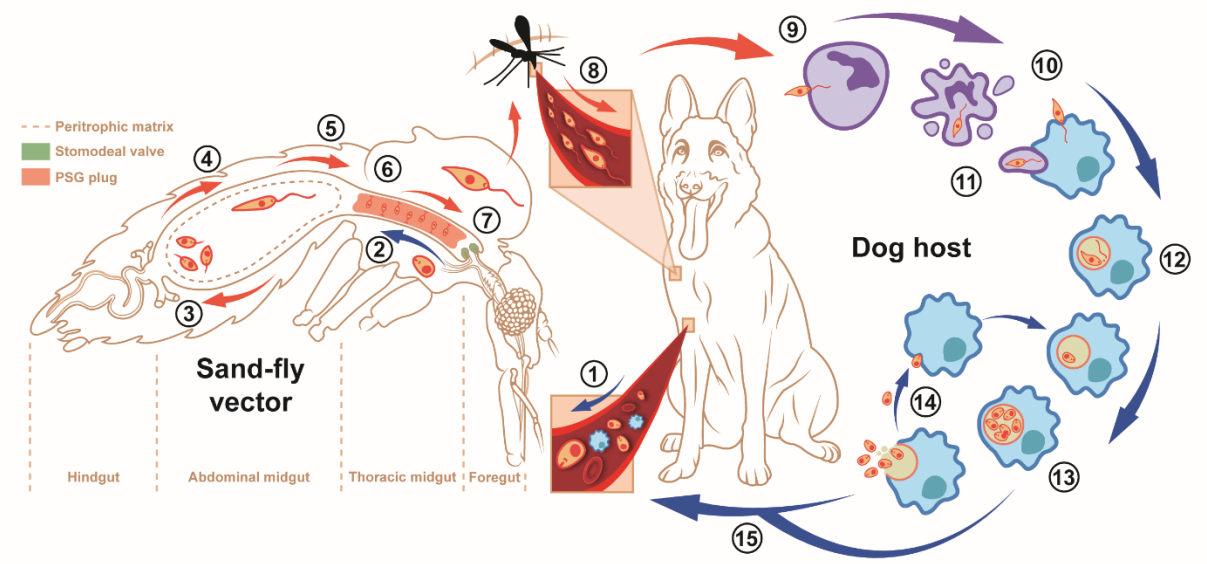

Figure 2. *Leishmania* life cycle in the phlebotomine vector and mammalian host
(1) When the sand-fly takes a blood meal from an infected mammalian host, it ingests macrophages with the amastigote form of *Leishmania*. (2) Amastigotes travel in the blood meal along the foregut and thoracic midgut to the abdominal midgut (3) where they differentiate into proliferative procyclic promastigotes. (4) Two to three days later, these forms differentiate into motile nectomonad promastigotes, which (5) break through the peritrophic matrix and move to the thoracic midgut. (6) Then, the parasite transforms into the replicative leptomonad promastigote, that along with haptomonad promastigotes attached to the midgut epithelium are responsible for the formation of the promastigote secretory gel (PSG) plug. (7) Leptomonad promastigotes differentiate into infective metacyclic promastigotes that accumulate near the stomodeal valve. (8) The female phlebotomine injects promastigotes into the skin of a mammalian host during a blood meal. (9) Neutrophils are rapidly recruited to the bite site and phagocytose metacyclic promastigotes. (10) Free promastigotes and promastigotes that evaded neutrophils are phagocytized by macrophages via a receptor-mediated process. (11) Infected neutrophils can also be internalized by macrophages through an efferocytosis process designated the "Trojan Horse" that transfers the parasite into macrophages. (12) Promastigotes lose the flagellum and differentiate into amastigotes inside the macrophage. (13) Amastigotes replicate inside the macrophage's parasitophorous vacuole until the host cell ruptures and are released into the extracellular space. (14) Released amastigotes can infect other phagocytes. (15) The cycle closes with a female sand-fly taking a blood meal from the infected vertebrate.

The replicative procyclic form slightly increases in size, develops a short flagellum, and presents poor mobility, starting a replicative process within the blood meal (Bates and Rogers 2005; Dostálová and Volf 2012). The blood meal is enclosed by a peritrophic matrix consisting of a chitin and protein mesh secreted by cells of the midgut epithelium (Bates 2007). Still within this matrix, 48-72 hours later, the replication of procyclic promastigotes slow down and parasites differentiate into long, highly mobile nectomonad promastigotes (Rogers et al. 2002; Secundino et al. 2006). These forms accumulate at the anterior end of the peritrophic matrix and trough the activity of a secretory chitinase break out of the blood meal and enter the abdominal midgut lumen (Schlein et al. 1991; Shakarian and Dwyer 2000). Three to seven days after blood feeding, nectomonad promastigotes move freely up to the thoracic midgut and towards the stomodeal valve, with some of the parasites attaching to the microvilli of the thoracic midgut epithelium (Bates and Rogers 2005; Bates 2007; Dostálová and Volf 2012). At this stage, the junction between the foregut and the thoracic midgut dissolves and is established the infection phase, which characterizes a true vector, since parasites were able to persist beyond the blood meal, avoiding expulsion during defecation (Bates 2007). Then, nectomonad differentiate into a third form, a leptomonad promastigote, another proliferative form that is also responsible for the secretion of the promastigote secretory gel (PSG), which plays an important role in transmission (Rogers et al. 2002). Some of the nectomonad/leptomonad promastigotes also attach to the cuticle-lined surface of the valve and differentiate into haptomonad promastigotes (Killick-Kendrick et al. 1974). These attachments are mechanically different from that observed in the midgut epithelium and are a consequence of the expansion of hemi-desmosome-like structures in the flagellar tip (Vickerman and Tetley 1990). Lastly, parasites differentiate into infective metacyclic promastigotes, which are inoculated into the skin of the vertebrate host during the next feeding (Sacks and Perkins 1985). It is at this stage that the above-mentioned PSG plays a crucial role. PSG, which is mainly composed of a filamentous proteophosphoglycan, a glycoprotein with high molecular weight (Ilg et al. 1996), creates a plug that fills the sand-fly's anterior midgut, causing a physical obstruction. This forces the female phlebotomine to regurgitate part of the PSG covered with metacyclic parasites, in order to feed on the blood meal, inoculating them in the fresh wound of the vertebrate host (Bates 2007).

In the vertebrate host, *Leishmania* metacyclic promastigotes meet the host's first line of immune defense, which consists of a neutrophil wave. Neutrophils are massively and rapidly recruited to the site of infection (Müller et al. 2001), were they play a critical role in parasite containment (Mócsai 2013). They have a vast arsenal of weapons that includes parasitic phagocytosis and subsequent degradation, production of ROS that can cause damage to the parasite, emission of neutrophil extracellular traps (NETs) (Brinkmann et al. 2004; Pereira, Alexandre-Pires, et al. 2019), which can inactivate extracellular parasites and release of

cytokines and chemokines (Regli et al. 2017) that can attract and activate other immune cells, exerting control of the initial infection. Monocytes in circulation infiltrate the host's dermis a few hours after intradermal inoculation of *L. infantum* and differentiate into functional macrophages (Santos-Gomes et al. 2000). Promastigotes that evade neutrophils or avoid being destroyed by these cells are internalized by macrophages (Peters et al. 2008) through a classic receptor-mediated process, involving the recognition of *Leishmania* surface molecules by macrophage innate receptors (Sampaio et al. 2007). Efferocytosis of infected neutrophils can also occur, named the "Trojan horse" mechanism, which can prevent the direct contact of the parasite with the macrophage's surface receptors and the activation of macrophage effector mechanisms, enabling an anti-inflammatory phenotype that ensures parasite viability and replication inside the macrophage (van Zandbergen et al. 2004). Promastigotes within the macrophage's parasitophorous vacuole lose their long flagellum and differentiate into the non-flagellated form, the amastigote, which replicates by binary fission until it causes lysis of the macrophage, releasing the produced amastigotes to the extracellular space, where they can be phagocytosed by other macrophages (Martínez-López et al. 2018). The life cycle ends when the sand-fly vector, inserts its saw-like mouthpieces into the skin of an infected vertebrate and agitate them to produce a small wound, into which the blood flows from superficial capillaries, bringing skin infected macrophages and/or free amastigotes into the blood pool, allowing their subsequent uptake into the abdomen of the sand-fly (Alemayehu and Alemayehu 2017).

The phlebotomine vector is of major relevance for the perpetuation of *Leishmania*'s life cycle and, although several authors have found the presence of this parasite in several species of arthropods, there is no clear evidence that vectors such as ticks and fleas are capable of naturally transmitting *Leishmania* to mammals in normal conditions (Coutinho et al. 2005; Coutinho and Linardi 2007). Considering the risk of transmission in the absence of the sand-fly as a negligible event, there have been numerous recorded cases of transplacental infection in dogs and humans (Díaz-Espiñeira and Slappendel 1997; Meinecke et al. 1999; Gaskin et al. 2002; Rosypal et al. 2005; Svobodova et al. 2017; Toepp et al. 2019) as well as cases of venereal transmission (Silva et al. 2009; da Silva et al. 2009), along with rare cases of transfer through bite wounds (Naucke et al. 2016). Blood transfusion in both humans and dogs should also be taken in consideration in endemic areas (Kaplanski et al. 1991; Owens et al. 2001; de Freitas et al. 2006), in particular the sharing of *Leishmania*-contaminated syringes amongst intravenous drug addicts, which can result in direct parasite maintenance in the human population (Cruz, Morales, et al. 2002). Although these non-sand-fly modes of transmission may not play a crucial role in the parasite's life cycle and in leishmaniosis epidemiology (Baneth et al. 2008), there is evidence of the maintenance of *Leishmania* in dogs by vertical transmission, as is the case of the foxhound population in North America that seems to perpetuate *L. infantum* infection via the transplacental route (Boggiatto et al. 2011).

1.3. Leishmaniosis, *L. infantum* infection, epidemiology and geographical distribution

Leishmaniosis is endemic in more than 98 countries, spread across three territories on four continents (Fig. 3), from the Old World (OW) in the Eastern Hemisphere and encompassing Asia, Africa, and southern Europe, and the New World (NW) that covers the Western Hemisphere, extending from south-central Texas to Central and South America (except Chile and Uruguay) (Kevric et al. 2015). This disease is not found in Australia, Antarctica or the Pacific islands. There are more than 12 million *Leishmania*-infected people and 350 million at risk of infection (WHO 2017), with estimates showing an incidence of 0.2-0.4 million cases of human visceral leishmaniosis (VL) and 0.7-1.2 million cases of cutaneous leishmaniosis (CL) each year (Alvar et al. 2012). According to the latest reports, seven countries (Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan) represented over 90% of global cases of VL in 2018 (WHO 2020a). Following conservative assumptions, there are an estimated 20,000 to 40,000 human deaths per year, mainly due to VL (Alvar et al. 2012).

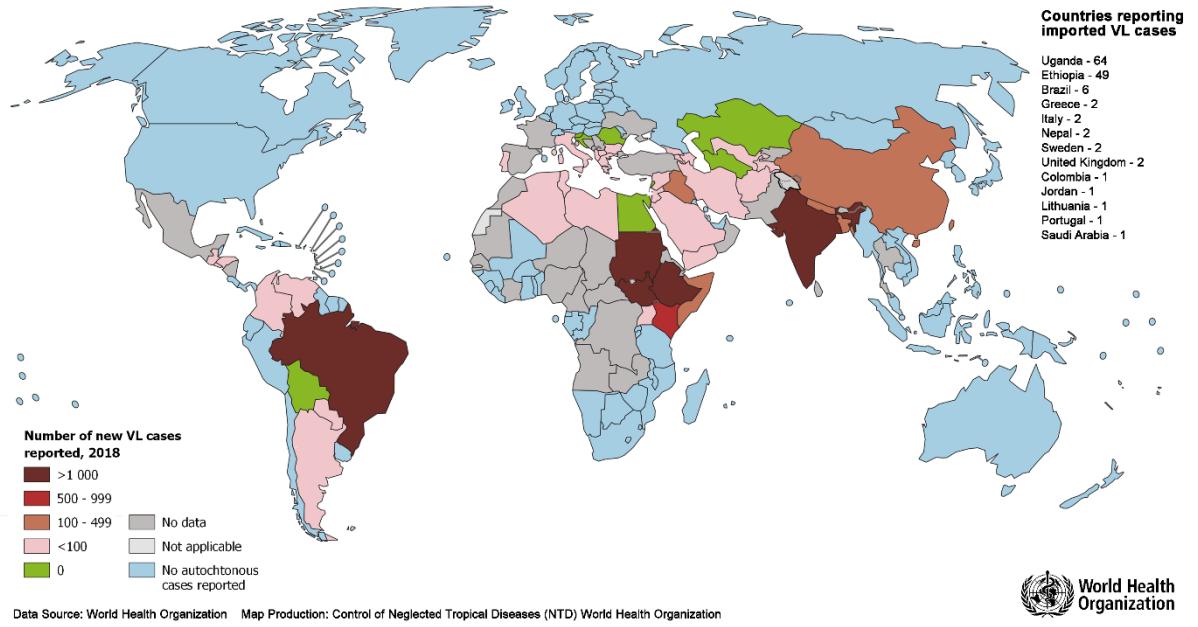

Figure 3. Status of endemicity of visceral leishmaniosis worldwide (2018)
Figure adapted from WHO (2019).

The first known case of human leishmaniosis in Portugal was reported by Dionísio Álvares in 1910, in a 9-year-old child residing in Lisbon (Álvares 1910). In the following year, Álvares and Silva reported results of a survey done in 300 dogs in the Metropolitan Region of Lisbon, eight of which had *Leishmania* parasites (Álvares and Silva 1911). In Portugal, human leishmaniosis has been considered an infant's disease, but with the emergence of HIV in the 1980s, there was a trend towards an increase of infection in adults and a decrease in child cases (Campino and Maia 2010). Since its mandatory declaration status in the 1950s, the

official numbers of human leishmaniosis reported by the Direcção-Geral de Saúde (DGS) have decreased over the years (Fig. 4) (Gaspar et al. 2017), but several studies have shown an underreporting of this disease.

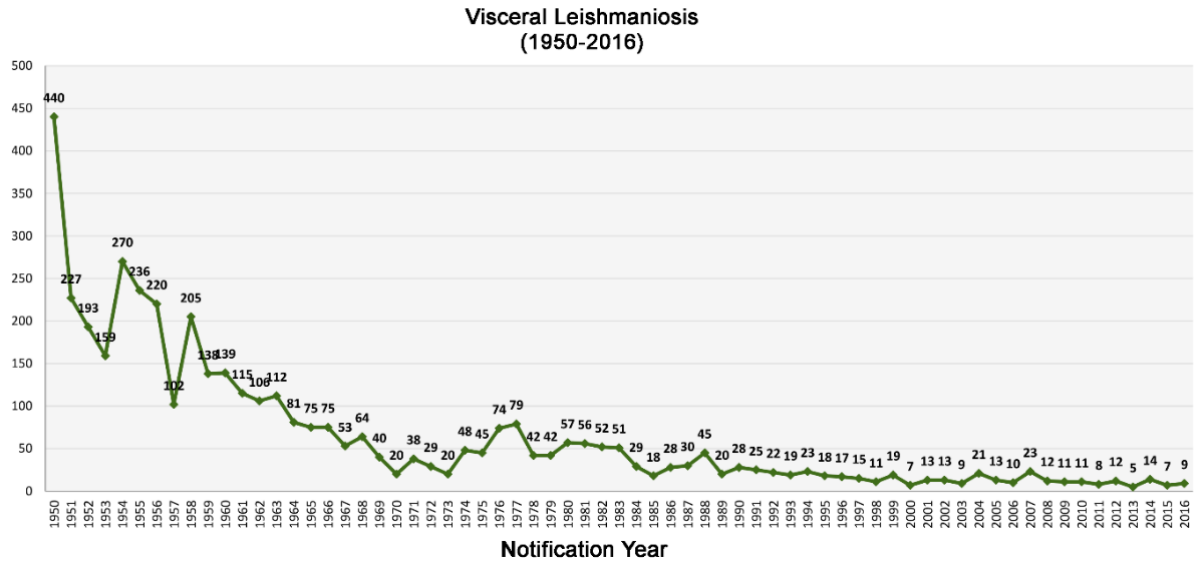

Figure 4. Notified cases of human Visceral Leishmaniosis in Portugal between 1950-2016
Graph obtained from Gaspar et al. (2017)

According to the DGS, a total of 132 cases of human VL were reported in the period of 2000-2009 (Gaspar et al. 2017). While during 1999-2009, of the 375 human cases with visceral leishmaniosis in various hospitals in mainland Portugal, only 38.6% were notified (Martins et al. 2014). This shows that there is a significant underreporting of this disease and according to the latest data from the World Health Organization (WHO) (Fig. 5) (WHO 2020b), in the last 5 years, there were only 5 reported cases of human visceral leishmaniosis in Portugal against the 30 cases reported between 2014-2016 in the latest DGS survey.

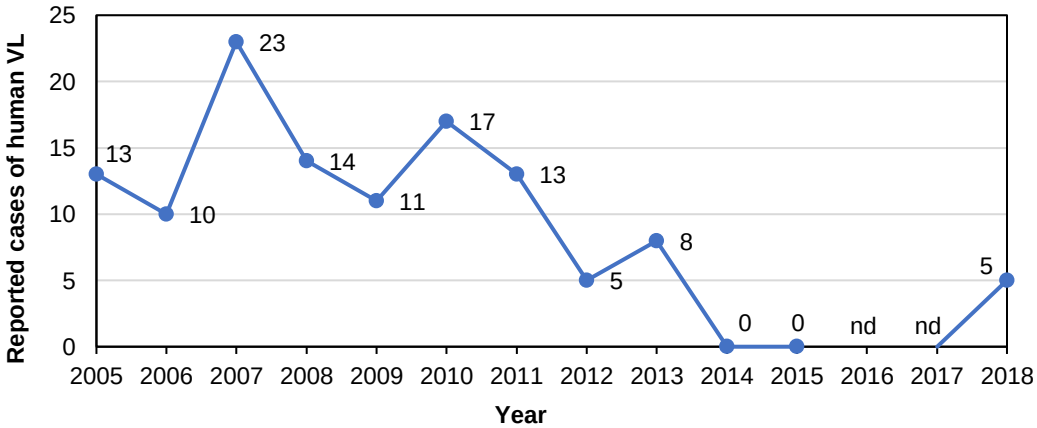

Figure 5. Cases of human Visceral Leishmaniosis reported in Portugal between 2005-2018 by the World Health Organization (WHO)
nd: no data available. Graph generated with data available in WHO (2020b).

CanL is a neglected disease, which has a significant role in Public Health, since the dog is the main reservoir of *L. infantum* (Abbate et al. 2019), and is in close contact with humans, whether in domestic or peridomestic contexts. CanL in Europe is mostly circumscribed to the south, encompassing the Mediterranean basin (Fig. 6) (Gramiccia and Gradoni 2005), including countries like Albania, Croatia, Cyprus, France, Greece, Italy, Malta, Portugal and Spain (Headington et al. 2002; Ferroglio et al. 2005; Živičnjak et al. 2005; Marty et al. 2007; Lazri et al. 2008; Mazeris et al. 2010; Cortes et al. 2012; Miró et al. 2013; Ntais et al. 2013), but in recent years a northward spread of leishmaniosis has been reported (Maroli et al. 2008). Variations in vector distribution as a result of climate change have been the assigned reasons to this spread, concurrently with increased travel and transportation of infected dogs from endemic countries (Teske et al. 2002; Menn et al. 2010). For example, in the United Kingdom (UK) several cases of dogs with clinical signs of CanL have been reported that have been imported from or travelled to CanL endemic countries (Shaw et al. 2009).

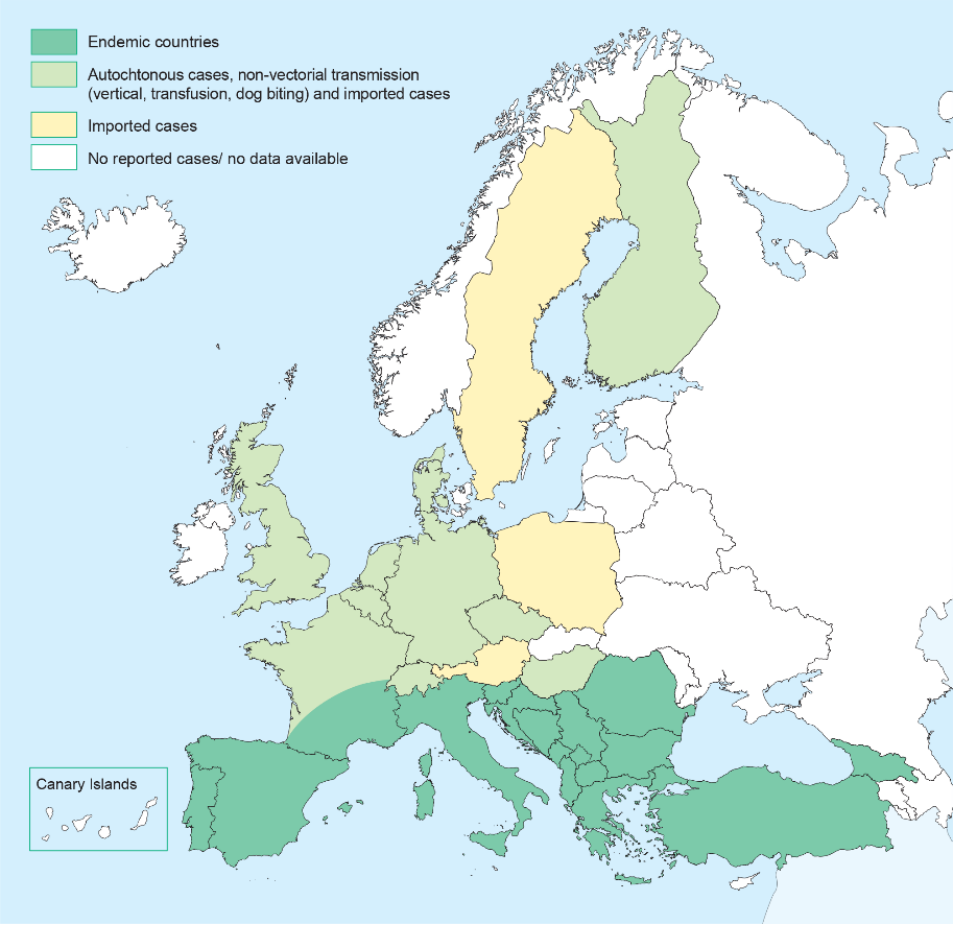

Figure 6. Distribution of *L. infantum* infection in dogs in Europe
Figure adapted from ESCCAP (2019).

It is estimated that in the Mediterranean basin, 2.5 million dogs of 15 million are seropositive for *Leishmania* infection (Moreno and Alvar 2002; Akhoundi et al. 2016). In South

America, the recorded seroprevalence values are even higher (Moreno and Alvar 2002). Despite this, a high infection of dogs does not necessarily imply a higher incidence of the disease in humans (Otranto and Dantas-Torres 2013). In any case, the absence of proper preventive measures in stray dogs can potentially play a role in maintaining *L. infantum* infection in areas where the disease is endemic. In Europe, many countries have municipal kennels, where dogs are kept throughout their lives in close contact with each other and sand flies, and where outbreaks of CanL can easily arise with infection rates of up to 35.3% in a single season (Otranto et al. 2013).

In Portugal, CanL is predominantly caused by *L. infantum* zymodeme MON-1, also widely present in the Mediterranean, with MON-24, MON-29 and MON-80 also present, but in a smaller percentage (Campino et al. 2006; Schönian et al. 2011; Alten et al. 2016). There are few recent epidemiological studies on CanL, none of them in the last few years, therefore, the following are the existing works. In 2002, this zoonosis was included in the group of compulsory notification infections alongside the Portuguese "Plano Nacional de Luta e Vigilância Epidemiológica da Raiva Animal e outras Zoonoses" (PNLVERAZ - National Plan for the Fight and Epidemiological Surveillance of Animal Rabies and other Zoonoses) from the Direção-Geral de Alimentação e Veterinária (DGAV) (Campino and Maia 2010; DGAV 2017). According to this report, that covers the period of 2010-2016 (Fig. 7), it was observed in 2012 a peak of treated dogs after being diagnosed with CanL (42.5% of positive dogs) followed by a progressive decrease. At the end of 2016, only 16.2% of positive dogs had been treated. On the contrary, an increase in the proportion of euthanized dogs can be seen in 2016, with a total of 83.8% of CanL positive dogs being culled.

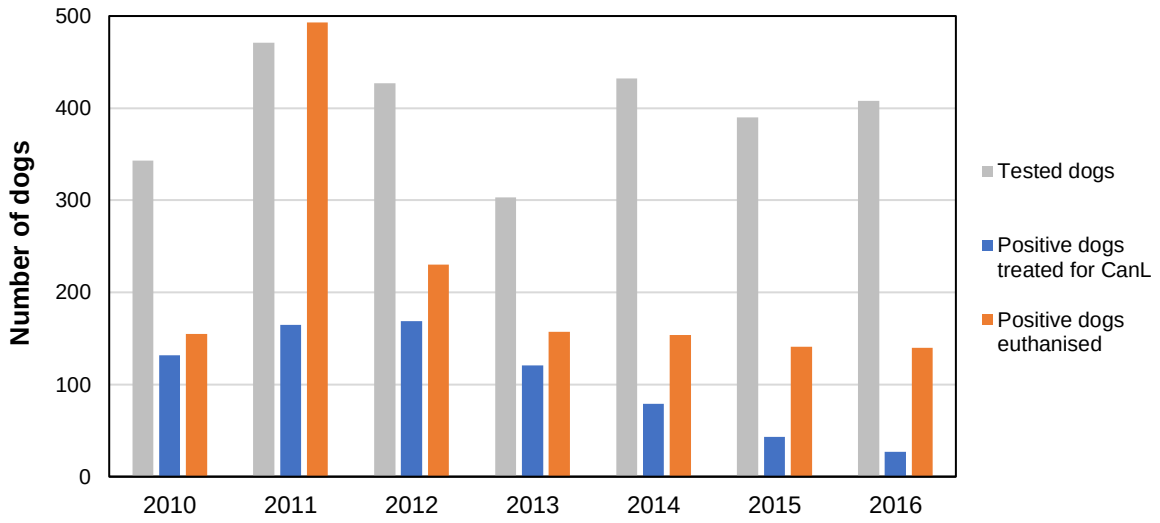

Figure 7. Results of a report on CanL, following the PNLVERAZ, between 2010-2016
Graph created from data obtained in DGAV (2017).

Despite its endemicity, the lack of information about CanL in Portugal led to the creation of the "Observatório Nacional de Leishmanioses" (ONLeish) in 2008. The first and only reported study from this project was carried out in 2009 and found a seroprevalence of 6% from a total of 4000 tested dogs (Maia et al. 2011). In the following year, an epidemiological network was developed in close association with veterinary medical care centers across the country. Between the period of April and August of 2010, of a total of 289 suspected animals, 137 dogs were positive for CanL, with 105 corresponding to new cases of CanL (Maia et al. 2011). The districts with most cases of CanL were Lisbon, Setúbal and Santarém in the center, Faro in the south and Coimbra, Viseu and Vila Real in the north (Fig. 8). These findings are in agreement with the overall view that the most endemic regions in Portugal are the Metropolitan Region of Lisbon, in the center, and the Algarve, in the south (Alten et al. 2016). A survey conducted in 2007 showed an increase in cases of CanL consulted by veterinarians across the country, with 21% having between 20-50 cases of CanL per year (Oliveira et al. 2010). Another study in 2012 found increased seroprevalence in Beja, Castelo Branco and Portalegre, pointing out to possible new endemic areas (Cortes et al. 2012).

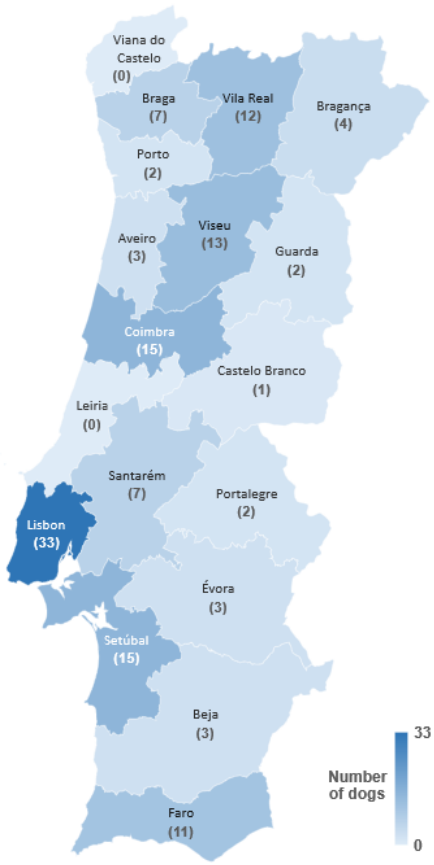

Figure 8. Number of dogs with CanL in a survey across the country from April to August 2010
Original map created from data obtained in Maia et al. (2011).

A study on the prevalence of various CVBDs, including leishmaniosis, carried out in Portugal between October 2010 and April 2011, showed that apparently healthy dogs presented some positivity to *L. infantum* serology in Lisbon (7.9%), in the Alentejo Region (5.9%), in the Algarve Region (3.8%) and the north (3.6%) (Cardoso et al. 2012). CVBD suspected dogs showed positive serology to *L. infantum* significantly higher and well distributed in all regions, with the following values: Lisbon (30.2%), Alentejo Region (27.2%), Algarve Region (25.7%), Centre (25.4%) and the North (18.6%).

In a questionnaire conducted over the period of 2004-2011 to local veterinarians in clinics in France, Portugal, Greece, Spain, Italy and Slovenia, Portuguese veterinarians reported an average of either 1-5 or 20-50 yearly cases of CanL (Bourdeau et al. 2014). The most frequently reported clinical signs in Portugal were renal disease, onychogryphosis, alopecia and skin ulcers. In all countries, the most frequent co-infections with CanL were canine ehrlichiosis (51%), followed by dirofilariosis (12%) and babesiosis (6%). And although most veterinarians recommended the use of insecticides/repellents for dogs living in endemic areas (96%), as well as the use of effective and safe vaccines (95%), among the countries present in the study, Portugal had the greater number of owners who were unaware of the public health implication of CanL (37%) and the lowest number of veterinarians who informed the owners of these implications (40%) (Bourdeau et al. 2014).

In 2013, a small retrospective study was carried out using information from the samples received for the routine screening of *Leishmania* infection, between 1997 and 2012, at the Laboratory of Parasitic Diseases of the Faculty of Veterinary Medicine, University of Lisbon (M.F. Santos et al. 2013). A total of 5207 dog samples were screened during this period, with 94.97% (n=4945) of the samples being tested by indirect immunofluorescence assay (IFAT), 10.56% (n=550) through direct observation of amastigotes in lymph node smears and 4.51% (n=235) in bone marrow smears. Dog samples were considered positive in 26.71% (n=1391) of the cases, and of the 1332 that were tested by IFAT, the antibody titers varied between 1:80 (17.04%, n=227), 1:160 (23.57%, n=314) and ≥1:320 (52.85%, n=704). From the 1391 positive samples, 186 (13.37%) were also revealed to be positive for other infectious diseases, with *Mycoplasma* spp., *Acanthocheilonema reconditum*, *Dirofilaria immitis*, *Babesia* spp. and *Ehrlichia* spp. being some of the most prevalent co-infections.

1.3.1. Vectors of *L. infantum* and their geographic distribution

Leishmania infection is mainly spread by the vector action of female sand flies of the genus *Phlebotomus* in the Old World and of the genus *Lutzomyia* in the New World (Table 2) (Kevric et al. 2015).

Table 2. Phlebotomine species of the genus *Phlebotomus* and *Lutzomyia* proven or suspected vectors of *Leishmania infantum*

Region	Species	Country
Old World	*Phlebotomus alexandri**	China[+], Iran, Iraq, Oman
	P. ariasi*	Algeria, France[+], Italy, Portugal[+], Spain[+], Morocco
	*P. balcanicus**	Armenia, Georgia[+]
	*P. brevis***	Caucasia, Greece, Iran, Malta, Turkey
	*P. chinensis**	China[+]
	*P. galileus***	Syria
	*P. halepensis***	Azerbaijan, Georgia, Syria
	*P. kandelakii**	Armenia, Azerbaijan, Georgia[+], Iran,
	*P. kyreniae***	Cyprus
	*P. langeroni**	Egypt[+], Spain, Tunisia[+]
	*P. longicuspis***	Algeria, Morocco, Tunisia
	*P. longiductus**	Kazakhstan[+], Kyrgyzstan, Ukraine, Uzbekistan
	*P. major s.l.**	Iran[+]
	*P. neglectus**	Albania[+], Cyprus, Croatia, Greece[+], Kosovo, Italy, Republic of Macedonia, Montenegro, Romania, Slovenia, Turkey, Ukraine
	*P. perfiliewi**	Albania, Algeria[+], Croatia, Greece, Israel, Italy[+], Malta, Morocco, Palestine, Republic of Macedonia, Romania, Tunisia, Turkey
	P. perniciosus*	Algeria[+], France[+], Italy[+], Malta[+], Monaco, Morocco, Portugal[+], Spain[+], Tunisia
	*P. sichuanensis**	China[+]
	*P. simici***	Greece, Iran, Turkey
	*P. smirnovi**	China[+], Kazakhstan
	*P. syriacus***	Greece, Israel, Lebanon, Palestine, Syria, Turkey
	*P. tobbi**	Albania[+], Croatia, Cyprus[+], Greece, Israel, Syria, Turkey[+]
	*P. transcaucasicus**	Azerbaijan, Iran[+], Turkey
	*P. turanicus**	Turkmenistan[+]
	*P. wui**	China[+]
New World	*Lutzomyia almerioi**	Brazil[+]
	*Lu. cruzi**	Brazil[+]
	*Lu. evansi**	Colombia[+], Costa Rica, Mexico, Nicaragua, Venezuela[+]
	*Lu. forattinii***	Brazil
	*Lu. longipalpis**	Argentina[+], Bolivia[+], Brazil[+], Colombia[+], Costa Rica, El Salvador, Guatemala, Honduras[+], Mexico, Nicaragua, Paraguay, Venezuela[+]
	*Lu. migonei***	Argentina, Brazil
	*Lu. pseudolongipalpis***	Venezuela
	*Lu. sallesi***	Brazil

*Proven vectors of *L. infantum*; **Suspected to be a *Leishmania* vector based on epidemiological evidence or because it is a proven vector elsewhere; [+]Countries in which the sand-fly species is a proven vector. Table adapted from information on Alten et al. (2016), Akhoundi et al. (2016), Killick-Kendrick (2002), Maroli et al. (2013) and Kasap et al. (2019).

Of a total of more than 800 known species of sand flies, approximately 375 species are found in the Old World and 464 species in the New World (Akhoundi et al. 2016). Of the two genera, *Phlebotomus* and *Lutzomyia*, several dozen species are proven or suspected vectors of *L. infantum* (Table 2), and particularly in the Mediterranean, various *Phlebotomus* species have been implicated in the transmission of CanL, namely: *P. ariasi*, *P. balcanicus*, *P.*

kandelakii, *P. langeroni*, *P. neglectus*, *P. perfiliewi*, *P. perniciosus* and *P. tobbi* (Alten et al. 2016).

In Portugal, Spain and France, *P. perniciosus* (Fig. 9) and *P. ariasi* (Fig. 10) are the main vectors implicated in CanL, with the first being the most widespread vector in Italy, where *P. perfiliewi* together with *P. neglectus* and *P. ariasi* represent regional threats (Maroli et al. 2013; Maia and Cardoso 2015; Alten et al. 2016). Although there are other species of the genus *Phlebotomus* in Portugal, such as *P. papatasi* and *P. sergenti*, these are not proven vectors of *L. infantum* (Maia et al. 2013). Likewise, while *L. major* was detected in *Sergentomyia diminuta* in Portugal, its role as a vector of *L. infantum* in Portugal has not been proven (Campino et al. 2013; Maia and Depaquit 2016).

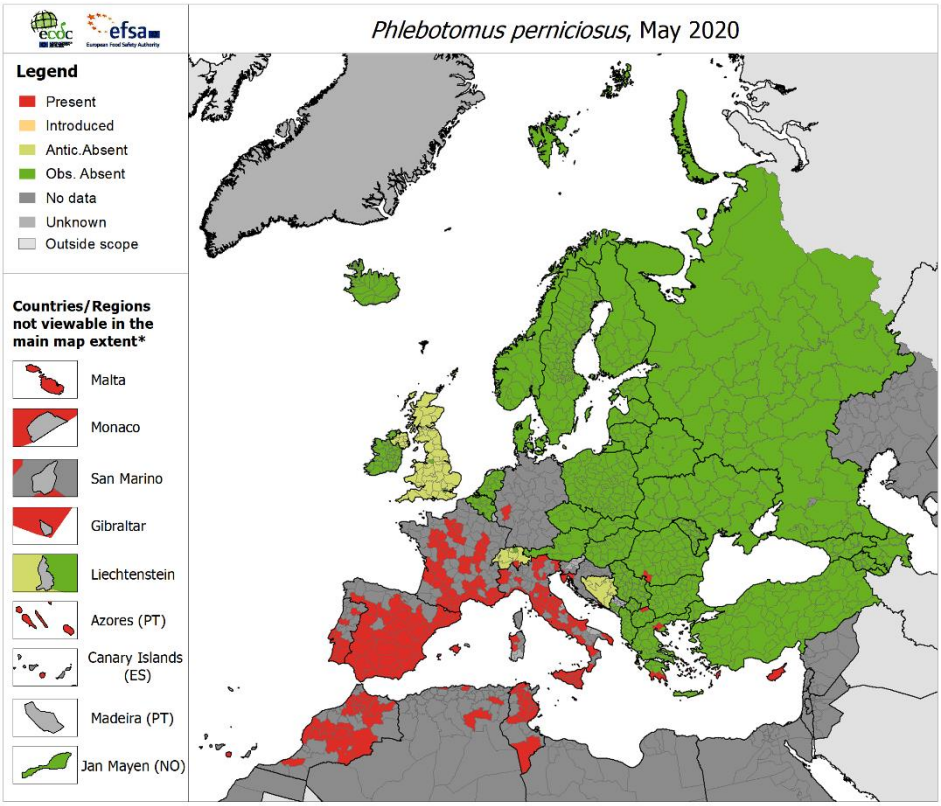

Figure 9. Geographical distribution of *Phlebotomus perniciosus* in Europe
Map adapted from European Centre for Disease Prevention and Control and European Food Safety Authority (2020).

Phlebotomine sand flies in the Mediterranean basin have a seasonal pattern, usually from spring to fall (Tarallo et al. 2010), with suitable temperature ranging between 15 ºC and 28 ºC, associated with high relative humidity and absence of extreme weather conditions, such as rain or strong winds (Maroli et al. 2013). The adults are particularly active in the evening, at night and early in the morning, although they can bite during the day if disturbed (Killick-Kendrick 2002). Female sand flies generally feed on areas on the dog's skin with little hair, such as the head, nasal bridge, ear pinnae, inguinal and perianal areas (Maroli et al. 2013).

Furthermore, under favorable conditions, a human host can be bitten as many as 658 times in one night (Killick-Kendrick and Rioux 2002; Askari et al. 2017).

Female sand flies usually lay between 30-70 eggs during a single gonotrophic cycle (i.e. egg development following a blood meal), which are deposited in cracks and holes in the ground or buildings, animal burrows and between tree roots (European Centre for Disease Prevention and Control 2019). The flight range of phlebotomine sand flies is typically very short (approximately 300 meters) (Maroli et al. 2013), but some species (namely *P. ariasi*) can fly distances of more than one kilometer (Rioux et al. 1979), suggesting that *Leishmania* parasites may have a greater spread.

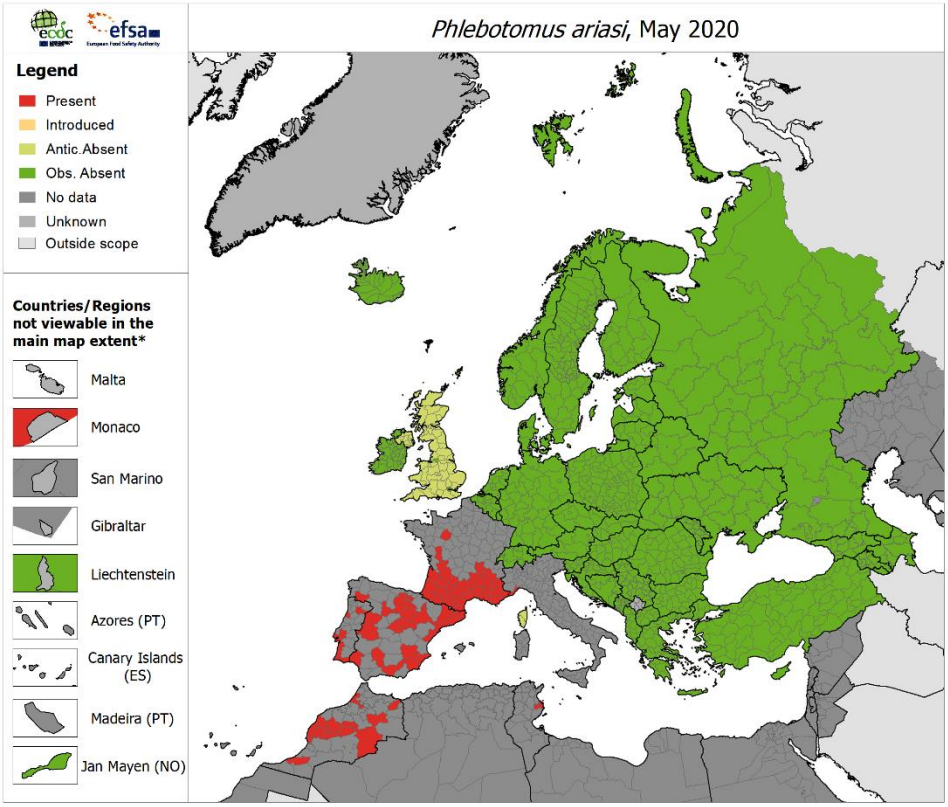

Figure 10. Geographical distribution of *Phlebotomus ariasi* in Europe
Map adapted from European Centre for Disease Prevention and Control and European Food Safety Authority (2020).

One factor that could increase the vector's dispersal capacity is climate change. With the temperature in northern Europe tending to become milder and increasing precipitation, together with winter temperatures rising at higher altitudes, a spread towards the north may become a future reality (European Centre for Disease Prevention and Control 2019). These changes in climate can lead to the expansion of phlebotomine sand flies in Europe, allowing them to survive in areas where they were previously absent. This includes the further expansion to large areas of northwest and central Europe, and to higher altitudes in areas where they are already established (Medlock et al. 2014). If climate change creates suitable temperatures and humidity for phlebotomine sand flies expansion, it is predicted that they

could establish in countries close to their current range, including inland Germany, Austria and Switzerland, as well as along the Atlantic coast (Naucke et al. 2008; Ready 2010; Naucke et al. 2011; Medlock et al. 2014).

1.3.2. *L. infantum* main reservoirs

Canids constitute the main reservoir of *L. infantum* in the Mediterranean basin, in China and in the Americas, with domestic dogs establishing the domestic cycle and stray dogs the peridomestic cycle (Moreno and Alvar 2002). Both sick and clinically healthy but infected dogs present high epidemiological relevance, as they are a source of infection to sand flies, posing a risk to other hosts, including humans (Maia and Cardoso 2015). Although dogs with active CanL are more likely to infect vectors, infected dogs but clinically healthy can also transmit *Leishmania* parasites to phlebotomine sand flies, contributing to the maintenance of the parasite's life cycle (Molina et al. 1994; Bongiorno et al. 2013). The infectivity to sand flies by infected dogs, whether symptomatic or asymptomatic, has yet to be established (Moreno and Alvar 2002). However, xenodiagnostic studies have shown that asymptomatic dogs (50–60% of all seropositive and 80% of all infected dogs) (Abranches et al. 1991; Solano-Gallego et al. 2001) are highly infective to both *P. perniciosus* (54%) (Molina et al. 1994) and *Lu. longipalpis* (Miles et al. 1991). Symptomatic dogs seem to be even more infective to insect vectors (70%) (Moreno and Alvar 2002), with a strong positive correlation between infectivity and serological response (Molina et al. 1994).

The above mentioned vector expansion to northern Europe (Maroli et al. 2008), as a consequence of climate change, can be reinforced by the movement of the main reservoir, the domestic dog, to, and especially, from endemic regions. The current increase in the mobility of dogs across borders, whether travelling with their tutors, through importation or relocation of infected animals from endemic countries to non-endemic regions, has led to increased risk of CanL introduction in *Leishmania*-free countries (Baneth et al. 2008; Otranto et al. 2009). In Europe, particularly in the UK, the Netherlands and Romania, dogs travelling from endemic countries, such as Portugal and Spain were diagnosed with CanL (Slappendel 1988; Teske et al. 2002; Shaw et al. 2003; Pavel et al. 2017). In Germany, in 2005, a serological survey of 291 dogs, either introduced from *Leishmania* endemic Mediterranean regions (particularly Spain) or travelled with their tutors to endemic regions revealed that 38% of the dogs had positive *L. infantum* ELISA titers (Mettler, Grimm, Naucke, et al. 2005). Also in Germany, a Spanish-born female Jack-Russell-Terrier, brought to Germany at the age of 2, inadvertently transmitted *L. infantum* through her bite wounds to another dog living in the same household (Naucke et al. 2016). Thus, CanL has been considered an emerging travel-associated disease in central Europe for some time (Otranto et al. 2009). Sudden outbreaks of CanL in non-

endemic regions can strengthen the above assumption, as is the case with dogs infected with zoonotic *L. infantum* in kennels in the eastern states of the United States of America (USA), where there are no known vectors or autochthonous human cases (Rosypal et al. 2003; Petersen and Barr 2009; Toepp et al. 2017).

But not even humans fail to become a “moving reservoir”, with numerous cases of humans travelling from *L. infantum* endemic regions, as is the recent case of seven immunosuppressed patients diagnosed with VL in Norway, with an history of travel from countries such as Portugal and Spain (Schwartz et al. 2019).

In the Mediterranean basin, several other hosts have been identified, whether in urban, rural or sylvatic areas, such as rodents, lagomorphs, equines, felids and other carnivores (Table 3).

Table 3. Urban, rural and sylvatic host species of *L. infantum* in the Mediterranean

Scientific name	Common name	Country	Reference
Apodemus sylvaticus	European wood mouse	Spain	(Fisa et al. 1999; Portús et al. 2002)
Canis aureus	Jackal	Israel	(Baneth et al. 1998)
Canis lupus	Wolf	Spain	(Sobrino et al. 2008)
Capra aegagrus hircus	Goat	Spain	(Portús et al. 2002)
Equus ferus caballus	Horse	Portugal	(Rolão et al. 2005)
Geneta geneta	Genet	Spain	(Portús et al. 2002; Sobrino et al. 2008)
Herpestes ichneumon	Mongoose	Spain	(Sobrino et al. 2008)
Lepus europaeus	European Hare	Italy, Greece, Spain	(Ruiz-Fons et al. 2013; Ebani et al. 2016; Tsokana et al. 2016)
Lepus granatensis	Iberian Hare	Spain	(Molina et al. 2012)
Lynx pardinus	Iberian Lynx	Spain	(Sobrino et al. 2008)
Martes martes	Pine Marten	Spain	(Millán et al. 2011)
Meles meles	Badger	Spain	(Portús et al. 2002)
Mus musculus	House mouse	Portugal	(Helhazar et al. 2013)
Mus spretus	Algerian mouse	Spain	(Fisa et al. 1999; Portús et al. 2002)
Oryctolagus cuniculus	European rabbit	Italy	(Abbate et al. 2019)
Ovis aries	Sheep	Spain	(Portús et al. 2002)
Rattus norvegicus	Brown Rat	Portugal	(Helhazar et al. 2013)
Rattus rattus	Black Rat	Italy	(Bettini et al. 1978)
Vulpes vulpes	Red Fox	France, Israel, Italy, Portugal, Spain	(Rioux et al. 1968; Abranches et al. 1984; Baneth et al. 1998; Fisa et al. 1999; Abbate et al. 2019)

Although some of these wild animals appear to spread the infection (Miró and López-Vélez 2018), their potential role as reservoir hosts of *L. infantum* is still under debate. In 2009, a leishmaniosis outbreak in the southwest area of Madrid (Spain) that lasted throughout 2012, was attributed to man-made environmental changes and caused 446 cases of human leishmaniosis (Arce et al. 2013). Studies of dog seroprevalence from the area revealed no direct correlation with the outbreak, while the monitoring of potential vectors showed high densities of *P. perniciosus*, which is an endemic species (Arce et al. 2013). Changes in the urban landscape due to the construction of urban parks around the outbreak area caused an overpopulation of hares that moved from woodland to urban habitat, facilitating their multiplication in the absence of natural predators and becoming the perfect source of infection

for the sand-fly population (Arce et al. 2013). Ever since, the status of the hare as a potential sylvatic reservoir has been supported (Molina et al. 2012; García et al. 2014).

Another reservoir closely related to the human and dog is the cat (*Felis catus domesticus*). Historically considered as an unusual host for *Leishmania*, but since its first report in 1912 (Sergent et al. 1912), there have been more and more clinical cases and infections (Ozon et al. 1998; Hervás et al. 1999; Poli et al. 2002; Pennisi et al. 2004; Savani et al. 2004; Rüfenacht et al. 2005; Maia et al. 2008; Maia and Campino 2011; Maia et al. 2015; Basso et al. 2016), with the most recent guidelines recognizing the importance of Feline leishmaniosis (LeishVet Guidelines 2018), not only for the cat population, but also for the possible epidemiological effect on human and dog populations.

1.4. Pathogenesis and clinical manifestations

In CanL, the traditional view that dogs infected with *L. infantum* would eventually develop severe clinical leishmaniosis after an uncertain incubation period has been disregarded (Ferrer et al. 1988), with several cases of spontaneous regression of the infection supporting this assumption (Fisa et al. 1999). This is a disease in which the infection does not correspond directly to clinical illness, showing a high prevalence of subclinical infections (Solano-Gallego et al. 2001; Baneth et al. 2008). CanL caused by *L. infantum*, among the possible visceral, cutaneous and mucocutaneous clinical forms, is often considered a form of Visceral Leishmaniosis, however, dogs eventually manifest clinical signs common to all three clinical forms, with no clear distinction between cutaneous, mucocutaneous or visceral infections (Spickler 2017).

A wide range of clinical signs is present in dogs, with infections ranging from subclinical, manifesting as a self-limiting disease, to even severe and fatal illness (Solano-Gallego et al. 2009). These opposite extremes result, respectively, from the ability or inability of the dog's immune system to counteract the *L. infantum* infection (Solano-Gallego et al. 2011). Although the canine mechanisms responsible for the protection or susceptibility to infection are not completely clear (Alvar et al. 2004), the classical hypothesis is that the self-limiting disease status corresponds to a protective canine immune response (Th1), mediated mainly by $CD4^+$ helper T cells through the release of interferon-gamma (IFN-γ), interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α) that induces macrophage's anti-*Leishmania* activity (Alvar et al. 2004; Solano-Gallego et al. 2009; Alexandre-Pires et al. 2010; Travi and Miró 2018).

The severe clinical form, in turn, is related to a predominant humoral response (Th2) and a reduced or depressed cellular immune response (Baneth et al. 2008; Solano-Gallego et al. 2009). Considering these strict relationships, an imbalance of the dog's immune response towards one of the poles could lead to either clinical cure or disease. However, taking into

account that subclinical animals can, at a certain point, develop disease due to immunosuppression or other concomitant diseases (Baneth et al. 2008; Solano-Gallego et al. 2009), it is difficult to obtain a true parasitological cure, with subclinical and clinically healthy infected dogs still constituting relevant reservoirs of *L. infantum* (Alvar et al. 2004). Even so, achieving clinical cure through appropriate control methods can reduce the parasite load, increase life expectancy, improve the dog's quality of life and enable the reduction of infectivity to sand-fly vectors (Ribeiro et al. 2018).

1.4.1. Canine leishmaniosis (CanL) clinical manifestations

CanL is a systemic disease that can involve any organ, tissue or body fluid, and where dogs can either present an asymptomatic profile (over 80% of cases in some areas) or can progress to a life-threatening disease, with a wide variety of clinical signs (Table 4) (Alvar et al. 2004; Solano-Gallego et al. 2011; Otranto et al. 2013).

After inoculation by the female sand-fly, *Leishmania* parasites are rapidly dispersed to the lymph nodes and spleen by the bloodstream or lymphatic system, and from there to the kidneys and liver (Alvar et al. 2004). Later, they spread to the reproductive organs, skin, bladder, digestive and respiratory systems (Alvar et al. 2004), demonstrating a widespread visceral infection. According to Alvar et al. (2004) and Moreno (2019) some of the early signs of the disease include loss of body weight (Fig. 11A and B) and cutaneous signs such as ulcerative and hyperkeratosis lesions (Fig. 11C and D), periorbital (Fig. 11G) and auricular alopecia, conjunctivitis and anemia.

Patent symptoms add to the previous ones and consist of changes in appetite, polydipsia, lymphadenopathy, hepatomegaly, splenomegaly, exfoliative and ulcerative dermatitis (Fig. 11E and F), skin lesions around the nose (Fig. 11G and H), ocular orbits and ears (Fig. 11G, H, M and P), nodular dermatitis (Fig. 11I, J and M), pustular dermatitis, keratitis (Fig. 11G), peeling (Fig. 11Q) and alopecia (Fig. 11R), epistaxis, muscular atrophy and onychogryphosis (Fig. 11K and L) (Slappendel 1988; Abranches et al. 1991; Alvar et al. 2004; Moreno 2019). In the final chronic stage of CanL, some of the additional symptoms include a widespread of ulcers and alopecia, cachexia, opportunistic infections and renal or hepatic failure (Moreno 2019). Chronic renal failure is one of the most serious results of disease progression and the main cause of mortality in CanL (Alvar et al. 2004; Solano-Gallego et al. 2011).

Table 4. Clinical manifestations representative of CanL caused by *L. infantum*, according to LeishVet Consensus and Canine Leishmaniosis Working Group Guidelines

	LeishVet Consensus Guidelines	Canine Leishmaniosis Working Group Guidelines
General	Generalized lymphadenomegaly	Mild to moderate enlargement of palpable lymph nodes
	Loss of body weight	Poor nutritional state or cachexia
	Lethargy	Lethargy
	Mucous membrane pallor	Pale mucous membranes
	Splenomegaly	Hepatosplenomegaly
	Fever	Fever
	Diarrhea (including chronic colitis)	Gastrointestinal involvement
	Vomiting	
	Polyuria and polydipsia	
	Decreased or increase appetite	
		Muscular hypotrophy
Cutaneous	Non-pruritic exfoliative dermatitis with or without alopecia	Desquamative dermatitis (localized or generalized)
	Erosive-ulcerative dermatitis	Ulcerative dermatitis with varying appearance and distribution (e.g. mucocutaneous junctions, skin covering the extremities and traumatized sites)
	Nodular dermatitis	Nodular dermatitis
	Papular dermatitis	Papular dermatitis
	Pustular dermatitis	Pustular dermatitis
	Onychogryphosis	Onychopathy
		Lupus- or pemphigus-like nasal lesions
		Nasodigital hyperkeratosis
Ocular	Blepharitis (exfoliative, ulcerative or nodular) and conjunctivitis (nodular)	Palpebral lesions
	Keratoconjunctivitis, either common or sicca	Corneal lesions, mainly associated with the conjunctiva (keratoconjunctivitis), nodular keratitis and keratoconjunctivitis sicca, diffuse or nodular conjunctival lesions
	Anterior uveitis	Diffuse or granulomatous lesions of anterior uvea and lesions of posterior uvea (chorioretinitis, hemorrhages and retinal detachments) Possible complications of uveal diseases (glaucoma and panophthalmitis)
	Endophthalmitis	Scleral lesions (diffuse or nodular scleritis and episcleritis) Granulomatous orbital lesions or myositis of extrinsic muscles
Other	Epistaxis	Epistaxis
	Lameness (erosive or non-erosive polyarthritis, osteomyelitis and polymyositis)	Lameness and joint swellings
	Neurological disorders	Neurologic involvement
	Atrophic masticatory myositis	
	Vascular disorders (systemic vasculitis and arterial thromboembolism)	
	Mucocutaneous and mucosal ulcerative or nodular lesions (oral, genital and nasal)	

Adapted from Paltrinieri et al. (2010) and Solano-Gallego et al. (2011).

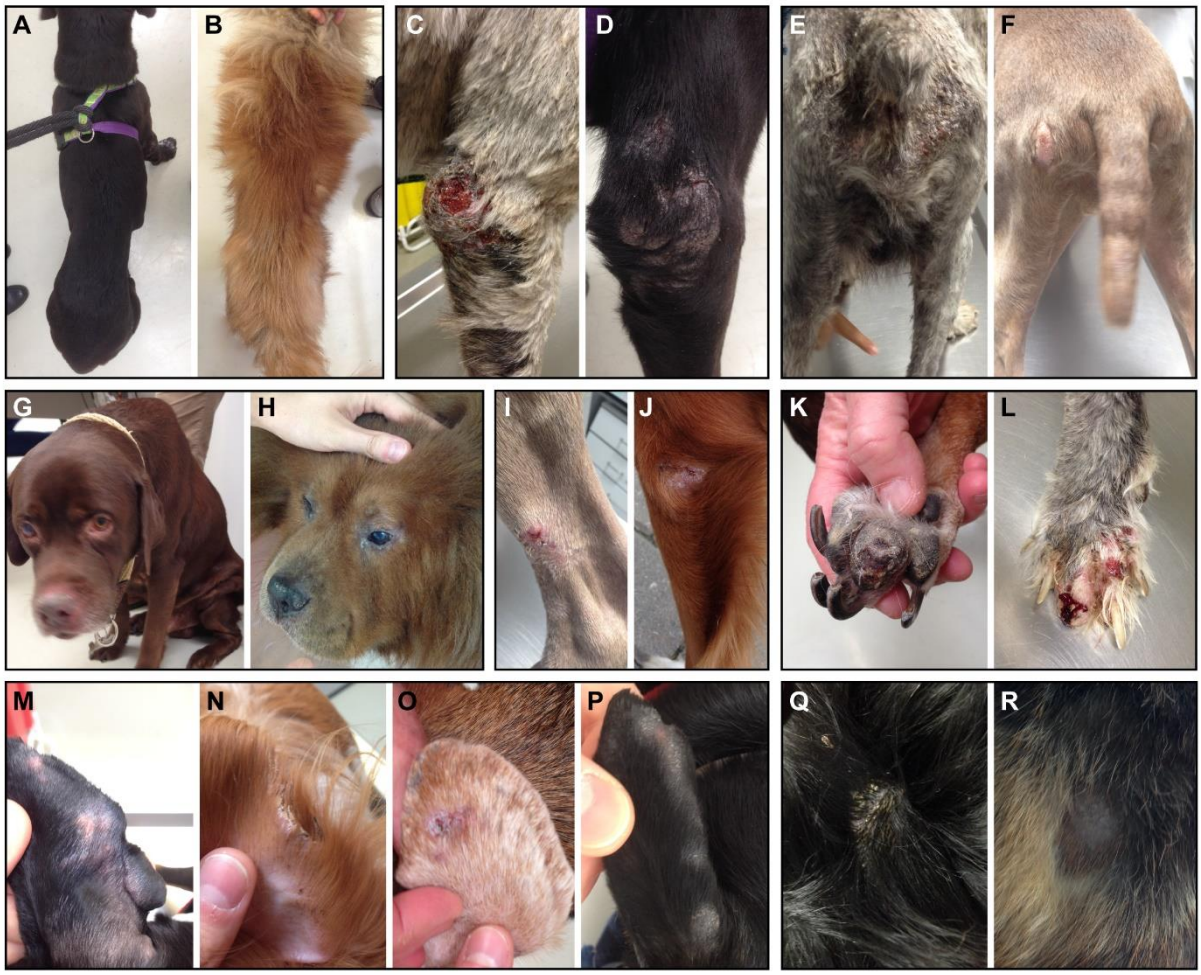

Figure 11. Representative images of CanL external clinical signs
(A, B) Emaciation; (C, D) Ulcerative and hyperkeratosis lesions in the elbow of the front limb; (E) Exfoliative dermatitis; (F) Ulcerative dermatitis; (G) Prostration, keratitis, periocular and nasal dermatitis; (H) Periocular dermatitis and nasal hyperkeratosis; (I, J) Erosive dermatitis; (K) Onychogryphosis and digital hyperkeratosis; (L) Onychogryphosis with hemorrhage; (M) Nodular dermatitis in the ear; (N) Erosive dermatitis in the ear; (O) Ulcerative dermatitis in the ear; (P) Exfoliative dermatitis in the ear; (Q) Squamative dermatitis; (R) Alopecia.

1.4.2. Hematological and biochemical abnormalities in CanL

Along with characteristic physical manifestations, some hematological and biochemical abnormalities can be found (Table 5). Laboratory analysis of parameters related to hematopoiesis, renal function and serum electrophoretic profile are used as complementary tools for clinical diagnosis (Ribeiro et al. 2018). Some of the tests used are: complete blood count (CBC), serum biochemical analysis, serum protein electrophoresis and urinalysis (Slappendel 1988; Koutinas et al. 1999; Paltrinieri et al. 2010).

Table 5. The most common laboratory abnormalities in CanL, caused by *L. infantum*, according to the two most recognized CanL guidelines

	LeishVet Consensus Guidelines	Canine Leishmaniosis Working Group Guidelines
Complete blood count (CBC)	Mild to moderate non-regenerative anemia	Poorly regenerative or nonregenerative anemia
	Leukocytosis or leukopenia: lymphopenia, neutrophilia, neutropenia	Neutrophilic and monocytic leukocytosis with lymphopenia and eosinopenia Leukopenia
	Thrombocytopenia	Possible thrombocytopenia
	Thrombocytopathy	
	Impaired secondary hemostasis and fibrinolysis	
		Possible regenerative anemia (due to immune-mediated processes)
Serum biochemical profile with protein electrophoresis	Hyperproteinemia	Hyperproteinemia
	Hyperglobulinemia (polyclonal beta and/or gammaglobulinemia)	Hyperglobulinemia Increased α_2-globulin concentration and polyclonal or oligoclonal gammopathy
	Hypoalbuminemia	Hypoalbuminemia
	Decreased albumin/globulin ratio	Altered albumin/globulin ratio
	Renal azotemia	Azotemia (high serum concentrations of urea and creatinine)
	Elevated liver enzyme activities	Increased hepatic enzyme activities
Urinalysis	Proteinuria (urine protein:creatinine ratio [UPC])	Proteinuria (determined by dipstick test and UPC ratio)
		Isosthenuria (specific gravity, 1.008 to 1.012) or poorly concentrated urine (<1.030)
Basic coagulation profile		Hyperfibrinogenemia and possible increase in prothrombin time and activated partial thromboplastin time

Adapted from Paltrinieri et al. (2010) and Solano-Gallego et al. (2011)

Anemia is one of the most frequent abnormalities found in dogs with CanL, being it most likely the result of various processes, such as hemorrhage, hemolysis, chronic renal failure, bone marrow hypoplasia or aplasia and decreased lipid fluidity of the erythrocyte membrane (de Luna et al. 2000; Ribeiro et al. 2013; Ribeiro et al. 2018). Following leishmaniosis infection, the generally intense polyclonal proliferation of B cells give origin to a humoral immune response with high production of antileishmanial antibodies, which results in visible changes in the electrophoretic plasma profile, and leads to the occurrence of damage in the kidneys, eyes and skin (Ribeiro et al. 2013). CanL is frequently characterized by an increase in total serum proteins (hyperproteinemia), azotemia, hypergammaglobulinemia (polyclonal B cell response), hypoalbuminemia (associated with renal and/or liver failure) (Paltrinieri et al. 2016), and values of Albumin-Globulin ratio below the lower reference limit. Renal disease in CanL can manifest as mild proteinuria to nephrotic syndrome or chronic renal failure, as a result of glomerulonephritis usually associated with the deposition of immune complexes in the kidneys (Paltrinieri et al. 2016). These parameters are considered good markers for diagnosis and therapeutic monitoring, as it is recognized that kidney damage associated with CanL is almost inevitable (Ribeiro et al. 2013). Hepatic enzymes are generally

within the reference values for dogs, although biochemical findings in infected dogs may include changes in alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (Heidarpour et al. 2012; Paltrinieri et al. 2016).

1.4.3. CanL guidelines

The two most recognized CanL guidelines are from the LeishVet Group (Solano-Gallego et al. 2011) and the Canine Leishmaniosis Working Group (CLWG) (Paltrinieri et al. 2010). Both guidelines propose a different staging of the dog's clinical condition based on several considerations, namely, the physical examination, clinicopathological abnormalities, histopathological findings, molecular diagnostics, anti-*Leishmania* antibody titer and assessment of renal function. These staging tools allow for a more uniform classification of the dog's condition and the determination of the appropriate treatment. The two guidelines therefore present a four-part staging of canine leishmaniosis, which are presented below:

- **LeishVet Group:**
  - **Stage I** (Mild disease) - Dogs with mild clinical signs, such as peripheral lymphadenomegaly or papular dermatitis. Generally, no clinicopathological abnormalities are observed. Negative to low positive antibody titers. Normal renal profile (creatinine <1.4 mg/dl; non-proteinuric: UPC < 0.5);
  - **Stage II** (Moderate disease) - Dogs with signs listed in stage I, along with diffuse or symmetrical cutaneous lesions, such as exfoliative dermatitis/onychogryphosis, ulcerations (nasal plane, footpads, bony prominences, mucocutaneous junctions), anorexia, weight loss, fever and epistaxis. Clinicopathological abnormalities, such as mild non-regenerative anemia, hyperglobulinemia, hypoalbuminemia, serum hyperviscosity syndrome. Low to high positive antibody titers. Normal renal profile to slight proteinuria (creatinine <1.4 mg/dl; UPC = 0.5-1);
  - **Stage III** (Severe disease) - Dogs with signs listed in stages I and II, which may present signs originating from immune-complex lesions: vasculitis, arthritis, uveitis and glomerulonephritis. Clinicopathological abnormalities listed in stage II. Medium to high positive antibody titers. Chronic kidney disease with UPC>1 or creatinine 1.4-2 mg/dl;
  - **Stage IV** (Very severe disease) - Dogs with signs listed in stage III, who may have pulmonary thromboembolism or nephrotic syndrome (UPC > 5) and end-stage renal disease. Clinicopathological abnormalities listed in stage II. Medium to high positive antibody titers. Chronic kidney disease with either creatinine 2-5 mg/dl or creatinine>5 mg/dl.

- **Canine Leishmaniosis Working Group (CLWG):**
  - **Stage A** (Exposed dogs) - Dogs with negative cytological, histological, parasitological, and molecular diagnostic findings, as well as low anti-*Leishmania* antibodies titers. Dogs are clinically normal or show clinical signs associated with other diseases. Generally, dogs exposed to *L. infantum* infection are those that live or have lived during one or more transmission seasons in a geographical region where the presence of *Leishmania* vectors has been confirmed;
  - **Stage B** (Infected dogs) - This category includes dogs in which the presence of parasites has been confirmed through direct methods (e.g. positive results from microscopic analysis, organism culture or PCR assay) and which have low anti-*Leishmania* antibodies titers. These dogs may be healthy or may have clinical or pathological signs associated with other illnesses. In endemic areas, a positive PCR assay of skin or peripheral blood in the absence of lesions and obtained during the infection transmission period may not be sufficient to consider a dog infected;
  - **Stage C** (Sick dogs) - This category includes dogs with positive cytological results, regardless of serological tests, dogs with high anti-*Leishmania* antibodies titers, and rarely, infected dogs. One or more clinical signs common to leishmaniosis are present (Table 4). Given the multifaceted manifestations of the disease, the signs indicative of disease can be different from those listed, as long as they can be clearly associated with the ongoing infection. In the absence of detectable signs on physical examination, such a dog should still be considered sick when it has hematological, biochemical and urinary alterations suggestive of leishmaniosis;
  - **Stage D** (Severely sick dogs) - Sick dogs with a severe clinical condition are included in this category, as indicated by one of the following: evidence of proteinuric nephropathy or chronic renal failure; concurrent problems, such as ocular disease causing functional loss or severe joint disease impairing mobility, related or not to leishmaniosis and requiring immunosuppressive treatment; presence of concomitant conditions, including various co-infections or neoplastic, endocrine, or metabolic diseases; and lack of clinical response to repeated cycles of anti-*Leishmania* drugs.

1.5. Laboratorial diagnosis

Current methods for diagnosing *L. infantum* infection and canine leishmaniosis are mainly restricted to reference hospitals or research centers with well-equipped laboratory settings (Akhoundi et al. 2017). Some of the exceptions are rapid serological tests commercially available for clinical use, but according to Solano-Gallego et al. (2017) these screening tests still do not appear to have ideal diagnostic performance when compared to conventional serological tests. In endemic areas, and after identifying clinical signs compatible with CanL, it is recommended to combine the clinical diagnosis and epidemiological information with several specific techniques, either to isolate and confirm the presence of the parasite or its components in biopsies (direct tests) and/or of the host's immune response to the parasite (indirect tests) (Paltrinieri et al. 2016; Miró and López-Vélez 2018). These methods cover a wide variety of techniques, from morphological identification of parasites by optical microscopy, detection of parasite deoxyribonucleic acid (DNA) by molecular biology assays and evaluation of humoral immune response by serology, and many types of samples, such as peripheral blood, lymph node, bone marrow, conjunctiva fluid, urine, skin and spleen (Solano-Gallego et al. 2017; Taylor 2018; Travi et al. 2018).

1.5.1. Microscopic examination

This technique confirms the presence of the parasite by direct observation of amastigotes on lymph node, bone marrow or conjunctival mucosa aspirates or biopsy aspirates of target tissues, such as the skin, liver, and spleen. Various staining techniques, such as Giemsa staining (Fig. 12), after observation under optical microscopy at ×400/×1000 magnification allow the morphological identification of amastigote forms within macrophages or free on smear due to cell disruption (Paltrinieri et al. 2016; Akhoundi et al. 2017; Taylor 2018). Liver and spleen biopsies have fallen into disuse, as they are highly invasive methods and present a risk of internal bleeding (Reis et al. 2013; Miró and López-Vélez 2018).

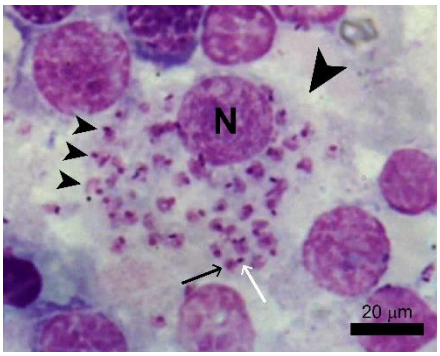

Figure 12. Amastigote forms of *L. infantum* within macrophage
Bone marrow infected macrophages (large arrowheads) containing numerous *Leishmania* amastigotes (small arrowheads) in the cytoplasm. Each amastigote has a nucleus (black arrow) and a rod-shaped kinetoplast (white arrow). Giemsa staining. N – Nucleus of macrophage.

Along with the presence of *Leishmania* amastigotes, the typical cytological patterns observed in CanL tissue biopsies are generally characterized by granulocytic-macrophagic or pyogranulomatous inflammation, associated with a moderate to severe lymphoplasmacytic infiltration in the skin or nodular lesions with atypical localization (Mylonakis et al. 2005; Saridomichelakis et al. 2005). In lymph nodes, reactive hyperplasia of variable degrees is usually observed, characterized by lymphoplasmacytic and macrophagic infiltration, often associated with numerous neutrophils (Mylonakis et al. 2005; Saridomichelakis et al. 2005). This is a fast and cheap approach, but invasive in some types of samples, requiring organ biopsy, which can be challenging to perform (Akhoundi et al. 2017). It is not a reliable quantitative technique, depending on the load and dispersion of the parasite, and the technical skills of the personnel performing the test, which accounts for its low sensitivity (Solano-Gallego et al. 2011; Akhoundi et al. 2017). In addition, it does not allow the distinction between *Leishmania* species (Solano-Gallego et al. 2011; Akhoundi et al. 2017). In the absence of parasite visualization, it requires the performance of other diagnostic tests, such as immunohistochemistry and/or PCR (Solano-Gallego et al. 2011).

Nonetheless, due to its cost-effectiveness and simplicity, microscopic examination is a technique widely used throughout endemic areas. Some of the best samples for this technique are injured skin, bone marrow and lymph node, with blood being less sensitive (Paltrinieri et al. 2010).

1.5.2. *In vitro* culture

The *in vitro* culture of *L. infantum* from aspirates, scrapings or tissue biopsies enables not only to confirm whether suspect dogs harbour parasites, but also whether parasites are viable (Paltrinieri et al. 2016). On the other hand, it is a time-consuming (up to 30 days) and expensive method, which requires a sophisticated laboratory setup in order to be carried out under strictly sterile conditions (Berman 1997; Paltrinieri et al. 2010; Akhoundi et al. 2017; Taylor 2018), being generally restricted to reference centers and with the intention of research, not being recommended for routine practice (Solano-Gallego et al. 2011; Paltrinieri et al. 2016). The isolation of parasites in culture from biopsy samples is no more than 70% effective, even though it is not very difficult to maintain viable and replicative *Leishmania* promastigotes (Akhoundi et al. 2017). However, parasite culture is a necessary technique before performing certain DNA and protein-based methods developed to discriminate *Leishmania* species (Akhoundi et al. 2017). Several select mediums used are blood agar-based, such as the NNN medium (Novy, McNeil and Nicolle), otherwise the brain-heart infusion (BHI) agar medium, EMTM (Evans' modified Tobie's medium) or Schneider medium supplemented with FBS (Fetal bovine serum) can also be used (Taylor 2018).

1.5.3. Polymerase chain reaction (PCR) based assays

Another direct test to confirm the presence of the parasite is PCR, which not only enables the diagnosis, but also the identification of *Leishmania* spp. from different samples, either fresh or frozen, formalin-fixed and paraffin-embedded tissue biopsies (Taylor 2018).

This technique is characterized by the amplification of a specific DNA target, using oligonucleotide sequences (primers) selected from the parasite's small-subunit ribosomal ribonucleic acid (RNA) gene (Mathis and Deplazes 1995), kinetoplast DNA minicircles (de Bruijn and Barker 1992) or other highly repetitive genomic DNA sequences (Bulle et al. 2002). This method is very sensitive, particularly when using multicopy DNA sequence targets (Paltrinieri et al. 2010). There are several available techniques within PCR technology for parasite detection. A nested PCR approach uses an additional set of primers, in addition to the initial ones, with homology to the previously amplified target, increasing the number of copies produced and subsequently enhancing the sensitivity of this technique (Cruz, Cañavate, et al. 2002; Paltrinieri et al. 2010; Taylor 2018). Adding to this, the use of fluorescent-labeled probes, such as Taqman™ probes, and intercalating fluorescent dyes, such as SYBR® Green, enables for an additional increase in sensitivity through quantitative real-time PCR (qPCR), where the equipment used provides real-time feedback on the amplification process and allows the possibility to estimate the number of amplified copies of the target gene and, therefore, the quantification of parameters, such as parasitic load (Paltrinieri et al. 2010; Solano-Gallego et al. 2011). Despite this high sensitivity of the technique, it should be considered that different samples can have different parasite concentrations and even variable chances of containing leishmanial DNA. For example, several samples used routinely in decreasing order of sensitivity are bone marrow or lymph nodes, skin, conjunctiva, buffy coat and the less sensitive are urine and peripheral whole blood (Maia and Campino 2008; Paltrinieri et al. 2010; Solano-Gallego et al. 2011; Lombardo et al. 2012; Solano-Gallego et al. 2017). This molecular methodology can also present high specificity, allowing species discrimination as is the case of PCR-restriction fragment length polymorphism (RFLP) analysis, in which the PCR products obtained are digested by appropriate restriction enzymes resulting in a specific pattern of restriction fragments (Minodier et al. 1997; Marfurt et al. 2003; Volpini et al. 2004; Montalvo et al. 2012). Multi-locus microsatellite typing (MLMT) and multi-locus sequence typing (MLST) are two other methods that allow strain classification by targeting repeated and polymorphic DNA sequences, such as those coding for the ribosomal internal transcribed spacer 1 (ITS1), cysteine protease B, kinetoplast DNA minicircles, surface glycoprotein 63, heat-shock protein 70, mini-exons and microsatellites (Mauricio et al. 2006; Reithinger and Dujardin 2007; Schönian et al. 2008; Kuhls et al. 2011).

Nonetheless, these molecular methodologies can present several cons, as is the case of false positive results that can occur due to DNA contamination, it is an expensive technique that requires specific reagents, specialized equipment and highly trained technicians, it requires the standardization of PCR based techniques between the diverse diagnostic laboratories, and should not be performed as the sole diagnostic test, because a positive result confirms only the presence of *Leishmania* DNA, which indicates a possible infection, but it is not necessarily an indicator of disease (Solano-Gallego et al. 2011).

1.5.4. Serological tests

In CanL diagnosis, some of the most widely used methods are quantitative serological techniques, such as the indirect fluorescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA) (Solano-Gallego et al. 2011). These tests by detecting the presence of antileishmanial antibodies in the serum of suspect dogs are therefore considered indirect techniques. IFAT is recommended by the World Organization for Animal Health (OIE) as the reference serological method for CanL (Taylor 2018). This serologic test presents a sensitivity and specificity close to 100% (Paltrinieri et al. 2016). It is a genus-specific technique, although cross-reactions with other genera, such as *Trypanosoma cruzi* in the New World, have been reported (Taylor 2018). In this test, serial serum dilutions of the suspect dog are overlaid onto promastigote-coated slides and antigen-antibody complexes are detected by adding a secondary antibody conjugated to a fluorochrome. The fluorescence is observed under a fluorescence microscope (Fig. 13A), which allows the estimation of the antibody titer, which is indicative of the relative concentration of antileishmanial antibodies (Paltrinieri et al. 2010). In CanL, IFAT antibody titers between 1:40-1:80 are suggestive of exposure to *Leishmania* parasites, not necessarily of infection. Titers of 1:160 and above are indicative of established infection and disease in clinically suspected dogs (Paltrinieri et al. 2010).

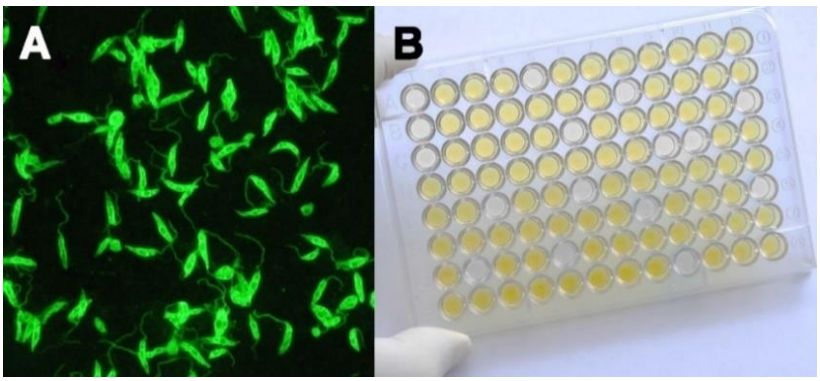

Figure 13. Images representative of a positive serum by IFAT (A) and of an ELISA microplate with positive samples (yellow wells) (B).

In the ELISA, the diluted serum is placed in *Leishmania* antigen–coated microplates. The complex antileishmanial antibody-antigen is subsequently detected by the use of a secondary antibody that has been conjugated to an enzyme. After the addition of the enzyme's appropriate substrate, a colorimetric reaction that can be identified by simple visualization, usually the conversion of a blue solution to a yellow shade, and quantified by spectrophotometry indicates a seropositive result (Fig. 13B), unlike the IFAT that depends on the technician's subjective observation of fluorescence under a microscope (Paltrinieri et al. 2010). ELISA is a specific test with sensitivity ranging from 86% to 99%, which can increase when multiple antigens are used, being an especially good tool for analyzing large quantities of samples and for sero-epidemiological surveys under field conditions (Taylor 2018). A major current problem with these serological techniques is the immune response developed by the administration of vaccines to prevent CanL, as these tests may not distinguish between naturally infected and vaccinated dogs (Solano-Gallego et al. 2017).

Additional serological tests, such as the direct agglutination test (DAT) and the immunochromatographic-based dipstick tests (ICT) are also used, particularly in veterinary clinics, being easier to use and providing quick qualitative results, but their performance is still not ideal (Mohebali et al. 2004; Mettler, Grimm, Capelli, et al. 2005; Ferroglio et al. 2007; Solano-Gallego et al. 2017).

1.5.5. Protein based methods

Additional methods for species identification of *Leishmania* parasites are the isoenzyme identification by multi-locus enzyme electrophoresis (MLEE) and the matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS). MLEE is the reference method for species identification (Rioux et al. 1990), where soluble enzymes extracted from cultured promastigotes are deposited in a matrix (usually made of starch gel, cellulose acetate, acrylamide or agarose) and submitted to electrophoresis generating a specific band pattern (zymodeme) (Taylor 2018). Extracts from reference strains are used to determine the zymodeme of the new strain (Taylor 2018). MALDI-TOF MS is a powerful tool for the identification of *Leishmania* species (Mouri et al. 2014). Proteins of cultured promastigotes are ionized in a specific acidic solution, then several laser beams from a spectrometer evaporate the sample towards the sensor, which measures the "time of flight" that is dependent on the molecular weight of the ionized molecules (Akhoundi et al. 2017). This protein spectral "fingerprint" of a strain can then be compared with the reference spectral database (Mouri et al. 2014). The major disadvantages of these methods are the requirement for specialized and expensive equipment, the need for highly trained technicians and the

requirement for mass cultures of parasites, making them unfeasible for clinical diagnosis, being usually restricted to epidemiological studies (Schönian et al. 2011; Akhoundi et al. 2017).

1.5.6. Xenodiagnosis

Xenodiagnosis is a useful method for the isolation of parasites in their natural arthropod vector and for testing the infectivity of the infected host (Nogueira et al. 2019). It is conducted by exposing possible infected tissues or lesions of a suspected dog to a competent phlebotomine vector and examination after feeding for the presence of *Leishmania* flagellates in the gut of the sandly (Sadlova et al. 2015). Although its high specificity and reasonable sensitivity, it is a time-consuming, non-quantitative and non-viable technique without animal/insectary facilities, being mainly restricted to research and not recommended for routine practice (Akhoundi et al. 2017).

1.6. Treatment

In CanL, the outcome of the infection and subsequent development of the disease depends on host factors, particularly its genetic background and immune status, and also the virulence of the parasite (Saridomichelakis 2009). These are some of the reasons why there is a wide array of clinical presentations, ranging from clinically healthy infected dogs, which do not require immediate treatment (Solano-Gallego et al. 2009; Solano-Gallego et al. 2011; Miró and López-Vélez 2018), to those who manifest a marked or severe illness and that should start therapy as soon as possible for a better response to treatment (Miró et al. 2008; Solano-Gallego et al. 2009). Nevertheless, clinically healthy but infected dogs should be monitored for early detection of possible clinical signs and/or laboratory abnormalities compatible with the disease (Miró and López-Vélez 2018). The use of unnecessary treatments can affect the balance of dog's immunocompetence (Miró and López-Vélez 2018).

The available drugs used to treat CanL can temporarily improve clinical signs or achieve a clinical cure, while lowering parasite burden (Miró et al. 2011), although none of these treatments seem to eliminate the infection (Miró et al. 2008). Table 6 shows some of the recommended compounds, used either in monotherapy or in combination, together with their potential adverse effects. The response to treatment is usually quick, with weight gain, reduction of cutaneous lesions and blood values tending to normality, leading to an overall improvement of the dog's condition (Alvar et al. 2004). If not, there may be a concomitant infection or a drug-resistant *Leishmania* infection (Alvar et al. 2004).

Nonetheless, it is advised that biochemical, clinical and parasitological examinations should be performed after treatment, and that treated dogs be periodically evaluated for disclosure of any relapses (Alvar et al. 2004; LeishVet Guidelines 2018).

Table 6. Recommended therapeutic protocols

Active ingredient	Therapeutic protocol	Potential adverse effects
Meglumine antimoniate*	100mg/kg once a day or 50mg/kg twice a day for 4-6 weeks, subcutaneously	Potential nephrotoxicity, pain and inflammation at injection site
Miltefosine*/**	2mg/kg once a day for 28 days, *per os*	Vomiting and/or diarrhea
Allopurinol	10mg/kg twice a day for at least 6-12 months, *per os*	Xanthine urolithiasis
Domperidone***	0.5mg/kg once a day for 1 month, *per os*	Galactorrhea
Amphotericin B deoxycholate	0.5mg/kg twice per week for 2 months, intravenously	Nephrotoxicity

*Registered for veterinary use in most European countries; both drugs are commonly recommended in combination with allopurinol. **The only approved anti-*Leishmania* drug for veterinary use in Brazil. ***Only considered for Stage I of LeishVet Guidelines. Table adapted from Ribeiro et al. (2018) and LeishVet Guidelines (2018).

1.6.1. Pentavalent antimonial compounds

In 1912, Gaspar Vianna reported the effectiveness of antimony potassium tartrate (emetic tartar), a trivalent antimonial, for the treatment of mucocutaneous leishmaniosis, but due to its severe side effects it was quickly abandoned (Vianna 1912; Frézard et al. 2009). Several decades later, in the 1940s, the less toxic pentavalent antimonial compounds were introduced in the treatment of human and canine leishmaniosis (Adler and Tchernomoretz 1946; João et al. 2006; Frézard et al. 2009).

In Europe, the most frequently used pentavalent antimonial against CanL is *N*-Methyl-D-glucamine, also known as meglumine antimoniate (Glucantime®), either alone or in combination with allopurinol (Solano-Gallego et al. 2011), and while it is considered the first-line treatment, its use in the clinical setting has several limitations (Frézard et al. 2009). These compounds must be given daily for 4 weeks through parenteral administration, which causes local pain and requires tutors to take the dog to the veterinarian every day for proper administration or, alternatively, for the tutor to learn and voluntarily administer the therapy. Typical reported side effects include nausea, vomiting, weakness and myalgia, abdominal colic, diarrhea, skin rashes and hepatotoxicity (Frézard et al. 2009), but are usually reversible (Alvar et al. 2004). Since that *Leishmania* infection can lead to hepatic and renal damage, it is difficult to determine whether changes during treatment are due to the chemotherapy or the parasite, since glomerulonephritis caused by antigen-antibody complex deposition may appear more frequently after treatment with these pentavalent antimonials (Alvar et al. 2004; Bonagura and Twedt 2013). Although these drugs have been used for more than six decades, the

pharmacological and toxicological mechanisms involved in their action are still unclear (Moreira et al. 2017). An early hypothesis proposed that these drugs interfere with the bioenergetic processes of *Leishmania* amastigotes, forming stable complexes with ribonucleosides, which interfere with the parasite's fatty acid-oxidation and glycolysis, promoting the depletion of adenosine triphosphate (ATP), an essential source of energy for the survival of the parasite (Berman 1997; Demicheli et al. 2002). Another hypothesis suggests that pentavalent antimonials act as a prodrug that is transformed into the more toxic trivalent form to exert its antileishmanial activity (Sereno et al. 1998; Frézard et al. 2001; Miekeley et al. 2002; Ferreira et al. 2003; Moreira et al. 2017). Despite these leishmanicidal effects, treated dogs may continue to harbor the parasite and be infectious to sand flies, although to a lesser extent when compared with untreated dogs (Ikeda-Garcia et al. 2007; Manna, Reale, Vitale, et al. 2008; Ribeiro et al. 2008; Miró et al. 2011). Coupled with the emergence of drug-resistance cases, this leads to the need for continued research on new compounds and formulations (Lira et al. 1999; Hefnawy et al. 2017).

Another less used pentavalent antimonial is sodium stibogluconate (Pentostam®), an active ingredient also administered subcutaneously at a dose of 20 mg/kg for 28 days, with suspected mechanisms of action similar to meglumine antimoniate (Stephen 2010; Sykes and Papich 2014; Sundar and Chakravarty 2015). This drug, compared to meglumine antimoniate presents severe side effects, such as nausea, diarrhea, muscle and joint pain, fatigue, serum transaminase elevations, pancreatitis and rarely myocardial, renal and hepatic damage (Stephen 2010; Ryan 2018).

1.6.2. Miltefosine

Miltefosine or hexadecylphosphocholine (Milteforan®) is a synthetic alkyl phospholipid developed in the early 1980s as an anti-neoplastic agent (Unger et al. 1989; Alvar et al. 2004; Haldar et al. 2011; Nogueira et al. 2019). In 1992, the effectiveness of this compound against *L. donovani* and *L. infantum* was demonstrated both *in vitro* and *in vivo* in mice (Kuhlencord et al. 1992). This drug exhibits a broad antimicrobial spectrum and has also demonstrated activity against *Leishmania*, being currently the only recognized oral agent used to treat various clinical forms of leishmaniosis (Haldar et al. 2011; Dorlo et al. 2012; Passero et al. 2018), either alone or in combination with allopurinol (Solano-Gallego et al. 2011). Some side effects include mild gastrointestinal adverse reactions, such as vomiting, nausea, diarrhea or abdominal pain (Passero et al. 2018). The mechanism of action of miltefosine is still unclear and, considering that not all *Leishmania* species are equally susceptible to this drug, it is possible that it has a multifactorial effect (Dorlo et al. 2012; Passero et al. 2018). One of the main proposed modes of action is the inhibition of the biosynthesis of the glycosylphosphatidylinositol (GPI) receptor,

a key molecule for intracellular survival of *Leishmania* (Berman 2008; Nogueira et al. 2019). It also appears to interfere with the synthesis of phospholipase and protein kinase C, as well as the biosynthesis of glycolipids and membrane glycoproteins of the parasite, along with DNA fragmentation, which leads to loss of parasite viability (Berman 2008; Nogueira et al. 2019). Several studies even propose that this drug may have immunomodulatory properties (Eue et al. 1995; Zeisig et al. 1995; Safa et al. 1997; Dorlo et al. 2012; Nogueira et al. 2019). While *in vitro* studies have shown for years that several *Leishmania* strains were resistant to miltefosine, cases in humans and dogs have been emerging in recent years (Proverbio et al. 2014; Srivastava et al. 2017). According to these studies, the resistance mechanisms generated *in vitro* are mainly related to decreased drug uptake, reducing the therapeutic effect (Berman 2008; Dorlo et al. 2012).

It should be mentioned that in 2017, miltefosine became the first authorized treatment of CanL in Brazil, a highly endemic country (Ribeiro et al. 2018). Although the results of a study carried out in Italy, over a period of 6 years, in dogs naturally infected with *L. infantum*, showed that treatment with meglumine antimoniate plus allopurinol presented better results than miltefosine plus allopurinol (Manna et al. 2015).

1.6.3. Allopurinol

Allopurinol or 4-Hydroxypyrazolo(3,4-d)pyrimidine (Zyloric®) is a purine analog used as a xanthine oxidase inhibitor to reduce the serum urate concentration. This oral drug has been prescribed for the treatment of gout in humans (Sivera et al. 2014) with its antileishmanial activity being first described in 1974 (Pfaller and Marr 1974). Allopurinol's mechanism of action consists of the inhibition of the leishmanial enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Pfaller and Marr 1974). This enzyme is important in the parasite's purine salvage pathway, converting dephosphorylated purines into nucleoside monophosphates (Chawla and Madhubala 2010). When allopurinol is phosphorylated by HGPRT, producing an inactive analog of inosine, it is incorporated into leishmanial RNA, causing disruption in protein translation (Baneth and Shaw 2002; Chawla and Madhubala 2010). But since this is not the parasite's only purine salvage pathway, its leishmanicidal effect is not very strong (Chawla and Madhubala 2010). That is why, although allopurinol is sometimes used in monotherapy, its effectiveness is questioned, never truly clearing the parasite from the host (Miró et al. 2011; Miró and López-Vélez 2018). For this reason, the therapeutic guidelines recommend the use of allopurinol in combination with either meglumine antimoniate or miltefosine (Solano-Gallego et al. 2011). While allopurinol is considered to be a safe drug for dogs, prolonged therapy has shown a predisposition to cause xanthinuria and xanthine urolithiasis, which is why the duration of therapy should be between 6 to 12 months

(Torres et al. 2016). There have also been reports of resistance to allopurinol in dogs, especially after disease relapse (Yasur-Landau et al. 2016). The gene METK, which codes for S-adenosylmethionine synthetase in *L. infantum*, seems to be connected to this resistance (Yasur-Landau et al. 2018).

1.6.4. Amphotericin B

Amphotericin B (AmBisome®, Fungizone®) is an anti-fungal drug produced by the actinomycete *Streptomyces nodosus* (Caffrey et al. 2001). This compound acts by binding to ergosterol in the parasite's cell membrane, causing structural disorganization and forming aqueous pores that lead to the loss of cellular constituents and subsequent death of the parasites by osmotic lysis (Baneth and Shaw 2002; Alvar et al. 2004; Miró et al. 2008). Because it also has affinity for cholesterol, the main sterol in mammalian cell membranes, a possible side effect is nephrotoxicity by renal vasoconstriction and possibly also by direct action on renal epithelial cells, endangering dogs who already have renal pathology (Baneth and Shaw 2002; Miró et al. 2008). Other side effects such as trembling, fever, nausea, vomiting, myalgia, arthralgia and progressive weight loss can occur during treatment, as well as increased levels of serum creatinine and urea nitrogen (Alvar et al. 2004). To counteract this, a less toxic liposomal formulation has been developed, still being administered parenterally, but less frequently (Baneth and Shaw 2002). Still, reports on the effectiveness of this formulation in CanL are contradictory and not yet conclusive (Baneth and Shaw 2002).

1.6.5. Other compounds

Pentamidine, an aromatic diamidine compound used to treat pneumocystosis, babesiosis and trypanosomosis, can be also applied to canine leishmaniosis (Berman 1997; Baneth and Shaw 2002; Bourdeau et al. 2014). The exact mechanism of action is still unknown, but it is believed to affect mitochondria, causing kinetoplast DNA disintegration and reducing the number of ribosomes (Alvar et al. 2004). The administration of the drug usually leads to muscular irritation at the injection site (Alvar et al. 2004), and its effectiveness is still questioned. Due to its toxicity it can induce anorexia, nausea, vomiting, abdominal pain, diarrhea, hypotension and tachycardia, among others (Jha 1983; Baneth and Shaw 2002). Records of infected dogs treated with pentamidine show that this drug improves the dog's clinical condition, but relapses several months after treatment are common (Baneth and Shaw 2002).

Aminosidine sulfate, an antibiotic of the aminoglycoside family is produced by the actinomycete *Streptomyces chrestomyceticus* and has been used in Africa and Europe for the

treatment of human visceral leishmaniosis (Chunge et al. 1990; Scott et al. 1992; Baneth and Shaw 2002). This injectable antibiotic acts in CanL by binding to the small ribosomal subunit, inhibiting protein synthesis by the parasite (Maarouf et al. 1997; João et al. 2006). The administration of this antibiotic presents several risks, such as the development of nephrotoxic and ototoxic reactions (Chunge et al. 1990; Alvar et al. 2004), and its effectiveness in clearing parasites from dogs has been contraindicated (Athanasiou et al. 2013).

Trifluralin, a dinitroaniline herbicide used against *Leishmania* (Bhattacharya et al. 2002), *Trypanosoma* (Traub-Cseko et al. 2001), *Toxoplasma* (Stokkermans et al. 1996) and *Plasmodium* (Bell 1998), has a high affinity for tubulins, the main component of microtubules, causing disruption and affecting *Leishmania* cell mitosis and mobility, inhibiting promastigote proliferation and reducing amastigote infectivity (João et al. 2006). These dinitroanilines are apparent potential drugs, for they are ineffective against mammalian tubulins, and therefore have selective activity against parasites (Chan and Fong 1990). However, due to their low solubility, liposomal delivery systems have been developed, but still without much therapeutic success (João et al. 2006; C. Marques et al. 2008).

Several anti-fungal oral drugs such as metronidazole, ketoconazole, fluconazole, itraconazole and secnidazole have been investigated for antileishmanial activity. Their mode of action is based on the inhibition of ergosterol synthesis, promotes the activation of phosphorylases and intensify glycogenolysis which reduces the parasite's glycogen reserves, in addition to inhibiting the synthesis of nucleic acids (Goad et al. 1985; Olliaro and Bryceson 1993). Unfortunately, some of these drugs are less effective than meglumine antimoniate in reducing the parasitic burden in *L. infantum* infected mice and dogs (Gangneux et al. 1999; Pennisi et al. 2005; Bahashwan 2011).

1.7. Prevention

1.7.1. Environmental vector control

The various preventive measures directed against CanL are based on the two main agents of this disease, the phlebotomine vector and the domestic reservoir dog (Vulpiani et al. 2011). In highly endemic areas, where the risk of transmission is high, the prevention of physical contact with the insect vector and the reduction in the number of possible infectious bites can be further achieved by:

- Physical barriers - These include protecting windows and doors of houses, shelters or kennels using fine mesh screens (Maroli et al. 2010);
- Chemical barriers - Through spraying residual insecticides or permethrin-treated screens in dwellings and surrounding areas (Basimike and Mutinga 1995; Quinnell and Courtenay 2009);
- Removal of sand-fly breeding locations - Such as organic peridomiciliary materials like compost, pruning scraps, bins, wood and stone piles, as well as any other materials favorable to sand-fly breeding near inhabited areas (Solano-Gallego et al. 2009; Ribeiro et al. 2018);
- Reduce contact with sand flies - During high-risk seasons, keeping dogs indoors from dusk to dawn, when female sand flies are more actively looking for blood meals (Miró et al. 2017).

In addition to these simple measures, there are also some natural compounds known to be insect repellent, although their true effectiveness against sand flies is unknown. Many of these products have not been tested on dogs, and the repellent's duration is believed to be very limited (Miró et al. 2017). The repellents that have been tested, like candles impregnated with citronella, linalool and geraniol extracts show a weak repellent effect against sand-fly bites (Müller et al. 2008).

1.7.2. Topical insecticides and other formulations

One of the simplest phlebotomine repellent products available is PVC collars impregnated with synthetic pyrethroids such as deltamethrin and flumethrin that used alone or in association with other insecticides can display a synergistic effect on insects (Ribeiro et al. 2018) (Table 7). These synthetic pyrethroids have the ability to alter the function of voltage-gated sodium channels in insect neuronal membranes, disrupting electrical signaling in the

nervous system, leading to paralysis (Soderlund 2010). They are classified as ectoparasiticides, affecting not only phlebotomine sand flies, but other ectoparasites, such as fleas, ticks and mosquitos (Brianti et al. 2016). Collars are considered slow-releasing products and, while full protective activity is achieved only approximately one week after application, these products have the benefit of providing protection between 4-8 months, depending on the components (Maroli et al. 2010; Ribeiro et al. 2018).

Table 7. Examples of current prophylactic impregnated PVC collars available for CanL prevention

Trade name	License	Pharmaceutical compounds	Duration	Efficacy in field studies	References
Scalibor®	MSD-Animal Health	4% deltamethrin	4-6 months	61.8%; 50-86%	(Maroli et al. 2001; Brianti et al. 2016)
Seresto®	Bayer Animal Health	4.5% flumethrin + 10% imidacloprid	8 months	88.3%	(Brianti et al. 2016)

Table adapted from Ribeiro et al. (2018).

The mechanism of action of these synthetic pyrethroids involves two main aspects: the sand flies that rest on the dog's skin long enough will absorb a lethal dose of insecticide, and those that have had only a brief contact with the insecticide-treated skin can still be affected by irritation and disorientation, which results in reduced blood feeding rates (Killick-Kendrick et al. 1997). As an example, a study using 4% deltamethrin-impregnated collars demonstrated potent non-feeding effects against *P. perniciosus*, with killing rates of 60% of the insects within 2 hours after exposure (Killick-Kendrick et al. 1997). Another study, using a commercially available collar with the same formulation (Scalibor®), showed a reduction of sand-fly *(P. perniciosu*s) feeding ≥ 94% compared to unprotected dogs (Paulin et al. 2018). A study using the same brand presented an efficacy of 61.8% in *L. infantum* infection prevention, while another brand (Seresto®) showed an 88.3% overall efficacy (Brianti et al. 2016). Safety tests carried out after application of these compounds on the skin of dogs revealed only rare and temporary skin reactions, such as itching and erythema, in some smaller breeds with thin and delicate skin (Maroli et al. 2001). Considering the long-term effect of collars, applying them to most dogs in endemic *L. infantum* regions can substantially reduce contact to vectors and diminish the risk of infection for dogs and humans (Killick-Kendrick et al. 1997; Maroli et al. 2010).

Another simple repellent application system is spot-on insecticides, which contains synthetic pyrethroids, such as permethrin, used alone or in combination with other insecticides (Ribeiro et al. 2018) (Table 8). While these spot-on formulations have the advantage of covering a large body surface, achieving full protective activity at approximately 24-48 hours after application, they offer protection for shorter periods compared to PVC collars, usually between 2 and 4 weeks, requiring frequent reapplication (Maroli et al. 2010; Ribeiro et al.

2018). A study using a 10% imidacloprid and 50% permethrin spot-on formulation showed a potent non-feeding effect on *P. perniciosus* above 90% during the first 3 weeks of application (Miró et al. 2007). Other spot-on formulations, as is the case of a solution containing 65% permethrin has been reported to be more than 90% effective against *P. perniciosus* bites for 4 weeks (Molina et al. 2001). Field studies using these formulations registered a significant reduction in the risk of infection in endemic areas (Giffoni et al. 2002; Otranto et al. 2007; Ferroglio et al. 2008).

Table 8. Examples of current prophylactic Spot-on insecticides for CanL prevention

Trade name	Company	Pharmaceutical compounds	Duration (weeks)	Efficacy in field studies	References
Advantix®	Bayer Animal Health	50% permethrin + 10% imidacloprid	3	88.9-90.4%	(Otranto et al. 2007)
Exspot®	MSD-Animal Health	65% permethrin	2-3	84%	(Ferroglio et al. 2008)
Frontect® or Frontline Tri-Act®	Merial	50.48% permethrin + 6.76% fipronil	3	100%	(Papadopoulos et al. 2017)
Effitix® or Fiprotix® or Fipratix®	Virbac	54.5% permethrin + 6.1% fipronil	4	-	-
Perfikan®	Clément Thékan	54.5% permethrin + 6.1% fipronil	4	-	-
Caniguard Line On®	Beaphar	40% permethrin	5	-	-
Vectra 3D®	Ceva	36.08% permethrin + 4.95% dinotefuran + 0.44% pyriproxyfen	4	-	-

Table adapted from Ribeiro et al. (2018).

Lastly, another alternative is systemic compounds in the form of chewable tablets (Table 9), usually containing isoxazolines, a novel class of compounds that targets the central nervous system and neuromuscular junctions of arthropod vectors, blocking ligand-gated chloride channels leading to the vector's death after the blood meal (Weber and Selzer 2016). Although these compounds are marketed as systemic anti-flea and anti-tick ectoparasiticides, several studies have demonstrated their effectiveness against phlebotomine sand flies. Such is the case of a study using an oral dose of fluralaner (Bravecto®) that resulted in 100% mortality of *P. perniciosus* after 24 hours in days 1 and 28 after the application, with significant insecticidal efficacy (>50%) still being observed on day 84 (Bongiorno et al. 2019). In another study, oral administration of afoxolaner (NexGard®) resulted in insecticidal efficacies against *P. perniciosus* of 100%, 95.9% and 75.2% after 48 hours on days 1, 14 and 28 after exposure, respectively, and 100%, 100% and 86.3% at 72 hours on days 1, 14, and 28 (Perier et al. 2019). In this formulation a single chewable tablet confers "protection" between 30 (NexGard®) and 84 (Bravecto®) days. The major disadvantage is that, while topical insecticides can act as a result of physical contact with the arthropod, systemic compounds involve the arthropod's bite and feed to act, thus not preventing the infection, but only further transmission (Jongejan

et al. 2016). To counteract this, these chewable tablets can be combined with topical insecticides, such as PVC collars (Walther et al. 2014).

Table 9. Examples of current chewable tablets with systemic compounds for *Leishmania* transmission prevention

Trade name	Company	Pharmaceutical compounds	Duration (days)	Efficacy in lab studies	References
Bravecto®	MSD-Animal Health	Fluralaner	84	100%	(Bongiorno et al. 2019)
NexGard®	Merial	Afoxolaner	30	86.3%	(Perier et al. 2019)

1.7.3. Immunomodulators and immunostimulants

Prophylactic medication is not commonly used in CanL, however, some products like Leisguard®, a domperidone-based oral solution, have been marketed for both prevention and treatment in several European countries (Table 10) (Gómez-Ochoa et al. 2009; Mattin et al. 2014; Travi and Miró 2018). Domperidone, a benzimidazole derivative, is a gastric prokinetic and anti-emetic drug with selective dopamine D2 receptor antagonistic activity, resulting in the release of serotonin, which in turn stimulates prolactin production (Barone 1999; Woosley 2004; Reddymasu et al. 2007; Ready 2017). Prolactin, a hormone excreted from the pituitary gland and generated by lymphocytes, is considered a pro-inflammatory cytokine and, although its mechanism of action is still largely unknown, it is believed to stimulate $CD4^+$ cellular immunity (Th1) by increasing the production of IL-2, IL-12, TNF-α and IFN-γ (Majumder et al. 2002; Travi and Miró 2018). This response can lead to the activation of phagocytic cells and potentiate the intracellular killing of parasites, which can help to prevent CanL and reduce the risk of developing clinical disease (Ribeiro et al. 2018). Although immunomodulators such as domperidone are commonly used as a preventive method (Ready 2017), since particular immune changes occur during CanL, their use associated with specific treatments has been suggested (Alvar et al. 2004; Solano-Gallego et al. 2017). Some of these compounds, which have multifactorial effects, act indiscriminately on cellular and humoral immunity (Alvar et al. 2004). Prednisone and prednisolone, for example, have been used to decrease the formation of antigen-antibody complexes, being only recommended when there are lesions following immunocomplex deposition (Alvar et al. 2004). Immunostimulants such as levamisole have been used occasionally, never alone and always associated with another conventional treatment, with the premise of cellular immunity and macrophage activation (Alvar et al. 2004; Ribeiro et al. 2018).

Table 10. Examples of current prophylactic immunostimulants for the prevention of CanL

Trade name	Company	Pharmaceutical compounds	Therapeutic protocol	Duration	References
Leisguard®	Esteve	Domperidone	0.5 mg/kg once a day for 30 days, *per os*	≤4 months	(Reguera et al. 2016)
Impromune®	Bioibérica	Nucleoforce® + AHCC	1 tablet once a day for at least 6 months	-	(Segarra et al. 2018)

Apart from some studies presented at congresses (Ceballos et al. 2011; Gómez-Ochoa et al. 2012), there are few studies on the effectiveness of prevention that domperidone confers, and the existing ones show similar efficacy with other prevention measures, such as impregnated collars and spot-on (Fernandez et al. 2018; Travi and Miró 2018). Some side effects associated with domperidone administration, such as polyuria, dysorexia, vomiting and diarrhea have been reported (Travi and Miró 2018), and as long as the presence of side effects is not properly studied, the reliance on these therapies is left to the clinician's personal experience and the tutors' decision.

Promising new immunomodulatory molecules are being tested, such as the protein aggregate magnesium-ammonium phospholinoleate-palmitoleate anhydride (P-MAPA) (Roatt et al. 2014; Hosein et al. 2017), which seems to promote improvement in clinical signs and a significant reduction in parasite load in the skin (Santiago et al. 2013). In peripheral blood mononuclear cell cultures, supernatants showed an increase in IL-2 and IFN-γ and a decrease in IL-10 levels, along with an increase in $CD8^+$ T cells (Santiago et al. 2013). Other studies point out a possible induction of the Toll-like receptor 2 (TLR2) in human embryonic kidney cells (Fávaro et al. 2012) and TLR2 and ROS production in infected canine macrophages (Melo et al. 2014). However, little is known about the effects and mode of action of this molecule.

Another recent compound is Setarud (IMOD™), an herbal immunomodulatory drug composed of a herbal mixture of *Rosa canina*, *Urtica dioica*, *Tanacetum vulgare* and selenium, which has shown significantly high efficacy in resolving the clinical signs of CanL and hematobiochemical factors when in combination with meglumine antimoniate (Malmasi et al. 2014). IMOD™ was patented in Europe for its potential to reduce oxidative stress and TNF-α activity, improve helper T lymphocytes in HIV-positive patients, effectiveness in experimental models of immunoinflammatory diseases and reduce mortality rates in cancer units, without mutagenic and genotoxic effects (Novitsky et al. 2007). This product appears to be well tolerated with no adverse effects on humans and animals (Hasani-Ranjbar et al. 2009; Khairandish et al. 2009; Mohammadirad et al. 2011).

Impromune®, a dietary supplement that contains a mixture of dietary nucleotides and an active hexose correlated compound (AHCC) is also a compound available on the market (Segarra et al. 2018). AHCC is a cultured extract of shiitake mushrooms (*Lentinula edodes*)

mycelia, used for its ability to stimulate the immune system, especially cellular immunity (Segarra et al. 2018). One of the proposed mechanisms of action involves a possible TLR-agonistic activity by certain bioactive components found in this compound (Lee et al. 2012; Ulbricht et al. 2013; Mallet et al. 2016). In a recent study, dogs with subclinical infection (infected dogs, but clinically healthy), which according to current guidelines are not recommended for treatment, were used to test the preventive capacity of this formulation over a period of one year (Segarra et al. 2018). According to the authors, a lower proportion of dogs treated with the supplement developed clinical signs in comparison to the placebo group, with significantly reduced antibody titers and disease scores (Segarra et al. 2018). Since a third of the tested dogs were excluded from the study, for various reasons, and this is the only study on the preventive effectiveness of this product, further field studies are needed. These recent studies also propose the use of this supplement for the treatment of canine and feline leishmaniosis in combination with first-line drugs instead of allopurinol, as it appears to have similar efficacy without leading to xanthinuria (Segarra et al. 2017; Leal et al. 2018).

Lastly, while these compounds reduce the risk of dogs developing clinical signs of CanL and help already infected dogs to control the progression of infection (Sabaté et al. 2014), they still do not eliminate their status as *L. infantum* reservoirs.

1.7.4. Vaccines

In the past decade, there has been an increasing focus on the development of vaccines that stimulate the dog's immune response and prevent animal infection and disease progression, thereby blocking the parasite's life cycle and reducing the prevalence and the incidence of CanL (Table 11) (Reguera et al. 2016). The first generation of vaccines against *Leishmania* infection emerged from leishmanization, which consisted of inoculating live virulent parasites from an active lesion in healthy patients, in order to develop a self-healing lesion and thus protect against future infections (Khamesipour et al. 2005). Some active principles were composed of heat or phenol-killed promastigotes associated with different adjuvants, such as BCG (*Mycobacterium bovis*, Bacillus Calmette-Guérin) or irradiated or attenuated live promastigote (Mayrink de Oliveira et al. 2019).

Vaccines of second-generation, in turn, include purified or recombinant *Leishmania* spp. proteins (Jain and Jain 2015). In Brazil, two CanL vaccines of second-generation, Leishmune® (Zoetis, Brazil) and Leish-Tec® (Hertape Calier, Brazil), were registered in 2003 and 2006 respectively (Jain and Jain 2015).

Table 11. Current prophylactic vaccines for the prevention of CanL

Trade name	Company	Antigens	Adjuvant	Efficacy in field studies	References
Leishmune®	Zoetis	Fucose-Mannose Ligand (FML) of *L. donovani*	QuilA®	76-80%	(Palatnik-de-Sousa 2012)
Leish-Tec®	Hertape Calier	Recombinant protein A2 of *L. donovani*	Saponin	71.4%	(Regina-Silva et al. 2016)
CaniLeish®*	Virbac	Excreted-secreted proteins of *L. infantum* (LiESP)	QA-21	68.4%	(Oliva et al. 2014)
Letifend®*	Laboratorios Leti	Recombinant Protein Q from *L. infantum*	None	72%	(Cotrina et al. 2018)

*Vaccines available in Portugal. Table adapted from Ribeiro et al. (2018) and LeishVet Guidelines (2018).

Leishmune®, the first available vaccine is composed of the purified GP36 fraction, which bears a fucose-mannose ligand (FML) isolated from *L. donovani* promastigotes, along with a saponin adjuvant (QuilA®) (Otranto and Dantas-Torres 2013; Jain and Jain 2015). The immunization schedule for this vaccine consists of a total of three doses administered subcutaneously once every 21 days in dogs from 4 months of age (de Lima et al. 2010; Zoetis 2014). Early field studies in dogs showed this vaccine to be safe, protective, highly immunogenic and capable of preventing parasite transmission (Dantas-Torres 2006). Leishmune® showed selective immune responses in dogs, including early phenotypic changes in neutrophils and monocytes, selective stimulation of $CD8^+$ T-cells with the induction of a specific pro-inflammatory response mediated by IFN-γ and nitric oxide (NO) (Araújo et al. 2011).

Leish-Tec®, in turn, is composed of the *L. donovani* recombinant A2 protein with the saponin adjuvant (Mayrink de Oliveira et al. 2019). For this vaccine, the immunization schedule consists of three subcutaneous applications of Leish-Tec®, once every 21 days, with an annual booster vaccination being recommended for a complete immunization in dogs aged 4 months and older (Reguera et al. 2016; M.P. de Campos et al. 2017). This A2 protein is a highly expressed amastigote surface antigen, having been the first virulence factor identified in the *Leishmania* genus and is an essential protein for parasite survival in the mammalian host, being also involved in pathogen visceralisation during infection (Zhang and Matlashewski 2001). A2 contains an immunogenic epitope for $CD4^+$ helper T cells and multiple repetitive units encoding $CD8^+$ cytotoxic T lymphocyte epitopes (Fernandes et al. 2012). Regarding efficacy, in a study in dogs immunized with Leish-Tec® and later experimentally infected with *L. infantum*, they only develop a partially protective immune response against CanL, showing positive bone marrow parasitism 9 months after the challenge (Fernandes et al. 2008). A more recent study in dogs in a highly endemic area did not show great vaccine efficacy in inducing clinical protection, as 43% of the vaccine recipients developed the disease over time (Grimaldi et al. 2017).

In 2011, the first commercially available CanL vaccine in Europe was registered and named CaniLeish® (Virbac, France) (Moreno et al. 2012). It is a formulation composed of *L. infantum* excreted/secreted recombinant proteins (LiESP) associated with a highly purified fraction of *Quillaja saponaria* saponin (QA-21) as an adjuvant (Moreno et al. 2012; Wylie et al. 2014). The immunization schedule for this vaccine is the same as the previous ones, with an additional annual re-vaccination for complete immunization in dogs aged 6 months or older (EMA 2010; Reguera et al. 2016). Dogs immunized with CaniLeish® and experimentally infected with *L. infantum* one year later demonstrated reduced parasitic load, stronger cell-mediated immune responses and lower probability of relapses in comparison to control dogs (Martin et al. 2014).

More recently, in 2016, the latest commercially available vaccine, LetiFend® (Laboratorios LETI S.L., Spain) was introduced in Europe (CVMP 2016; Fernandez et al. 2018; Mayrink de Oliveira et al. 2019). LetiFend® active ingredient is Protein Q, a recombinant chimeric protein composed by the fusion of five epitopes of *L. infantum* acidic ribosomal proteins LiP2A, LiP2B, LiP0 and histone H2A without any adjuvant (Soto et al. 1998; Cotrina et al. 2018). For this vaccine, the immunization schedule consists of a single subcutaneous injection each year for dogs aged 6 months and older (CVMP 2016). In a large-scale canine population, this vaccine has been shown to be safe and effective in the active immunization of uninfected dogs, reducing the risk of developing CanL after natural infection by *L. infantum* (Cotrina et al. 2018).

All of these commercially available vaccines can only be used in healthy seronegative dogs and they are not 100% reliable in preventing infection, but aid in controlling disease progression and reduce the likelihood of developing clinical signs (LeishVet Guidelines 2018). In addition, current diagnostic methods based on serology do not allow the distinction between vaccinated dogs and naturally infected dogs (Gradoni 2015; Manna et al. 2015; Ceccarelli et al. 2016; Gavazza et al. 2016; Hosein et al. 2017; Miró et al. 2017; Solano-Gallego et al. 2017), except for those vaccinated with Letifend® (Corrales et al. 2016; Segarra et al. 2018; LeishVet Guidelines 2018). Nonetheless, recent studies have shown the possibility of differentiating healthy dogs vaccinated with CaniLeish® from vaccinated and parasitized dogs by using the relationship between the seroreactivities of two different antigens (SPLA and rK39) (Lima et al. 2019).

Lastly, current guidelines recommend that the future for CanL control should be an integrated approach to prevention that includes vaccination against *L. infantum* with an effective canine vaccine and the use of long-acting topical insecticide applications (Solano-Gallego et al. 2011; Ribeiro et al. 2018). Thus, the vaccine would prevent the establishment of infection introduced by the bites of any sand flies that escaped from the insecticide's action (Solano-Gallego et al. 2011).

2. DOG'S IMMUNE RESPONSE TO *L. INFANTUM* INFECTION

The immune system is the body's defense mechanism. Beyond the physical barriers that prevent the penetration of invading microorganisms, its main function is to differentiate the self from the non-self, promoting an immune response in case of imminent infection and/or prevent its exacerbation (Tizard 2012). Immediate responses generated by the innate immune response, such as inflammation, the complement system and antimicrobial molecules, are nonspecific and aid in the control of infection, while the adaptive immune response is a long-term mechanism aimed towards a specific pathogen defense through clonal expansion of T and B lymphocytes (Tizard 2012). In CanL, the classical consensus is that a dog's likelihood of developing disease depends mainly on its immune response to *Leishmania* infection, with a protective Th1 response usually leading to clinical cure, while a non-protective Th2 response impairs a good defense against this pathogen (Strauss-Ayali et al. 2005).

2.1. Innate immune response

2.1.1. Neutrophils

The most predominant blood leukocytes and also the first line of defense against microbial infections are neutrophils (Fig. 14) (Tizard 2012). They account for 60 to 75% of the total leukocytes in an adult dog and, after leaving the bone marrow where they are produced, circulate in the bloodstream with a life span of only a few days (Borregaard 2010; Tizard 2012). Neutrophils have several mechanisms to help contain the infection, which includes phagocytosis, the release of neutrophil extracellular traps (NETs), production of ROS and exocytosis of granular molecules, in addition to the production of cytokines (Tizard 2012; Regli et al. 2017; Pereira, Alexandre-Pires, et al. 2019).

2.1.2. Monocytes/Macrophages

Assisting neutrophils are monocytes, bone marrow-derived cells that circulate in the bloodstream for about 3 days before entering the tissues where they differentiate into active macrophages (Fig. 14) (Tizard 2012). These cells represent 5% of the total blood leukocyte population and, in addition to effective phagocytosis, they produce macrophage extracellular traps (METs), perform crucial antigen presentation and release cytokines, for example, IL-1, IL-6, IL-12, IL-18 and TNF-α (Tizard 2012; Pereira, Valério-Bolas, et al. 2019; Pereira, Alexandre-Pires, et al. 2019). These cells are part of the innate immune system and express

many different pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) common to many microorganism, such as bacteria, viruses and protozoa, allowing them to phagocytize and destroy a diversity of pathogens (Kawai and Akira 2010; Tizard 2012).

2.1.3. Natural Killer (NK) and Dendritic Cells (DC)

Natural killer and dendritic cells are two other cell types belonging to the innate system (Fig. 14). NK cells have receptors for surface molecules present in normal cells, and when these molecules are modified or absent in infected or altered cells, NK cells can induce cytolysis or apoptosis of these altered cells to eliminate pathogens or defective cells (Tizard 2012; Belizário et al. 2018).

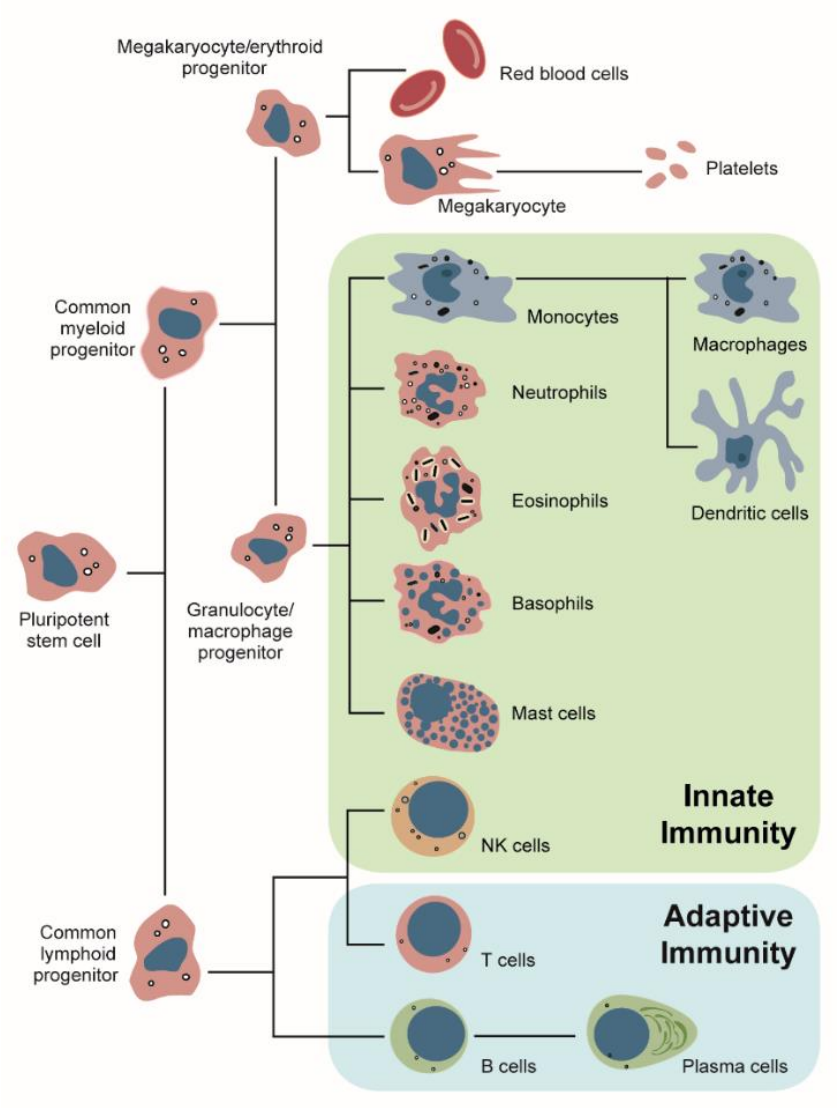

Figure 14. Bone marrow derived immune cells
Lymphoid cells originate from stem cells other than cells of the myeloid system. Cells, such as eosinophils and basophils, are probably closely related despite significant morphological differences. NK – Natural killer. Adapted from Tizard (2012).

DCs are antigen-presenting cells (APCs), which alongside macrophages and mast cells are considered sentinel cells scattered throughout the body, being found in larger numbers just below the skin, where it is more likely to find invading microorganisms (Liu and Uzonna 2012; Tizard 2012). As sentinel cells, they are important recognizers of PAMPs through their PRRs and, together with macrophages, stimulate neutrophil migration from blood vessels to the infection site, the development of inflammation and above all phagocytosis of pathogens and apoptotic cells (e.g. pathogen-infected neutrophils), digest them into small antigenic fragments and expose the antigens on the cell surface together with major histocompatibility complex (MHC) molecules (Tizard 2012; Belizário et al. 2018). These MHC molecules exist in two classes: class I molecules of MHC (MHC-I) that are present in all nucleated cells and bind endogenous antigens (foreign proteins produced by cells commanded by intracellular pathogens, e.g. viruses) and class II molecules of MHC (MHC-II) that are only present in specialized antigen-processing cells like DCs, macrophages and B cells and bind exogenous antigens (proteins from exogenous pathogens, e.g. bacteria and protozoa) (Hewitt 2003; Tizard 2012; Roche and Furuta 2015). These APCs and their antigens can subsequently be recognized by naïve lymphocytes, thus forming a connection between the innate and adaptive immune response (Tizard 2012).

2.2. Adaptive immune response

2.2.1. Lymphocytes

Lymphocytes, such as T and B cells, belong to the adaptive immune response (Figs. 14 and 15), and are found throughout the body in lymphoid organs, blood and spread under mucosal surfaces (Tizard 2012). The adaptive immune response, in turn, is subdivided into functional groups representing humoral and cellular immunity (Fig. 15). Humoral immunity involves B lymphocytes (B cells), which synthesize and secrete antibodies, while cellular immunity involves effector T lymphocytes (T cells), which secrete immune mediators after interaction with APCs that present foreign material to lymphocytes (Actor 2014). Common lymphoid progenitor cells produced in the bone marrow migrate to the thymus, giving rise to mature T cells. These cells constitute up to 60-80% of lymphocytes in the dog's bloodstream (Tizard 2012). B cells can develop either in the bone marrow, bursa or Peyer's patches and account for 10 to 40% of blood lymphocytes (Tizard 2012). The adaptive immune response developed by these cells is extremely efficient because, unlike the innate response, it is a specific response to the invading pathogen. Furthermore, after the encounter with the antigens of pathogenic agents, these cells generate immune memory cells. The downside is that it takes longer than the innate response. To elicit this type of response, antigens complexed with MHC

on APC's surface need to be recognized by lymphocytes through specific receptors, such as the T cell receptors (TCR) and B cell receptors (BCR) (Janeway et al. 2001).

2.2.2. T lymphocytes

In the cell membrane of T lymphocytes, connected to the TCR, is expressed a transduction complex called cluster of differentiation 3 (CD3), signaling the cell when antigen binding occurs (Kuhns et al. 2006). Along with TCR, T cells can also present either the co-receptor CD4 or CD8, which further divides T lymphocytes into two different subpopulations, T helper (Th) and T cytotoxic (Tc) cells, respectively. This way, naïve $CD4^+$ Th cells recognize antigens complexed with MHC-II presented by APCs while naïve $CD8^+$ T cells recognize antigens complexed with MHC-I (Tizard 2012). In addition to this binding between the TCR and the MHC-peptide complex, additional signals are required to activate a T cell-mediated immune response, such as cytokines produced by APCs, which in turn lead to cytokine secretion by the T cells and differentiation into effector and memory cells (Gutcher and Becher 2007; Tizard 2012).

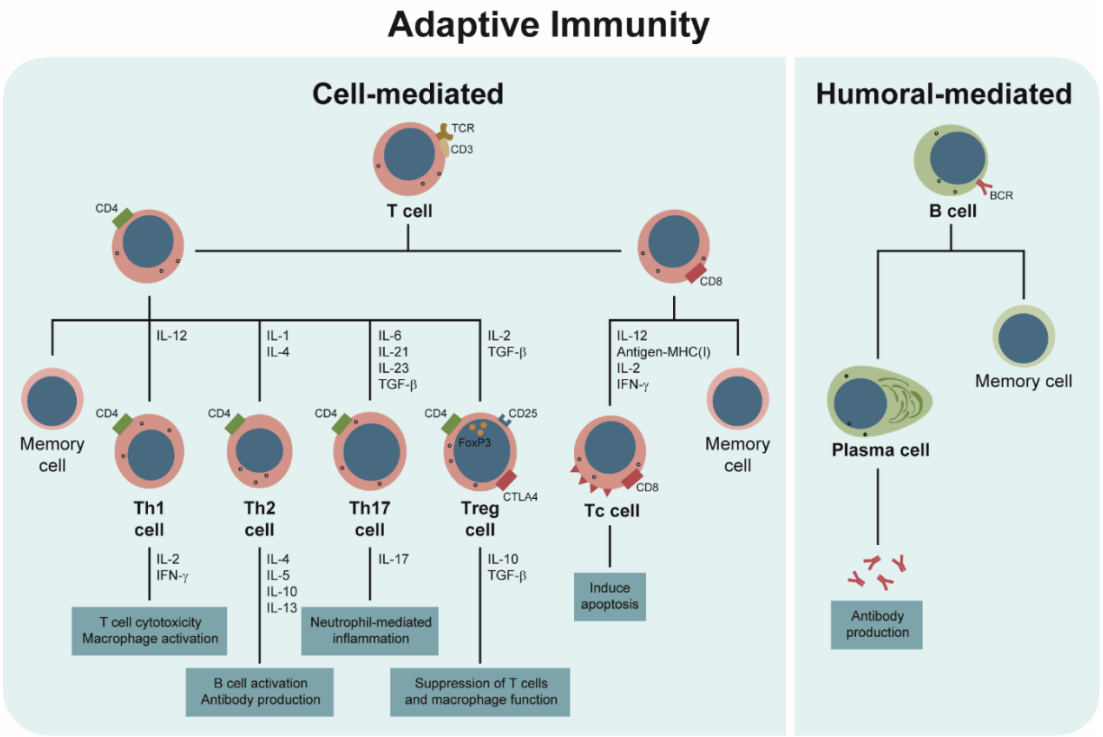

Figure 15. B and T cell populations, the respective cell subsets and the immune mediators involved in the adaptive immune response

Generally, progenitor T cells produced in the bone marrow migrate and enter the thymus, where they expand and begin to express TCR (Overgaard et al. 2015). During development in the thymus, these thymocytes transiently express both CD4 and CD8 co-receptors on their cell surface being characterized as double-positive (DP) (Germain 2002). At

this stage, DP thymocytes survive 3-4 days until the negative selection stage differentiates them into either $CD4^+$ or $CD8^+$ single-positive cells, becoming mature peripheral T cells (Weiss et al. 1998). This idea of separate lineages is generally considered to be fixed, but despite that, $CD4^+CD8^+$ (DP) T cells have been reported in the blood and peripheral lymphoid tissues in several species, such as humans (Nascimbeni et al. 2011), mice (Das et al. 2003), rats (Kenny et al. 2000), chickens (Morgan et al. 2005), monkeys (Akari et al. 1997), pigs (Saalmüller et al. 1987) and dogs (Alexandre-Pires et al. 2010), as well as in numerous disease settings, such as HIV, cancer and autoimmune diseases (Overgaard et al. 2015). Initially suggested to be thymocytes, which escaped prematurely from the thymus, recent studies have shown these extrathymic $CD4^+CD8^+$ T cells in peripheral sites express T cell maturation markers and lack thymic-stage markers, revealing to be a mature population in the periphery, alongside conventional $CD4^+$ and $CD8^+$ T cells (Bismarck et al. 2012).

While the presence of DP T cells has been found in several different species and settings, their function remains poorly described and controversial, with reports of enhancement of cytotoxic responses during viral infections, as well as suppressive potential (Overgaard et al. 2015).

2.2.3. Helper T cells (Th)

Mature Th cells can be further classified into Th1, Th2 or Th17 and distinguished by the profile of cytokines they produce (Shibuya and Hirohata 2005). Th1 cells are stimulated by IL-12 and produce the pro-inflammatory cytokines IL-2 and IFN-γ, promoting cell-mediated immune responses (e.g. macrophage activation) and generating immunity against intracellular organisms, such as mycobacteria and viruses (Shibuya and Hirohata 2005; Tizard 2012). Th2 cells develop in the absence of IL-12 and are stimulated by IL-1 and IL-4, and are producers of the anti-inflammatory cytokines IL-4, IL-5, IL-10 and IL-13 (Shibuya and Hirohata 2005). These cells generally promote a humoral immune response by stimulating B cell proliferation and immunoglobulin (antibody) production, being associated with enhanced immunity against extracellular invaders (e.g. parasitic worms) but with decreased resistance to mycobacteria and other intracellular organisms (Coffman et al. 1993; Shibuya and Hirohata 2005). Lastly, Th17 cells are stimulated by IL-6, transforming growth factor-β (TGF-β), IL-21 and IL-23, and produce IL-17 that promotes neutrophil-mediated inflammation (Tizard 2012; Halwani et al. 2017). These cells play an important role in the protective response to extracellular Gram-negative bacteria and assist in the clearance of fungi. The type of activated Th response is dependent on the characteristics of the immune synapse and the type of cytokines present in the microenvironment (Tizard 2012).

2.2.4. Cytotoxic T cells (Tc)

The maturation of Tc cells requires three key signals: the first being IL-12 from activated dendritic cells, next the binding of antigen to the MHC-I complex on infected or abnormal cells, and finally, IL-2 and IFN-γ secreted by Th1 cells (Von Essen et al. 2012). After these three signals, naïve $CD8^+$ Tc cells become activated, inducing the apoptosis of cells infected with intracellular pathogens (e.g. viruses and mycobacteria) or other abnormal cells (e.g. leukemic cells) (Tizard 2012).

2.2.5. Regulatory T cells (Treg)

Another subpopulation of T cell is suppressor or regulatory T cells (Treg). These cells play a key role in regulating the immune system, by maintaining immune tolerance and homeostasis, being particularly relevant in preventing autoimmunity (Taams et al. 2006; Cortese et al. 2015). Regulatory T cells (Tregs) are lymphocytes that characteristically express CD4 and CD25 (the α chain of the IL-2 receptor) molecules on the cell surface and can be broadly divided into two categories: natural Treg (nTreg) cells derived from the thymus and inducible Treg (iTreg) cells that are derived from the periphery and generally develop in response to antigens and co-stimulation by IL-10 and TGF-β (Tizard 2012; Bhattacharya and Ali 2013; Ferreira et al. 2019). These signals induce the transcription of the Forkhead box Protein 3 (FoxP3), a characteristic marker of Treg cells, which induces the transcription of CTLA-4 genes (also known as CD152, a suppressor of T cell activation) and production of the regulatory cytokines TGF-β and IL-10 (Tizard 2012). Treg cells are spread throughout the dog's body and represent about 5% of circulating T cells and 10% of lymph node T cells (Tizard 2012). Although traditionally only the $CD4^+$ fraction has Tregs, there are $CD8^+$ T lymphocytes that express CD25 and FoxP3. And because $CD4^+CD25^+FoxP3^+$ T cells are potent suppressors of the activation of $CD8^+$ T cells (Piccirillo and Shevach 2001), $CD8^+$ T suppressor cells have not been well studied, with their mode of action and purpose not yet fully understood (Shevach 2006; Zhang et al. 2018). Some studies have shown that resting $CD4^+$ lymphocytes are resistant to $CD8^+CD25^+FoxP3^+$ Treg cells, so the initiation of T-cell response is unlikely to be affected by $CD8^+$ Treg cells (Hu et al., 2012). On the other hand, these $CD8^+$ Treg cells may play an important role in suppressing ongoing $CD4^+$ T-cell responses (Hu et al., 2012). For instance, Jarvis et al. (2005) found that human $CD8^+$ T cells stimulated with activated DCs, followed by cloning, resulted in several $CD8^+$ T cell clones that responded to stimulation with DCs, produced IL-4, IL-5 and IL-13, but not IFN-γ and expressed CTLA-4 and FoxP3. These

cells suppress IFN-γ production and proliferation by $CD4^+$ T cells in a contact-dependent manner that could be reversed by anti-CTLA-4.

2.2.6. B lymphocytes

The last group of lymphocytes belonging to adaptive immunity are B cells. Through their BCR they can recognize most antigens without prior processing, although, to obtain an optimal response, co-stimulation with Th cells and cytokines is required (Tizard 2012; Nera et al. 2015). Once stimulated, B cells differentiate into memory cells and plasma cells, and exhibit a humoral-mediated immune response, differentiating into larger secretors of soluble BCRs, i.e. immunoglobulins (Ig), initially of the IgM class and then of IgG, IgA and IgE (Tizard 2012; Nera et al. 2015).

2.3. Cytokines, clusters of differentiation and transcription factors

Cytokines are a diverse group of soluble peptides that allow signaling between cells and elicit biological responses, including, but not limited to, cell activation, proliferation, growth, differentiation, migration and cytotoxicity (Tarrant 2010). These include chemokines, interleukins, tumor necrosis factors and interferons (Ferreira et al. 2019). Interferon was the first cytokine to be described (Isaacs and Lindenmann 1957) and, since then, many other cytokines have been discovered, along with their functions and effects, some of which are listed in Table 12. Classically, the main role of cytokines is closely linked to the management of inflammation and the immune response (Tarrant 2010). Cytokines act on many different types of cells, and cells rarely secrete just one cytokine at a time, being redundant in their activity, which means that similar functions can be stimulated by different cytokines (Zhang and An 2007). They can act in the cell where they are produced (autocrine action), in nearby cells (paracrine and juxtracrine action) or distant cells (endocrine action) (Tarrant 2010; Ferreira et al. 2019). This complexity results in a cytokine network, a sometimes seemingly contradictory web of different signals transmitted between cells of the immune system, mediated by complex mixtures of cytokines (Turner et al. 2014).

The cytokines produced by T lymphocytes can also be further divided according to the subgroups of cells by which they are governed, namely:

- Th1 cells – IL-2, IL-6, IL-12, TNF-α and IFN-γ;
- Th2 cells – IL- 4 and IL-5;
- Th17 cells – IL-17, IL-21 and IL-22;
- Treg cells – IL-10 and TGF-β.

Table 12. Examples of cytokines, producing cells, target cells and main functions

Cytokines	Produced by	Target cells	Main function
IL-1	Family of cytokines produced by macrophages, DCs, T cells, B cells, NK cells, vascular endothelial cells, fibroblasts and keratinocytes.	Th2 cells, B cells, NK cells, neutrophils, eosinophils, DCs, fibroblasts, endothelial cells and hepatocytes.	Pyrogenic, pro-inflammatory mediator, stimulator of Th2 cells and of bone marrow cell proliferation.
IL-2	Th1 cells and NK cells.	T cells, B cells and NK cells.	Activates Th, Tc and NK cells, and stimulates T cell proliferation and cytotoxicity.
IL-4	Th2 cells, mast cells and activated basophils.	T cells, B cells, mast cells, macrophages, endothelial cells and fibroblasts.	Stimulates growth and differentiation of B cells, Tc cells, expression of MHC class II and IgG and IgE production.
IL-5	Activated Th2 cells, mast cells and eosinophils.	T cells, B cells and eosinophils.	Promotes the differentiation of B cells into plasma cells and IgA and IgM production.
IL-6	Activated macrophages, T cells, B cells, mast cells, vascular endothelial cells, fibroblasts, keratinocytes and mesangial cells.	T cells, B cells, hepatocytes and bone marrow stromal cells as well as the brain.	Pro-inflammatory mediator, promotes the differentiation of B cells into plasma cells and IgG production.
IL-10	Mainly produced by Th2 cells but also by M2 cells, NK cells and some DCs.	Th1 cells, B cells, macrophages, NK cells and mast cells.	Immunosuppressive and anti-inflammatory cytokine that suppresses inflammation.
IL-12	Monocytes and macrophages, DCs, B cells and keratinocytes.	Major activator of Th1 cells and NK cells.	Pro-inflammatory mediator and NK cells activator.
IL-18	Member of IL-1 family and produced, like IL-1, by antigen-presenting cells.	Activates Th1 cells.	Promotes the production of IFN-γ, TNF-α, IL-1 and several chemokines, leading to positive feedback where IL-18 and IFN-γ reinforce each other's activities.
TNF-α	Macrophages, mast cells, Th1 cells, B cells, endothelial cells, adipocytes and fibroblasts.	Macrophages, mast cells, Th1 cells, NK cells, endothelial cells and neutrophils.	Pro-inflammatory mediator, central inducer of inflammation and phagocyte cell activator.
TGF-β	Platelets, T cells, B cells, neutrophils and activated macrophages.	Act on most cell types, including T and B cells, DCs, macrophages, neutrophils and fibroblasts.	Immunosuppressive cytokine that regulates cell division, inhibits T and B cell proliferation, enhances extracellular matrix proteins deposition and promotes wound healing.
IFN-γ	$CD4^+$ Th1 cells, by some $CD8^+$ T cells, and NK cells.	Acts on B cells, T cells, NK cells and macrophages.	Key mediator of cell-mediated immune responses.

Table adapted from information in Tizard (2012) and Turner et al. (2014).

Moreover, macrophages and other APCs, as well as damaged cells secrete chemotactic (chemokines) and pro-inflammatory cytokines to elicit the innate immune response to sites of active inflammation (Tarrant 2010). The process by which cytokines work is perfectly illustrated by the inflammatory response, where a cascade of cytokines of various types act sequentially and in parallel to develop an immune response, but also to prevent exacerbation of this response (Tarrant 2010). In this process, primary pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, are sequentially expressed and amplify cell activation and recruitment to generate additional cytokines and chemokines (Tarrant 2010). Anti-inflammatory cytokines, such as IL-10, are produced early to downregulate pro-inflammatory cytokines, while the expression of TGF-β contributes to resolution and tissue repair (Tarrant 2010). These cascades result in a staging of the appearance and disappearance of cytokines in the local and systemic environments. Deregulation of these cascades can lead to autoimmune disease and hypersensitivity.

The cluster of differentiation (CD) is a cell surface molecule that identifies a particular differentiation lineage recognized by a group of monoclonal antibodies (Lai et al. 1998; Brody 2016). For each unique molecule a different number has been assigned (*e.g.* CD1, CD2, CD3), allowing the identification of different subpopulations of immune cells according to the presence or absence of these markers (Table 13) (Lai et al. 1998; Actor 2014). There are more than 350 officially recognized CD molecules, many still have no known function, while others do not play a significant role in the immune system (Tizard 2012; Actor 2014). These molecules are widely used as cellular markers in techniques such as Flow Cytometry, in a process defined as immunophenotyping, for the identification of cell populations and their distribution. They are also used for measuring changes in the proportion of cells carrying these markers in patients with disease.

Other molecules used in immunophenotyping are transcription factors (Lafarge et al. 2007), such as nuclear factor kappaB (NF-κB), signal transducer and activator of transcription (STAT) and FoxP3 (Table 13), the latter which, together with CD25, is used as a biomarker to identify Tregs and to distinguish them from other types of lymphocytes (Shevach and Thornton 2014). Tregs are important for the health of the body, as they prevent an inadequate immune response against normal cells and subsequent autoimmunity (Taams et al. 2006).

Table 13. Examples of clusters of differentiation and transcription factor present in immune cells and their main role

		Expressed in	Role
Cluster of Differentiation	CD3	Only found on T cells.	Collective designation for the signal transducing molecules of the TCR.
	CD4	Helper T cells, thymocytes, and monocytes.	Co-receptor for MHC-II molecules that plays a key role in the recognition of processed antigens by helper T cells.
	CD8	Cytotoxic T cells.	Co-receptor for MHC-I molecules that plays a key role in the recognition of endogenous antigens by cytotoxic T cells.
	CD25	Activated T cells, B cells and monocytes. Feature of regulatory T cells.	The α chain of the IL-2 receptor, with a role in lymphocyte differentiation and activation.
	CD45	They are found on all cells of hematopoietic origin except red cells.	A pan-leukocyte marker from the family of tyrosine phosphatases, some of which are required for signaling through the TCR.
Transcription Factor	FoxP3	Regulatory T cells.	Transcription factor that activates a set of genes and converts the cell into a regulatory T cell that suppresses immune responses.

Table adapted from information in Lai et al. (1998) and Tizard (2012).

Immunophenotyping using CD molecules is a method that allows the detection and distinction of cell groups through a process of progressive selection (Fig. 16) (Naeim 2008).

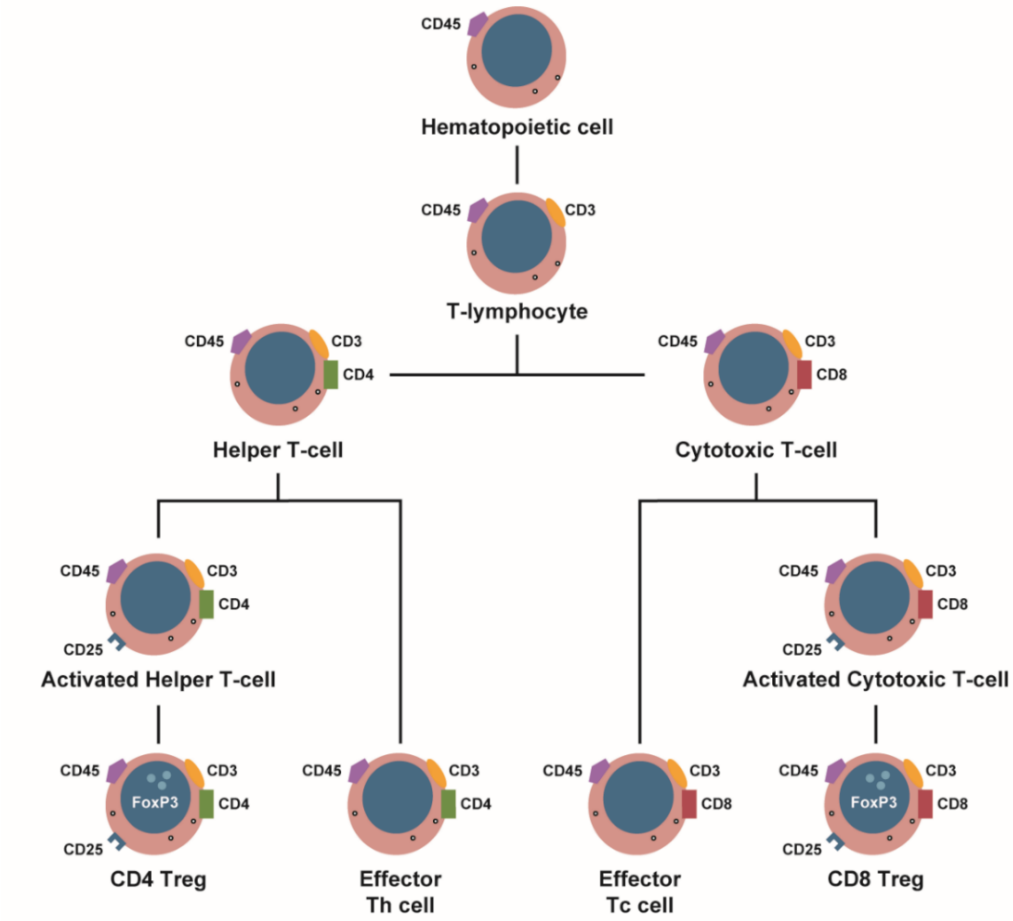

Figure 16. Immunophenotyping process of progressive selection of cell subsets

In this way, CD45, expressed in all hematopoietic cells (being considered a pan-leukocyte marker), allows the exclusion of red blood cells from the remaining cells of hematopoietic origin, with cells such as granulocytes, monocytes, B cells and T lymphocytes expressing CD45 in their membranes and being considered $CD45^+$ cells or leukocytes (Naeim 2008). Subsequently, CD3 is a pan-T-cell marker found only in T cells, which in turn are considered $CD45^+CD3^+$ cells, allowing the exclusion of the remaining cell types (Naeim 2008; Tizard 2012). In T cells, CD4 and CD8 allow for additional separation into $CD4^+$ ($CD45^+CD3^+CD4^+$ cells) and $CD8^+$ ($CD45^+CD3^+CD8^+$ cells), respectively (Tizard 2012). Within these two groups, CD25 and FoxP3 allow the distinction of two more subpopulations, those that express CD25 and FoxP3 and are considered Tregs ($CD45^+CD3^+CD4^+CD25^+FoxP3^+$ and $CD45^+CD3^+CD8^+CD25^+FoxP3^+$) (Shevach and Thornton 2014), and those that do not express CD25 and FoxP3 and are considered activated effector cells, either Th cells ($CD45^+CD3^+CD4^+CD25^-FoxP3^-$) or Tc cells ($CD45^+CD3^+CD8^+CD25^-FoxP3^-$).

2.4. Immune response against *L. infantum* infection

In CanL, as in many other infectious diseases, the first line of defense that the invasive promastigote forms of *L. infantum* encounter in dog skin are neutrophils (Tizard 2012). These cells resort to mechanisms, such as NETs release to capture parasites, exocytosis of granules with microbicidal agents and phagocytosis to try to contain the infection (Santos-Gomes et al. 2000; Peters et al. 2008; Borregaard 2010). Neutrophils parasitized by *Leishmania* appear to have their apoptotic death program delayed from the usual 6-12h cycle to up to 42h (Aga et al. 2002). This, together with the increased production levels of monocyte attracting chemotactic factors like MIP-1β, allows the recruitment of circulating monocytes, which infiltrate the canine dermis a few hours after the initial intradermal inoculation and differentiate into functional macrophages (Santos-Gomes et al. 2000). Parasites that escape or avoid being destroyed by neutrophils until their apoptosis can be readily engulfed by these macrophages through a classic receptor-mediated process, usually involving *Leishmania* and macrophage surface receptors (Sampaio et al. 2007; Peters et al. 2008; Pereira, Alexandre-Pires, et al. 2019). Otherwise, the delayed apoptotic death program in neutrophils allows an apparently temporary safe hideaway of the immune system (van Zandbergen et al. 2004). Through a process named efferocytosis, in which macrophages engulf infected neutrophils with their membranes still intact, the intracellular parasites have no direct contact with macrophage surface receptors and, consequently, there is no activation of the macrophage (van Zandbergen et al. 2004). This “Trojan Horse” mechanism silences the macrophage, induces the release of the anti-inflammatory cytokine TGF-β that promotes tissue repair, and no effector mechanisms are activated against the intracellular *Leishmania* that are free to differentiate into the amastigote form and replicate (van Zandbergen et al. 2004; Tizard 2012).

These early interactions between *Leishmania* parasites and APCs are what profoundly impacts the following adaptive immune response. To avoid being destroyed, the promastigote uses its surface glycolipid lipophosphoglycan (LPG) to inhibit the biogenesis of the phagolysosome, by alteration of the membrane’s fusogenic properties through periphagosomal accumulation of F-actin and disruption of phagosomal lipid microdomains (Desjardins and Descoteaux 1997). LPG also inhibits phagosome maturation, by impairing the assembly of NADPH oxidase that prevents the generation of ROS and the exclusion of the vesicular proton-ATPase in the early stages to allow differentiation of promastigotes into amastigotes (Moradin and Descoteaux 2012; Tizard 2012). Besides, *Leishmania* can also modulate the repertoire of cytokines secreted by infected macrophages and their ability to act like an APC, by suppressing the expression of MHC-II, preventing the adequate generation of the adaptive immune response (Cecílio et al. 2014; Martínez-López et al. 2018). Finally, once established, the amastigote forms rapidly divide within the macrophage phagolysosomes until

the cells rupture, releasing parasites that are then phagocytized by neighboring macrophages and DCs (Tizard 2012).

Depending on the capacity of the host's immune system, resistant dogs may be able to circumscribe parasites to the skin and remain either healthy or develop a mild, self-limited cutaneous disease, or infected DCs can migrate to the lymph nodes and enter the circulation and lodge in internal organs, such as the spleen and bone marrow, developing a disseminated visceral disease (Tizard 2012; Reguera et al. 2016). In resistant dogs, IL-12 production by APCs, such as DCs, is essential for the polarization of naïve $CD4^+$ T cells towards a Th1 subset and subsequent IFN-γ production alongside NK cells (Strauss-Ayali et al. 2005; Liu and Uzonna 2012; Rodrigues et al. 2016). IFN-γ activates infected macrophages into M1 cells (Figs. 17), which produce inducible nitric oxide synthase (iNOS), an enzyme that catalyzes L-arginine into NO, a toxic molecule essential for active killing of intracellular parasites like *Leishmania* (Nathan and Hibbs 1991; Liu and Uzonna 2012). In addition to the production of IFN-γ, Th1 response is also mediated by an increase in the production of IL-2, IL-12 and TNF-α by $CD4^+$ T cells (Strauss-Ayali et al. 2005). Given this predominant cellular immune response, these dogs usually exhibit a weak antibody response with low antibody titers against *L. infantum* (Pinelli et al. 1994; Rodríguez-Cortés et al. 2016).

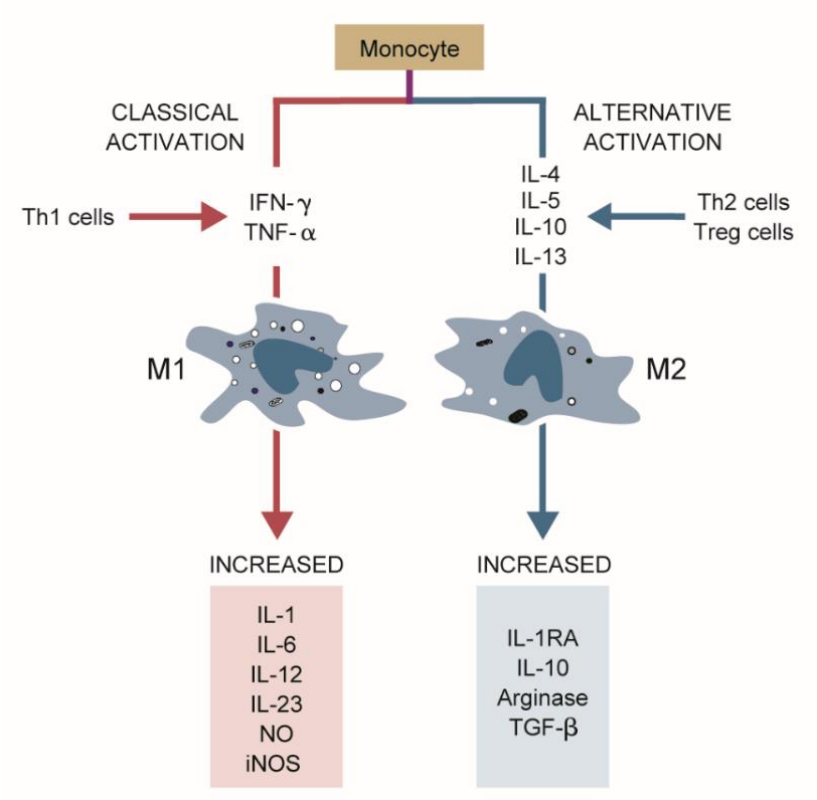

Figure 17. Macrophage activation pathways.
Depending on their cytokine exposure, macrophages can be classically activated (M1 cells) or become alternatively activated (M2 cells). M2 cells have an important regulatory role and are critical to granuloma formation and wound healing. These cells produce different combinations of cytokines. Adapted from Tizard (2012) .

In susceptible dogs, in contrast, the absence of IL-12 production by DCs, together with the production of IL-4, leads to the polarization of naïve $CD4^+$ T cells towards a Th2 subset and subsequent production of IL-4, IL-5, IL-10, IL-13 and TGF-β (Alexander and Bryson 2005; Tripathi et al. 2007; Hosein et al. 2017). These cytokines stimulate B cell proliferation and immunoglobulin secretion, but do not affect delayed hypersensitivity or other cell-mediated reactions (Tizard 2012). IL-4 promotes the growth and differentiation of B cells, IgG and IgE production and the inhibition of IL-2 and IFN-γ expression, while IL-5 promotes the differentiation of activated B cells into plasma cells (Tizard 2012). These cytokines, in turn, activate infected macrophages into M2 cells (Figs. 17 and 18), which produce arginase, an enzyme involved in proline (essential for the production of extracellular matrix by fibroblasts) and polyamine synthesis (required for cell proliferation), instead of NO, together with large quantities of IL-10, interleukin-1 receptor antagonist (IL-1RA) and TGF-β, resulting in a regulatory and anti-inflammatory profile, which favors the survival and growth of parasites (Bhattacharya and Ali 2013; Dayakar et al. 2019).

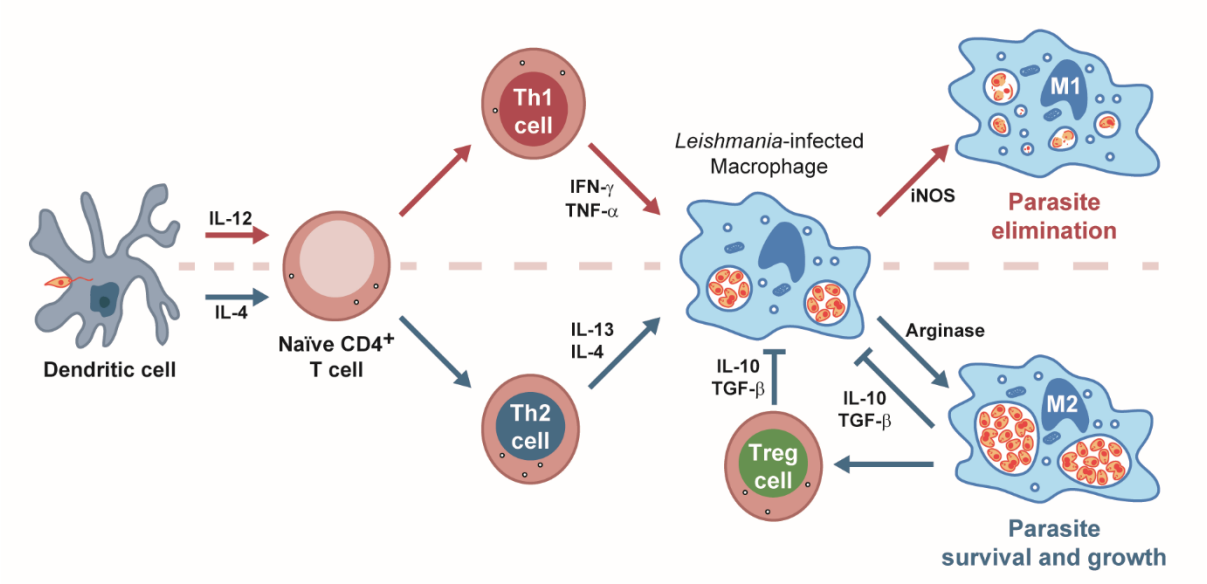

Figure 18. Role of dendritic cells and macrophages in the outcome of *Leishmania* infection. Following infection, macrophages and DCs phagocytize *Leishmania,* leading to different functional outcomes. Infected DCs produce IL-12, which is critical for the development of TNF-α and IFN-γ-producing $CD4^+$ Th1 cells. IFN-γ and TNF-α act on infected macrophages, leading to their activation (classical activation, M1), upregulation of iNOS, and production of nitric oxide and other free radicals that are important for intracellular parasite killing. In contrast, the production of IL-4 by other types of cells (including keratinocytes) supports $CD4^+$ Th2 development. Th2 cells produce IL-4 and IL-13, which leads to the upregulation of arginase activity, alternative macrophage activation (M2), and the production of polyamines that favor intracellular parasite proliferation. Besides, naturally occurring regulatory T cells (Treg) and infected macrophages also produce some immunoregulatory cytokines, including IL-10 and TGF-β, which further deactivate infected cells, leading to impaired parasite killing. Adapted from Liu and Uzonna (2012).

In these dogs, a progressive chronic disease develops, where highly parasitized macrophages accumulate and spread throughout the body, resulting in a widespread infection

(Rodrigues et al. 2016). Some of the clinical signs present in CanL are directly linked to this immune response (Tizard 2012), such as severe generalized nodular dermatitis, granulomatous lymphadenitis, splenomegaly and hepatomegaly. Excessive production of immunoglobulin can lead to hypergammaglobulinemia, lesions associated with type II and type III hypersensitivity, development of immune-mediated hemolytic anemia, thrombocytopenia and the production of antinuclear antibodies (Rodrigues et al. 2016). Chronic immune complex deposition can result in glomerulonephritis, uveitis and synovitis, leading to renal failure and death (Alvar et al. 2004).

From early studies that this Th1/Th2 dichotomy has been widely accepted (Sadick et al. 1986; Bretscher et al. 1992; Menon and Bretscher 1998), but since many of these studies have been and still are performed in mice with *L. major* infection, and considering that it is increasingly understood that human and canine leishmaniosis are far more complex, further research is needed (Hosein et al. 2017). For example, studies such as those by Kropf et al. (2003) demonstrated that the reported role of IL-4 in susceptibility to *Leishmania* infection by downregulating the Th1 response in mice may depend on the specific strain of *Leishmania*. Nevertheless, it is clear that the cytokine environment plays an important role in defining the immune response, with the polarization of whether a Th1 or Th2 immune response being influenced by cytokines during the first hours after infection (Sokol et al. 2008; Cummings et al. 2010).

Considering more recent studies, the dog's immune response against leishmaniosis is more similar to human infection (Hosein et al. 2017) with initial studies describing a Th1 protective cell-mediated immune response with production of IL-2, IFN-γ and TNF-α, and the active disease being characterized by a mixed Th1/Th2 response (Santos-Gomes et al. 2002; Carrillo and Moreno 2009).

However, many of these studies were only performed in peripheral blood and further reports indicate that the immune response to *Leishmania* is in fact organ-specific (Reis et al. 2009), with Th1, Th2 or mixed Th1/Th2 immune responses being observed in different organs of dogs with CanL. These results showed that the cytokine environment and the phenotypic cell profiles involved in the immune response, in the different compartments where parasites are known to replicate, have variable effects on local parasite control, highlighting the complexity of the cellular immune responses in *L. infantum* infection (Hosein et al. 2017).

2.5. Compartmentalized immunity in CanL

Given the extensive systemic profile of CanL and the presence of *L. infantum* in various organs of the dog, including skin, lymph nodes, bone marrow, liver and spleen, there have been more and more studies showing differences in the immune response between each compartment (Hosein et al. 2017; Giunchetti et al. 2019). Nevertheless, there has been much debate in the scientific community about the specific immune responses in each organ to *L. infantum* infection, with different studies showing conflicting results without reaching consensus. Furthermore, since many of these studies use different methods, as well as *L. infantum* infected dogs at different stages, it is difficult to compare and define an overall pattern.

2.5.1. Skin

Although the skin is essential for the natural transmission of *L. infantum* through phlebotomine sand flies, there is limited data on the cytokine profile and cell populations present in this tissue in CanL. According to Brachelente et al. (2005), the cytokine environment in the skin of naturally infected dogs seems to be defined by the severity of clinical signs and parasitic burden, with *Leishmania*-infected dogs presenting a mixed Th1/Th2 response with high expression of TNF-α, IFN-γ and IL-4 when compared to healthy dogs. In turn, increased expression of IL-4, IL-13 and TNF-α, leading to a Th2-biased humoral immune response, was present when plasma cells outnumbered T lymphocytes in the dermal infiltrate (Brachelente et al. 2005). In another study (Menezes-Souza et al. 2011), a similar profile of mixed cytokines was reported, with high levels of expression of IL-13, TNF-α and IFN-γ, together with the transcription factors GATA-3 and FoxP3, being highly expressed in asymptomatic dogs. Increased levels of IL-10 and TGF-β1 associated with low expression of IL-12 were also observed in dogs with high skin parasitism, possibly representing a key condition that allows persistence of parasite replication in this tissue (Menezes-Souza et al. 2011). In a study with experimentally infected dogs, Rodríguez-Cortés et al. (2016) reported that, although there was no significant expression of cytokines in the skin of these animals 6 months after *L. infantum* inoculation, after 16 months a mixed pro-inflammatory/regulatory immune response with increased expression of IFN-γ, TNF-α, IL-10 and TGF-β was recorded together with increased parasite load. These results show that there seems to be a “silent phase” in the skin, where parasite invasion occurs without disturbing cytokine expression, allowing the parasite to survive and establish itself in the dermis (Rodríguez-Cortés et al. 2016), similar to *L. major* infection in mice (Belkaid et al. 2000). These findings also explain why clinically healthy and naturally infected dogs are still infectious to sand flies (Molina et al. 1994).

With regard to cellular immunophenotyping of the skin in CanL, few studies are available. Moreira et al. (2018) observed in the skin of naturally infected dogs with low parasitic burden, and without external signs compatible with CanL, high density of $CD4^+$ and $CD8^+$ T cells, while symptomatic dogs showed increased $CD4^+$ T cells. Furthermore, another study by the same authors, reported the maintenance of M2 macrophages in the skin of *Leishmania*-infected dogs, favoring the parasite multiplication in this tissue (Moreira et al. 2016). Rossi et al. (2016), in turn, reported an unspecific chronic inflammatory infiltrate in the superficial dermis of dogs with clinical signs, characterized by the presence of increased numbers of macrophages, T lymphocytes ($CD4^+$ and $CD8^+$ T cells) and iNOS-producing cells. Fondevila et al. (1997) reported that dogs with alopecic dermatitis seem to develop an effective control of infection with presence of Langerhans cells and MHC-II^+ keratinocytes, both APCs, associated with mild T cell infiltration and without a significant number of parasites. On the contrary, dogs with generalized nodular disease appear to mount an impaired immune response, with a lack of the previous APCs and massive infiltration of macrophages and parasites in the dermis (Fondevila et al. 1997). Lastly, according to Papadogiannakis et al. (2005) in the skin of dogs suffering from exfoliative dermatitis, $CD8^+$ T cells outnumbered $CD4^+$ T cells, MHC-II expression in epidermal keratinocytes was increased and $CD45RA^+$ (naïve cells) and $CD21^+$ (complement receptor type 2, CR2) cells were also present in high numbers.

2.5.2. Peripheral blood

The peripheral blood, despite being the most analyzed tissue, is also the one with the most contradictory results. Initial studies with experimentally infected dogs reported that, 3 years after experimental infection, the asymptomatic dogs (considered as resistant dogs) responded to *L. infantum* antigen in *in vitro* lymphocyte proliferation assays and in delayed-type hypersensitivity reactions, without production of anti-*Leishmania* antibodies (Pinelli et al. 1994). On the other hand, symptomatic dogs (considered as susceptible dogs) failed to respond to the parasite's antigen in both *in vitro* and *in vivo* cell-mediated assays and had higher serum antibodies against *Leishmania* (Pinelli et al. 1994). Lastly, these authors found significantly higher levels of IL-2 and TNF-α in stimulated peripheral blood mononuclear cells (PBMC) supernatants from asymptomatic dogs compared to those from symptomatic and control uninfected dogs, while IL-6 showed no significant difference between groups (Pinelli et al. 1994). On the contrary, de Lima et al. (2007) found in sera from dogs with the active disease an increase in systemic IL-6 production when compared to healthy dogs, while TNF-α showed no significant difference between the two groups. Another study by Pinelli et al. (1995) reported in the following year that PBMC from experimentally infected asymptomatic dogs produced IFN-γ after specific stimulation with the parasite antigen, while lymphocytes from symptomatic

dogs did not. Chamizo et al. (2005) reported that T lymphocytes from experimentally infected asymptomatic dogs expressed IFN-γ, IL-2, TNF-α, IL-18 and IL-10 levels similar to uninfected dogs, while almost no expression of IL-4 was detected when compared to control dogs. They also observed that PBMC *in vitro* stimulation with SLA greatly induced the expression of IFN-γ and IL-2, along with some increase in TNF-α, IL-18, IL-4, IL-6 and IL-10, revealing a mixed Th1/Th2 immune response (Chamizo et al. 2005). These authors propose that, although both Th1 and Th2 cytokines were produced in asymptomatic *Leishmania*-infected dogs, there was a predominant Th1 cytokine response that conferred immunity to the parasite. Manna et al. (2006) showed that, although initially naturally infected asymptomatic dogs, who later developed symptoms, did not show significant cytokine expression beyond IL-18, six months later with the onset of clinical signs, their cytokine profile developed into a mixed Th1/Th2, with significant expression of IL-2, IL-4 and IL-10, and some expression of IFN-γ, IL-12 and IL-18. On the other hand, early observations on asymptomatic dogs without clinical signs for a prolonged time, showed a Th1 response mediated by IL-2, IFN-γ and IL-18, which six months later presented additional expression of IL-4 and IL-10 (Manna et al. 2006). In a study using an amastigote antigen from *L. pifanoi*, the P-8 proteoglycolipid complex (P-8 PGLC), the authors showed that it was able to induce the up-regulation of IFN-γ and TNF-α in asymptomatically infected dogs three to four times higher than that induced by SLA (Carrillo et al. 2007). When measurable induction of IL-10 and IL-18 was not observed, low levels of IL-4 mRNA were found in response to both P-8 and SLA antigens, establishing that both antigens can elicit a potential protective Th1-like immune response in asymptomatic infected dogs (Carrillo et al. 2007). Following some of these studies, it is possible to observe that, while for some authors IL-6 (Chamizo et al. 2005; de Lima et al. 2007) and IL-18 (Chamizo et al. 2005) constitute markers of active disease or asymptomatic infection, for others IL-6 (Pinelli et al. 1994) and IL-18 (Manna et al. 2006; Carrillo et al. 2007) have no apparent role.

In a study using experimentally infected dogs (Travi et al. 2009), the authors observed that, in the early stages of infection, 67% of symptomatic dogs produced high levels of IFN-γ in the blood, with the quantity of dogs producing this cytokine increasing over time, revealing that IFN-γ production seemed to be insufficient to prevent disease. Moreover, both asymptomatic and symptomatic dogs produced IL-10, but the latter tended to produce more of this cytokine (Travi et al. 2009). Another study in experimentally infected dogs by Sanchez-Robert et al. (2008) reported similar results, where a significant increase in IFN-γ was associated with an increase in parasite load and the symptomatic clinical status. They also showed that, while these symptomatic dogs showed some expression of IL-4 and IL-13 in the first four months after infection, the asymptomatic group showed no expression of these cytokines. In contrast, Barbosa et al. (2011) reported that asymptomatic dogs showed high gene expression of IL-4 and low IFN-γ and IL-2, while symptomatic dogs presented a mixed

Th1/Th2 cytokine profile with the expression of IL-12, IL-2, IFN-γ and IL-4. Another study also described a mixed Th1/Th2 cytokine profile (Panaro et al. 2009) in both asymptomatic and symptomatic naturally infected dogs, which expressed TNF-α, IFN-γ, IL-4 and IL-10. These cytokine mRNA levels presented a significant increase in symptomatic dogs, 8 months after the initial diagnosis. Following the growing reports of CanL in the United States, a study in a foxhound population found that disease progression was correlated with decreased proliferative response, accompanied by decreased production of IFN-γ and increased IL-10 release, and consistent detection of parasite kDNA in whole blood (Boggiatto et al. 2010).

In a longitudinal study using experimentally infected dogs, Santos-Gomes et al. (2002) observed a distinct temporal pattern during *L. infantum* infection. A long initial phase of pre-patent infection (8 months) in which dogs were asymptomatic, with low cytokine expression by both non-stimulated and stimulated cells, revealing a ‘‘silent establishment’’ of the parasite. Followed by a short pre-patent phase, in which dogs remained asymptomatic, but presented increased expression of IFN-γ and IL-2 and low IL-6 and IL-10, and, finally, a patent phase, where dogs showed clinical signs and reduced expression of cytokines. Across these phases, dogs maintained specific humoral immune responses, general abrogation of specific lymphocyte proliferation to parasite antigen and the presence of parasites in the skin, showing that dogs were able to transmit the parasite (Santos-Gomes et al. 2002). Lastly, the observation of a relatively long ‘‘silent’’ period, without induction of host cell-mediated immunity, nor development of pathology, and during which parasite multiplication occurred, has also been reported in mice infected with *L. major* (Belkaid et al. 2000).

The absence of a cell-mediated immunity appears to be a key aspect in the establishment of *L. infantum* infection, with $CD4^+$ T cells representing the central cell fraction in the development of a protective response. From initial studies, dogs naturally infected with active leishmaniosis have been found to have a significantly lower presence of peripheral blood $CD4^+$ T lymphocytes than healthy dogs (Bourdoiseau, Bonnefont, Magnol, et al. 1997). Moreover, some authors confirmed that the loss of $CD4^+$ T-cells is a process that begins soon after infection and continues during the incubation period (Alvar et al. 2004). Through direct xenodiagnoses of infected dogs, Guarga et al. (2000) were able to observe a significant association between their infectious capacity and the percentage of helper T cells ($CD4^+TcR\alpha\beta^+$ and $CD4^+CD45RA^+$), in which the lower the $CD4^+$ T cell count, the greater the infection rate in the vector. Bourdoiseau, Bonnefont, Hoareau, et al. (1997) reported a striking reduction in B ($CD21^+$) and T ($CD4^+$ and $CD8^+$) cells in symptomatic dogs in comparison to asymptomatic dogs, with drug therapy being able to restore these subsets of cells. Pinelli et al. (1995) observed that PBMCs stimulated by *Leishmania* antigen from experimentally infected asymptomatic dogs were able to lyse infected macrophages via $CD8^+$ T cells, through MHC, while PBMCs from symptomatic dogs did not proliferate and were unable to lyse infected

macrophages. However, they also reported that some asymptomatic dogs exhibited $CD4^+$ T cells that lysed infected macrophages (Pinelli et al. 1995). Other studies reported an increased number of $CD4^+$ and $CD8^+$ T cells as a predominant feature in asymptomatic dogs, while $CD4^+$ and $CD8^+$ T cells were reduced in symptomatic dogs (Reis et al. 2006; Reis et al. 2009; Coura-Vital et al. 2011). On the contrary, Cortese et al. (2013) described a significant increase of $CD3^+CD8^+$ T lymphocytes in the blood of dogs with active CanL, in the presence of normal levels of T lymphocytes and regardless of the IFAT titer or the presence of clinical signs of disease. These authors also refer to the reduced percentage of $CD3^+CD4^+Foxp3^+$ T regulatory cell subset that could be enabling the increased level of $CD8^+$ T cells and IFN-γ^+IL4^- producing lymphocytes (Cortese et al. 2013). In another study performed by the same group (Cortese et al. 2015) on the effect of an immunomodulatory diet in CanL, the authors found a decreased CD4/CD8 ratio in the blood of symptomatic dogs, associated with a significant increase of $CD8^+$ T cells, along with a decreased percentage of $CD3^+CD4^+Foxp3^+$ Tregs. Furthermore, Papadogiannakis et al. (2010) reported a significant decrease in circulating $CD4^+$ T cells in sick dogs, together with increase of $CD8^+$ T cells, which resulted in a decrease in the CD4/CD8 ratio. Some authors also describe a decline in $CD3^+$ lymphocytes in PBMCs of CanL symptomatic dogs, as a direct consequence of reduction of $CD4^+$ T cells (Moreno et al. 1999; Alexandre-Pires et al. 2010), while other authors, on the contrary, have found a significant increase in $CD3^+$ and $CD4^+$ T cells in sick dogs, especially in dogs considered severely affected (Miranda et al. 2007). Another study carried out in the blood of naturally infected dogs found no correlation between the percentage of $CD4^+$ Tregs, producing TGF-β or IL-10, and the parasitic load (Silva et al. 2014). Lastly, a study in a USA foxhound population found that *L. infantum* infection led to significant $CD8^+$ T cell exhaustion, along with increased surface expression of Programmed Death 1 (PD-1), occurring before the onset of symptomatic disease, followed by $CD4^+$ T cell depletion, decreased IFN-γ production and increased IL-10 production (Esch et al. 2013). Antibody block of PD-1 ligand, B7.H1, significantly allowed for the recovery of $CD4^+$ and $CD8^+$ T cell proliferation and IFN-γ production by $CD4^+$ T cells in response to *L. infantum* antigen, together with reduced presence of IL-10 in cell culture supernatants (Esch et al. 2013).

Following all these contradictory results between the studies, with a wide range of cytokines and blood cell populations being reported in dogs with CanL, the concept of different phases in *L. infantum* infection with different patterns of immune response seems to be highly present in blood, and considering that this is not the tissue of choice for parasite replication and persistence, the difficulty of reaching consensus is understandable.

2.5.3. Lymph node

In popliteal lymph nodes, a predominant pro-inflammatory environment has been reported as resistance to *L. infantum* infection, with asymptomatic dogs presenting high expression of pro-inflammatory cytokines, such as IFN-γ and TNF-α, associated with low parasitic burden (Alves et al. 2009). In the same study, the regulatory cytokines IL-10 and TGF-β were, in turn, correlated with high parasite burden and disease progression. Rodríguez-Cortés et al. (2016) reported in experimentally infected dogs the up-regulation of IFN-γ in the lymph node six months after infection, with an additional increase of this cytokine, as well as IL-10 and TGF-β, 16 months after infection, which correlated positively with increased parasitic load and clinical score. These authors associate the positive regulation of these anti-inflammatory/regulatory cytokines in the lymph node at a later time (16 months after inoculation) with the possible peripheral nature of this organ, since after intravenous administration of the parasite, this organ is invaded later than organs such as the liver, spleen and bone marrow. These findings suggest that the spread of *L. infantum* follows a sequential compartmentalized pattern, in which lymphoreticular organs reach higher burdens in the earlier stages of the infection than the lymph node and the skin (Travi et al. 2001). Another study by Barbosa et al. (2011) reported high expression of genes encoding the pro-inflammatory cytokines IL-2 and IL-12 in asymptomatic dogs, while symptomatic dogs showed high gene expression of IL-2 and TNF-α. Other studies present a mixed response, with the lymph node of experimentally infected dogs showing a balance between TNF-α and IL-10, in association with low parasite burden and absence of clinical signs (Maia and Campino 2012).

Regarding cell populations, Alexandre-Pires et al. (2010) demonstrated that $CD8^+$ T cells in the lymph nodes of treated dogs were significantly lower than in asymptomatic untreated dogs. Besides, these authors reported that in both treated and asymptomatic dogs, the $CD4^+$ T cell subset was significantly higher than in uninfected control dogs. Giunchetti et al. (2008), in turn, reported that in the lymph node of dogs with CanL, $CD8^+$ T cells are present in greater concentration compared to uninfected animals, with the highest levels of $CD8^+$ T cells being present in animals with the utmost skin parasite load, which led to the hypothesis that $CD8^+$ T cells may be involved in a distinct activation status and are probably associated with immunomodulatory or suppressor cell activity. These findings possibly indicate that an increase in lymph node $CD8^+$ T cells is associated with parasite persistence, while $CD4^+$ T cells expansion favors a protective response and parasite control.

2.5.4. Bone marrow

Bone marrow aspirates of naturally infected dogs presented high expression of IFN-γ and IL-4, IL-10 and IL-18 when compared to uninfected animals (Quinnell et al. 2001). Only dogs with severe clinical signs showed detectable IL-4 mRNA levels, revealing a relationship between this cytokine and disease severity. In a study performed on experimentally infected dogs, Rodríguez-Cortés et al. (2016) reported that the bone marrow of these dogs developed a predominantly pro-inflammatory environment, namely due to IFN-γ and TNF-α, with high parasite load, but low detection of IL-10 and TGF-β. Another study reported a mixed pattern of pro-inflammatory, namely TNF-α, and regulatory cytokines such as IL-10 and TGF-β, with increasing presence of iNOS in the bone marrow of experimentally infected asymptomatic dogs (Maia and Campino 2012). A study by Barbosa et al. (2011), in turn, did not report significant mRNA accumulation of IL-2, IL-12, IFN-γ and IL-4 cytokines in either asymptomatic or symptomatic dogs, suggesting the absence of a specific immune response against *Leishmania*. Treated dogs, on the other hand, revealed an increased expression of IL-12 mRNA (Barbosa et al. 2011). Silva et al. (2019) reported higher expression of TNF-α and IL-4 in naturally infected dogs when compared with the healthy control group, but there were no significant differences for IL-2, IL-10, IL-17 and IFN-γ. They also showed that dogs with severe CanL showed higher expression of TNF-α and IL-6.

Considering cell populations, Alexandre-Pires et al. (2010) reported that symptomatic and asymptomatic animals exhibited a significant increase in MHC-II expression in bone marrow lymphocytes, reflecting a possible presentation of *L. infantum* antigens. Subsequently, treated dogs showed increased MHC-II expression in lymphocytes and monocytes, pointing towards an increase in antigen presenting activity, probably due to the availability of parasitic antigens as a consequence of treatment. No significant differences were observed in $CD4^+$ and $CD8^+$ T cell populations in the bone marrow of sick and treated dogs, with the authors arguing that infection control in this tissue could be unrelated to the expansion of these cell subsets.

2.5.5. Liver

Studies on the cytokine profile in the liver of dogs with CanL are considerably scarce, especially given the intrinsic need for liver biopsy or even dog euthanasia, in order to measure the presence of cytokines. Initial studies reported an increase in the production of IFN-γ, IL-10 and TGF-β in the liver of naturally infected asymptomatic dogs, while symptomatic dogs had only an increase of IL-10 concentration (Corrêa et al. 2007). Considering that IL-10 and TGF-

β were present in particularly higher amounts than IFN-γ, the authors suggested that a predominant Treg immune response was present in the liver of infected dogs (Corrêa et al. 2007). A major shortcoming in this study is the lack of a control group of healthy animals to establish what normal, decreased or increased values are to be noted. In a study with experimentally infected dogs (Maia and Campino 2012), liver cells expressed some levels of iNOS, IL-10 and TGF-β and a particularly low expression of IFN-γ, without expression of TNF-α. These results are similar to those by Corrêa et al. (2007), but, like these, there was no control group to correctly assess this immune response. In liver cells of naturally infected dogs, Michelin et al. (2011) found that TNF-α and IL-4 levels were increased in asymptomatic and symptomatic dogs in comparison to healthy dogs, with IL-10 levels being also increased and showing a linear correlation with the level of parasite load in the liver. Nascimento et al. (2015) documented that in naturally infected dogs, disease progression was characterized not only by the downregulation of Th1-related cytokines (IFN-γ and TNF-α), but also of genes encoding IL-17A, iNOS and IL-10 in the liver of symptomatic dogs compared with asymptomatic dogs. And since IL-17A gene transcription level was positively correlated with mRNA expression of iNOS and IFN-γ, the authors considered that Th1 and Th17-related cytokines appear to play a role in restricting parasite growth via iNOS activation in this organ (Nascimento et al. 2015). Moreover, a study using experimentally infected dogs found that in liver samples down regulation of transcription was present for IL-22, an inflammatory cytokine with a controversial and poorly defined role in *Leishmania* infection (Hosein et al. 2015). In a study by Rodríguez-Cortés et al. (2016), experimental infection of dogs with *L. infantum* led to a mixed Th1/Th2 immune response in the liver, with a significant increase in IL-10 and IFN-γ, the latter correlated with both parasite load and *Leishmania*-specific IgG and IgA antibody levels. In a recent study by Vasconcelos et al. (2019), the authors highlighted that the hepatic tissue presented high expression of IFN-γ, IL-4, IL-6 and IL-10 associated with high parasite loads, but no expression of IL-12 or iNOS.

In terms of the cellular immune response, Pinho et al. (2016) observed that there was a slight predominance of Kupffer cells in asymptomatic dogs and of granulomas in the liver of CanL symptomatic dogs, but with similar proportions of $CD4^+$ and $CD8^+$ T cells, with the role of these cells being apparently unrelated to the clinical status of the dogs. In another study by Moreira et al. (2018) the liver presented the lowest parasitic load, along with low proportion of $CD4^+$ and $CD8^+$ T cells, with asymptomatic dogs showing higher number of $CD8^+$ T cells than symptomatic dogs.

2.5.6. Spleen

Just like in the case of the liver, there are few studies evaluating the cytokine profile and cellular immune response of the spleen in CanL. Corrêa et al. (2007) reported an increase in the production of IL-10 and TGF-β in the spleen of naturally infected asymptomatic dogs, while symptomatic dogs showed an important presence of IFN-γ along with particularly higher amounts of IL-10 and TGF-β, suggesting a predominant Treg immune response in this organ (Corrêa et al. 2007). Again, one of the major shortcomings of this study is the lack of a healthy dog control group to properly compare the results. In another study by Strauss-Ayali et al. (2007), cytokine expression in the spleen revealed an initial elevation of IL-4 one month after infection, followed by IFN-γ increase in both experimentally and naturally infected dogs. No significant changes were recorded for IL-12, TNF-α, IL-5, IL-10 and TGF-β during infection, with only the latter increasing at later stages (Strauss-Ayali et al. 2007). In turn, Lage et al. (2007) found an increase in IL-12 in the spleen of dogs with CanL, along with a positive correlation between the expression of IL-10 and disease progression, as well as a correlation between IFN-γ and increased parasitic load, suggesting a balanced Th1/Th2 immune response in this tissue upon *Leishmania* infection. In a study with experimentally infected dogs (Maia and Campino 2012), spleen cells expressed increased levels of iNOS and IL-10, with some expression of TGF-β and IFN-γ, but no expression of TNF-α. These results are similar to that reported by Corrêa et al. (2007), but still, like these, there is no control group to correctly assess this immune response. Another study by Michelin et al. (2011) reported that the level of splenic TNF-α correlated with the parasite load, and could represent a marker of infection evolution along with IL-10. Cavalcanti et al. (2015), in turn, reported that in naturally infected dogs parasites caused the breakage of splenic architecture, which resulted in a negative correlation with pro-inflammatory (IFN-γ, IL-2 and IL-6) and anti-inflammatory cytokines (IL-10 and TGF-β). Also in naturally infected dogs, Nascimento et al. (2015) reported that CanL progression was characterized by the down regulation of IFN-γ, IL-10, IL-17A and iNOS in the spleen of symptomatic dogs when compared with asymptomatic dogs. Spleen cells of experimentally infected dogs, in turn, showed significant down regulation of IL-22 transcription with disease progression (Hosein et al. 2015). Rodríguez-Cortés et al. (2016) observed a mixed Th1/Treg immune response in the spleen of experimentally infected dogs, with a significant increase in TGF-β and IFN-γ that correlated with parasite load. In the latest study, Vasconcelos et al. (2019) emphasize the high expression of IFN-γ, IL-6 and IL-4, along with no expression of IL-12 and iNOS, in the spleen of highly parasitized dogs. The authors also refer that granulomas were detected in this organ, but when absent, they were associated with increased IL-6 levels, pointing to an anti-inflammatory role for this cytokine.

Regarding the cellular immune response in the spleen of sick dogs, Moreira et al. (2018) reported that while the spleen of naturally infected symptomatic dogs showed the highest levels of parasite DNA, it also had significantly reduced levels of $CD4^+$ and $CD8^+$ T cells. Likewise, da Silva et al. (2018) reported a decrease in the amount of $CD4^+$ lymphocytes in the spleen, with the splenic white pulp microarchitecture evidencing disorganization, possibly preventing the migration of these $CD4^+$ T cells to their specific compartments within the white pulp. Lastly, Silva et al. (2014) verified that, although there was no significant difference in the percentage of $CD4^+FoxP3^+IL\text{-}10^+$ cells between infected and controls dogs, an increase in IL-10 production by these cells was present in the spleen of naturally infected dogs. Concurrently, there was a decrease in the total number of T cells in these dogs compared to healthy dogs, with no association being determined between parasite load and the percentage of spleen Treg cells producing IL-10 and TGF-β (Silva et al. 2014).

2.6. Effect of the main antileishmanial drugs in the dog's immune system

Considering the limited data on the cytokine and cellular immune profile in dogs with CanL, there are fewer reports on the effects of CanL treatments on the dog's immune response, with the following studies representing most of the available information. However, as many do not test the direct effect of these drugs on cell populations, the question remains: do these changes occur due to the direct action of the drugs or as a consequence of the death of *Leishmania* parasites and the dog's "natural" immune response?

2.6.1. Pentavalent antimonials

Several studies have pointed out the effects of pentavalent antimonials, many in human and mice models, such as sodium antimony gluconate (SAG), which seemingly interferes with the host's immune system by activating macrophages, through induction of the expression of MHC-I molecules, probably stimulating $CD8^+$ T cells that can induce the apoptosis of infected cells (Haldar et al. 2011; Passero et al. 2018). This drug also seems to promote the generation of ROS, such as NO, in order to cause oxidative damage, by driving the production of IL-12 and, subsequently IFN-γ which in turn activates macrophages, that through the induction of extracellular signal-regulated kinase 1 (ERK-1) and ERK-2 phosphorylation leads to the production of ROS (Basu et al. 2006) and parasite death. Following these and other studies, pentavalent antimonials, although directly microbicidal in both *in vitro* and *in vivo*, have failed to treat visceral leishmaniosis in human patients who are also infected with HIV or receiving immunosuppressive therapy (Haldar et al. 2011), with a complete cure being dependent on a Th1 response by T cells (Murray et al. 1989; Murray et al. 1991).

Concerning meglumine antimoniate and its effects on the dog's immune response, there are only a few reports. Vouldoukis et al. (1996) observed that macrophages from CanL dogs successfully treated with meglumine antimoniate seemed to be capable of inducing antileishmanial activity via IFN-γ in the presence of autologous lymphocytes, along with induction of NO synthase pathway. In dogs treated with a combination of meglumine antimoniate and allopurinol, Barbosa et al. (2011) observed an increased expression of IL-12 mRNA in lymph node and bone marrow, revealing a possible involvement in the activation of macrophages and an increase in their microbicidal activity in these tissues. Martínez-Orellana et al. (2017) found an increased IFN-γ concentration in stimulated blood cells of naturally infected dogs that were under long-term treatment with meglumine antimoniate and allopurinol. Moreover, the expression of MHC-II by monocytes in the lymph node and bone marrow of dogs was reported as being significantly increased after treatment, probably reflecting a rise in the presentation of *Leishmania* antigens (Alexandre-Pires et al. 2010). In the same study, treated dogs also showed an expansion of $CD4^+$ T cells subpopulations in the lymph nodes, revealing an important contribution of these cells in controlling local parasite replication. Several authors also refer a significant increase in the percentage of $CD4^+$ lymphocytes after treatment with meglumine antimoniate, when compared to healthy dogs (Moreno et al. 1999; Miranda et al. 2007). Other authors, in turn, while observing a reduced count of $CD4^+$ T cells in the peripheral blood of sick animals, reported that this T cell population returns to normal values after treatment with meglumine antimoniate (Bourdoiseau, Bonnefont, Hoareau, et al. 1997), but with no further increase beyond normal values.

Altogether, these findings indicate that antimonial drugs appear to have multifactorial activity, directly influencing the parasite or, on the other hand, indirectly affecting the parasite's survival by modulating the host's immune response (Passero et al. 2018).

2.6.2. Miltefosine

Several studies have reported the immunomodulatory properties of miltefosine, with *in vitro* studies showing the induction of TNF-α and NO release by peritoneal macrophages from BALB/c mice (Zeisig et al. 1995) and enhancement of IFN-γ receptors, thus restoring responsiveness to IFN-γ in *L. donovani*-infected macrophages and promoting a IL-12 dependent Th1 response (Wadhone et al. 2009). From experiments on healthy human PBMCs, it was found that miltefosine was able to enhance the production of IFN-γ as long as IL-2 was added exogenously, acting as a co-stimulator of the IL-2-mediated T cell activation process, along with increased expression of CD25 (α chain of the IL-2 receptor) and HLA-DR (human MHC-II cell surface receptor), evidencing the possible immunomodulatory activity of miltefosine (Vehmeyer et al. 1991). Other authors also report miltefosine as being capable of

enhancing the immune response of human IL-2-stimulated mononuclear cells, resulting in increased IFN-γ gene expression and production (Hochhuth et al. 1992), in addition to induced MHC-I production in human monocytes (Eue 2002). In turn, in a study in *L. major*-infected mice (Griewank et al. 2010), it was shown that, while miltefosine is able to eliminate the parasite, it did not up-regulate MHC-II or any costimulatory molecules that influence the maturation of DCs, nor did it alter the release of IL-10, IL-12 or TNF-α cytokines. In mice models, miltefosine does not appear to require T cell-dependent immune mechanisms in order to act (Murray 2000), indicating that this drug may be used in cases of T cell deficiency (N. Marques et al. 2008; Haldar et al. 2011).

In dogs, the immunological effects of treatment with miltefosine have been reported in only two studies. Manna, Reale, Picillo, et al. (2008) observed an increased IFN-γ expression in the peripheral blood of dogs during miltefosine and combined treatment. In Brazil, Andrade et al. (2011) found in the peripheral blood of naturally infected dogs that IFN-γ levels tended to increase during the follow-up period, while IL-4 and IL-10 levels showed a decrease, regardless of the miltefosine dose administered. However, while these authors reported a significant reduction in parasite load after 3 months, 6 months after treatment the animals relapsed with a progressive increase of parasitic burden and recurrence of anti-inflammatory cytokine production (IL-4 and IL-10), showing that in this case treatment with miltefosine did not result in parasite clearance (Andrade et al. 2011).

Taken together, these findings suggest that miltefosine appears to induce a general activation of Th1 cytokines, particularly represented by the increase in IFN-γ and IL-12 (Palić et al. 2019). However, more studies are needed to clarify the effects of this drug in CanL.

2.6.3. Allopurinol

Although allopurinol is sometimes administered in monotherapy, even in dogs (Vercammen and de Deken 1995), the effectiveness of this drug has been questioned, especially since this compound does not exert a therapeutic effect like meglumine antimoniate or miltefosine (Miró et al. 2011; Miró and López-Vélez 2018). Therefore, there are only a couple of studies on the immune response following treatment with allopurinol alone. Strauss-Ayali et al. (2007) who observed that initially high levels of IFN-γ after experimental infection decreased significantly in the spleen of dogs after treatment. In turn, in a study using naturally infected dogs, Papadogiannakis et al. (2010) reported that treatment with allopurinol in monotherapy improved the number of circulating $CD4^+$ T cells, but did not restore their number within the normal range. This may explain the ineffectiveness of allopurinol monotherapy, leading to the conclusion that dogs with CanL receiving prolonged allopurinol monotherapy may present a risk of infectivity to sand flies (Papadogiannakis et al. 2010).

3. OBJECTIVES

In canine leishmaniosis, the dog's immune response is a central point around which the ability to overcome the infection is centered. Allied to this, is the administration of several treatment protocols that aim to help reduce the parasitic burden and allow the immune system to fully act. Unfortunately, many of the dog's immune response mechanisms to *L. infantum* infection are not yet fully known, in the various organs in which the parasite is present, as well as whether the action of these treatment protocols results in any change in this response. On this note, the main objectives of the present study were to evaluate:

1. The gene expression of pro-inflammatory (IL-2, IL-12, TNF-α and IFN-γ), anti-inflammatory (IL-4 and IL-5) and regulatory (IL-10 and TGF-β) cytokines in blood, popliteal lymph node and bone marrow of dogs with CanL and during treatment with either meglumine antimoniate or miltefosine in combination with allopurinol, for a three-month period;
2. The profile of $CD4^+$ and $CD8^+$ T-cell subsets in peripheral blood, lymph node and bone marrow of dogs with CanL and during treatment with either meglumine antimoniate or miltefosine in combination with allopurinol, for a three-month period;
3. In addition to gathering and consolidating the most recent insights from our working group in the field of animal leishmaniosis, in order to better understand the dog's immune response against CanL, namely, the role of polymorphonuclear neutrophils, hepatocytes and Kupffer cells, as well as the effect of therapeutic protocols and the importance of feline leishmaniosis, among others.

The current study was carried out on dogs naturally infected with *L. infantum* and observed at the School Hospital of the Faculty of Veterinary Medicine, University of Lisbon (FMV-UL). The tutors of the dogs were informed and gave their consent by signing an informed consent statement (ANNEX 1). Animal handling and collection of biological samples (peripheral blood, lymph node and bone marrow) was carried out by the Veterinary team of the Teaching Hospital of the FMV-UL. The present work followed the Council of the European Union Directive 86/609/EEC and was approved by the Ethics and Animal Welfare Committee (Comissão de Ética e Bem-Estar Animal - CEBEA) of the FMV-UL (ANNEX 2).

CHAPTER II:

Meglumine antimoniate and miltefosine combined with allopurinol sustain pro-inflammatory immune environments during canine leishmaniosis treatment

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2.1. Abstract

Canine leishmaniosis (CanL) caused by *Leishmania infantum* is a zoonotic disease of global concern. Antileishmanial drug therapies commonly used to treat sick dogs improve their clinical condition, although when discontinued relapses can occur. Thus, the current study aims to evaluate the effect of CanL treatments in peripheral blood, lymph node, and bone marrow cytokine profile associated with clinical recovery.

Two groups of six dogs diagnosed with CanL were treated with miltefosine combined with allopurinol and meglumine antimoniate combined with allopurinol (MT+A and MG+A) respectively. At diagnosis and after treatment, during a three-month follow-up, clinical signs, hematological and biochemical parameters, urinalysis results and antileishmanial antibody titers were registered. Furthermore, peripheral blood, popliteal lymph node, and bone marrow samples were collected to assess the gene expression of IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α, TGF-β and IFN-γ by qPCR. In parallel, were also evaluated samples obtained from five healthy dogs.

Both treatment protocols promoted the remission of clinical signs as well as normalization of hematological and biochemical parameters and urinalysis values. Antileishmanial antibodies returned to non-significant titers in all dogs. Sick dogs showed a generalized upregulation of IFN-γ and downregulation of IL-2, IL-4 and TGF-β, while gene expression of IL-12, TNF-α, IL-5 and IL-10 varied between groups and according to evaluated tissue. A trend to the normalization of cytokine gene expression was induced by both miltefosine and meglumine antimoniate combined therapies. However, IFN-γ gene expression was still up-regulated in the three evaluated tissues. Furthermore, the effect of treatment in the gene expression of cytokines that were not significantly changed by infection, indicates that miltefosine and meglumine antimoniate combined therapy directly affects cytokine generation. Both combined therapies are effective in CanL treatment, leading to sustained pro-inflammatory immune environments that can compromise parasite survival and favor dogs' clinical cure. In the current study, anti-inflammatory and regulatory cytokines do not seem to play a prominent role in CanL or during clinical recovery.

Keywords: Canine leishmaniosis; Peripheral blood mononuclear cells; Lymph node; Bone marrow; Cytokine gene expression; Meglumine antimoniate; Miltefosine; Allopurinol.

2.2. Introduction

Leishmaniosis constitute a group of parasitic diseases of worldwide concern, that are considered by the World Health Organization as neglected tropical diseases (WHO 2010). Canine leishmaniosis (CanL) caused by the intracellular protozoan *Leishmania infantum* is a zoonotic disease endemic to several southern European countries, including Portugal. In CanL, a wide range of non-specific clinical signs can be present (Solano-Gallego et al. 2009), posing difficulties to a correct diagnosis. Previous studies differentiated sick dogs into symptomatic, oligosymptomatic and polysymptomatic (Manna et al. 2009; Mateo et al. 2009; Miró et al. 2009; Woerly et al. 2009) although more recently it has been proposed an improved system to stage dog's clinical condition (Solano-Gallego et al. 2011; LeishVet Guidelines 2018). This classification system takes into account the physical examination, clinicopathological abnormalities, anti-*Leishmania* antibody titer, and the evaluation of renal function according to the International Renal Interest Society guidelines (International renal interest society 2016). Other proposals also consider a first stage of exposed dogs as those living or that have lived in geographic regions in which the presence of vectors has been confirmed (Paltrinieri et al. 2010). CanL conventional treatments improve the dog's clinical condition, reducing skin parasite load and consequently the risk of *Leishmania* transmission. Although it is not definitively proved that treatment completely eliminates the parasite (João et al. 2006), and relapses are common when therapy is discontinued (João et al. 2006; Ikeda-Garcia et al. 2007; Manna et al. 2009) it remains crucial to improve the efficiency of protocols used for CanL treatment. The main protocols for dog treatment usually include meglumine antimoniate (N-methylglucamine antimoniate), miltefosine (1-O-hexadecylphosphocholine) and allopurinol. Meglumine antimoniate is a pentavalent antimonial-based drug whose precise mechanism of action is not yet well understood but being considered a multifactorial drug with probable activity on parasite molecular processes, and influence in macrophage microbicide activity (Frézard et al. 2009; Mcgwire and Satoskar 2014). Miltefosine is an alkylphosphocholine compound able to induce apoptosis by mechanisms still not entirely clear (Pérez-Victoria et al. 2006; Sundar and Olliaro 2007; Bianciardi et al. 2009; Dorlo et al. 2012). Allopurinol is a purine analog of adenosine nucleotide, which blocks RNA synthesis, inhibiting *Leishmania* growth (Denerolle and Bourdoiseau 1999). Up to date, meglumine antimoniate in combination with allopurinol is considered the first line of treatment in Europe (Solano-Gallego et al. 2009), while miltefosine plus allopurinol has being the second line of treatment. However, miltefosine therapy has been gaining more attention (Manna et al. 2009; Mateo et al. 2009; Miró et al. 2009; Woerly et al. 2009), being recently authorized in 2017 for CanL treatment in Brazil (Ribeiro et al. 2018), a highly endemic country for both canine and human leishmaniosis.

Nevertheless, with the arising of more reports of drug resistance that lead to either therapeutic failure, unresponsiveness or relapse, whether it be in humans or dogs, a deeper understanding of the usual therapies is imperative (Pérez-Victoria et al. 2006; Frézard et al. 2009; Haldar et al. 2011; Yasur-Landau et al. 2016).

The immune response of dogs evidencing leishmaniosis clinical signs has been usually characterized by higher levels of specific antibodies, along with a type-2 T-helper (Th2) response associated with the expression of interleukin (IL)-4, IL-5, and IL-6 (Mosmann and Moore 1991; Pinelli, van der Kaaij, et al. 1999; Santos-Gomes et al. 2002). On the contrary, protective immunity is thought to be dependent on a strong type-1 T-helper (Th1) response characterized by IL-2, IL-12, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ production (Mosmann and Moore 1991; Santos-Gomes et al. 2002). Furthermore, parasites may suppress host immunity by engaging regulatory T-cells (Treg) thus enabling the persistence of the infection (Rodrigues et al. 2009), with one study showing clearance of *Leishmania* infection after depletion of Treg populations in mice (Belkaid et al. 2002). Moreover, higher expression of regulatory cytokines (IL-10, transforming growth factor [TGF-β]) associated with high parasite burden observed in dogs presenting clinical signs (Alves et al. 2009) suggest a non-negligible role of these cytokines in disease progression. To the best of our knowledge, there is no study defining the ideal approach to CanL treatment based on the knowledge of the immune response elicited by the different treatment protocols, and there is only one study analyzing more than one parasite target organ in non-treated CanL (Rodríguez-Cortés et al. 2016). Therefore, further studies are essential to clarify how treatments affect dogs' ability to develop a protective immune response or, on the contrary, to elicit immune suppression of effector cells. In the present study, the influence of two different treatment protocols on disease evolution of naturally infected dogs and on immune response was evaluated by assessing the clinicopathological changes, and the gene expression of pro-inflammatory (IL-2, IL-12, TNF-α, IFN-γ), anti-inflammatory (IL-4, IL-5) and regulatory (IL-10, TGF-β) cytokines in blood, popliteal lymph node and bone marrow during a three-month period.

2.3. Materials and methods

2.3.1. Dog selection

Twenty-three dogs with at least 1.5 years of age, weighing more than 5 kg, not having been vaccinated for CanL and diagnosed with CanL clinical stage I/II, according to the LeishVet Consensus Guidelines (Solano-Gallego et al. 2011), and stage C in agreement to the Canine Leishmaniosis Working Group (CLWG) Guidelines (Paltrinieri et al. 2010) were selected from

a total of 170 household dogs living in the zoonotic visceral leishmaniosis endemic area of the Metropolitan Region of Lisbon (Portugal). Twelve of those 23 dogs had not undergone any treatment in the last 8 months that could interfere with the immune response (such as antibiotic and corticosteroid therapy or administration of immunomodulators), and were negative for circulating pathogens potentially responsible of canine vector-borne diseases (CVBDs), were selected to participate in the current study. Five clinical healthy dogs not having been vaccinated for CanL, negative for *Leishmania* antibody test and CVBDs were also included in the present study as a control group (Fig. 19). All dog owners gave written consent after being informed about the objectives of the study and every procedure, ensuring that clinical results were made available. Selected dogs include 13 males and 4 females of various breeds with ages ranging between 2-9 years and weight between 7.6-32.1 kg. Animal handling and sample collection procedures were done by the Veterinary team of the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon (Lisbon, Portugal). The present study followed the Council of the European Union Directive 86/609/EEC and was approved by the Ethics and Animal Welfare Committee of the Faculty of Veterinary Medicine, University of Lisbon.

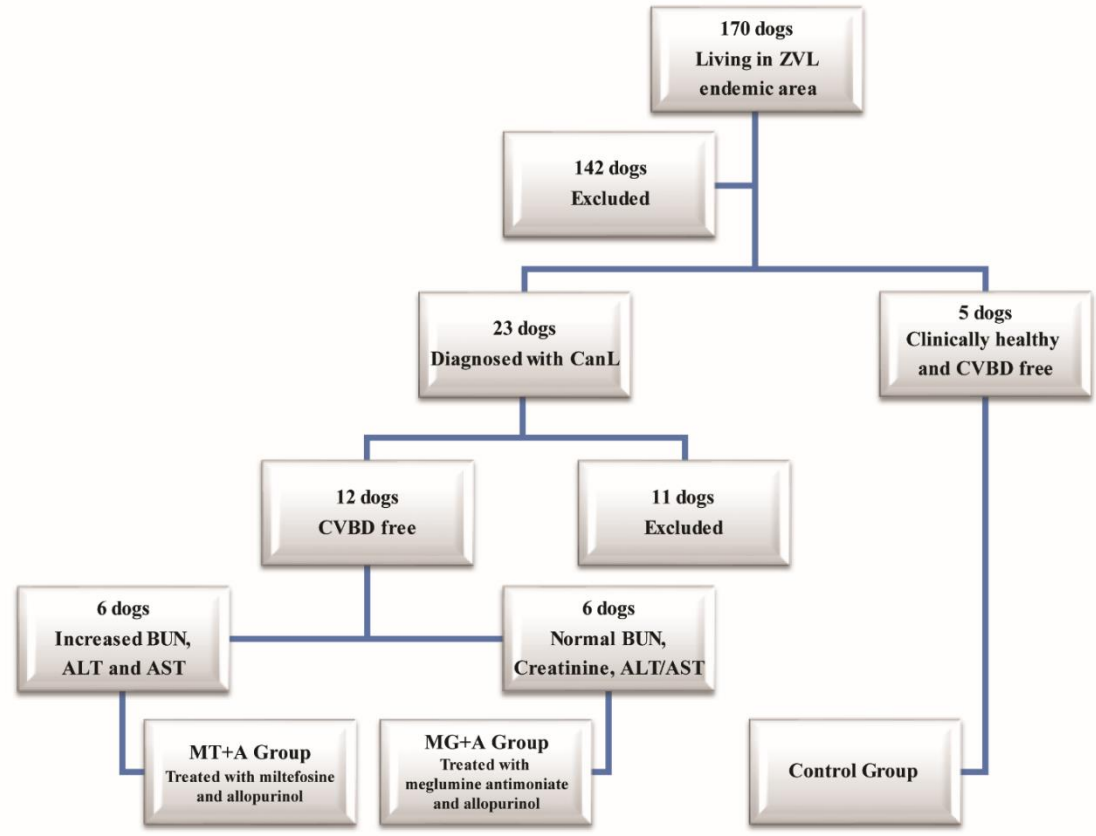

Figure 19. Flowchart representing the dog's selection process used in the current study
From a total of 170 dogs, living in an endemic area of zoonotic visceral leishmaniosis, two groups of dogs with canine leishmaniosis (CanL) were constituted, and were treated with either miltefosine in combination with allopurinol (MT+A) or with meglumine antimoniate in association with allopurinol (MG+A) along with one group of clinically healthy dogs (Control Group - CG). These dogs were negative for Canine Vector-Borne Diseases (CVBD). ALT - Alanine aminotransferase; AST - Aspartate aminotransferase; BUN - Blood urea nitrogen.

2.3.2. Experimental design

To reduce discomfort and ensure dog's well-being, the amount of sample collections and its periodicity were reduced to a minimum. Blood, popliteal lymph node and bone marrow samples were collected from healthy (control group) and sick dogs prior the onset of treatment (Tp0) and then from sick dogs, one (Tp1), two (Tp2) and three months (Tp3) after the beginning of treatment. The samples collected from sick dogs at Tp0 were used, not only, to establish the baseline levels of cytokine mRNA accumulation, but also, for ethical reasons, to serve as controls of themselves, avoiding the need of an extra group of sick animals without any treatment. Treatment success was clinically and serological re-assessed six months after the initial diagnosis for each treated animal (Fig. 20). Each dog was enrolled in one of the two treatment protocols (Fig. 19), according to the following criteria:

(i) Dogs presenting increased blood urea nitrogen (BUN), creatinine and/or alanine aminotransferase (ALT), aspartate aminotransferase (AST) and UCP between 0.2-0.6, pointing to the possibility of developing hepatic and renal lesion were treated with miltefosine (Milteforan®, Virbac S.A, France; 2 mg/kg *per os*, *semel in die* - SID - for 4 weeks) in association with allopurinol (Zyloric®, Laboratórios Vitória, Portugal; 10 mg/kg, *per os*, *bis in die* - BID - for at least 6 months) (MT+A);

(ii) Dogs presenting changes in biochemical and hematological parameters, serum proteins and UCP between 0.2-0.4 were treated with meglumine antimoniate (Glucantime®, Merial Portuguesa, Portugal; 100 mg/kg SID for 4 weeks) in association with allopurinol 10 mg/kg, *per os*, BID for at least 6 months (MG+A).

Deltamethrin-impregnated collars were applied to all dogs to prevent infections or re-infections during the current study and also in order to avoid *Leishmania* dissemination to sand flies. Blood samples were used for determination of hematological and biochemical parameters, and serological and molecular tests. Popliteal lymph node, bone marrow, and peripheral blood were used to examine cytokine gene expression. Urine samples were collected into sterilized containers for urinalysis and determination of protein/creatinine ratio (UPC).

2.3.3. Sample collection, hematological and biochemical analysis and serological tests

Peripheral blood (20 ml) was collected into syringes containing citrate phosphate dextrose adenine (CPDA-1, Medinfar Sorológico, Portugal). Popliteal lymph node aspirates were collected into syringes containing 0.8 ml of saline solution (0.9 % NaCl) in order to avoid

cell disruption and were then transferred to ethylenediaminetetraacetic acid (EDTA) tubes to avoid coagulation. After cutaneous anesthesia with a Xylocaine 10% Pump Spray (AstraZeneca, UK), bone marrow aspirates were collected from the distal area of the costal ribs, between the 9th and the 11th, into syringes containing 0.8 ml of saline solution. An additional 4 ml of blood was collected in EDTA tubes and dry tubes to be used for hematological (complete blood count), biochemical analysis (serum measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, bilirubin, blood urea nitrogen (BUN), creatinine, inorganic phosphorus, calcium, sodium, potassium, chlorides), serum proteinogram electrophoresis and CVBD screening. Peripheral blood samples were also used for the isolation of mononuclear cells. Popliteal lymph node and bone marrow were used for detection of *Leishmania* amastigote forms and isolation of mononuclear cells.

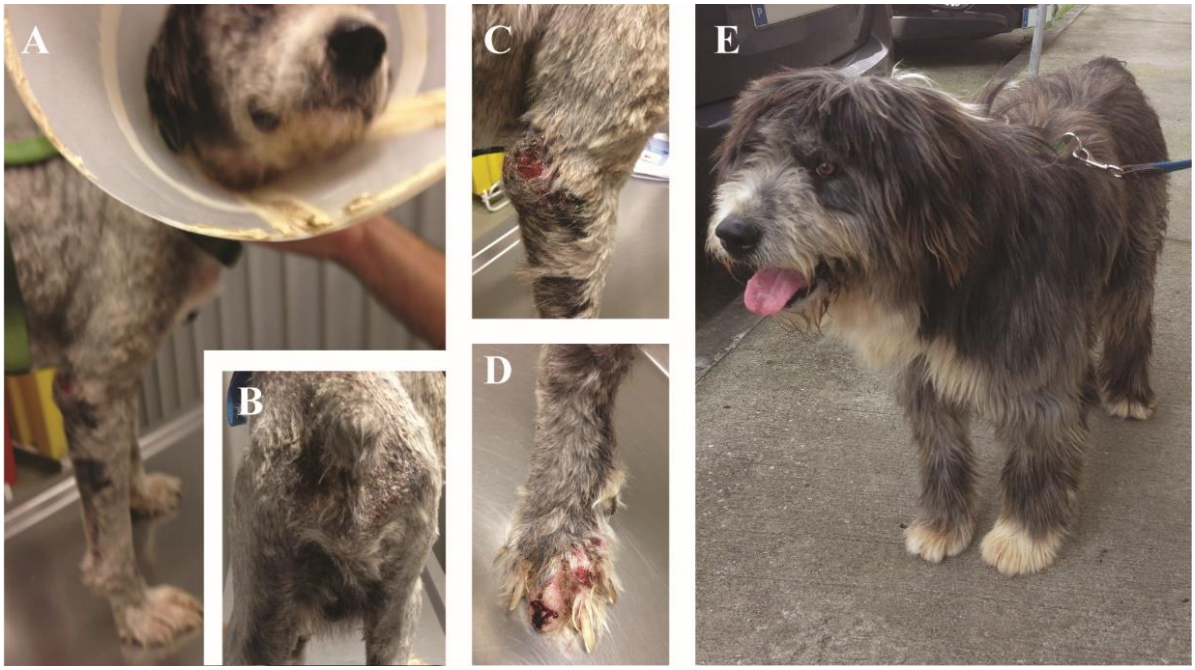

Figure 20. Clinical manifestations of a dog naturally infected with *Leishmania infantum*
(A,B) - Dog presenting evident loss of weight, lethargy, cutaneous alopecia and exfoliative dermatitis; (C) - Ulcerative and hyperkeratosis lesions in the elbow of the front limb; (D) - Onychogryphosis with severe bleeding; (E) - Dog from the MT+A group 6 months after the diagnosis with full remission of clinical signs. Photos by Marcos Santos.

2.3.4. *Leishmania* screening

Serum samples were used for detection of anti-*Leishmania* antibodies by IFAT assay (*Leishmania*-Spot IF, BioMérieux, France) using *L. infantum* promastigotes as antigen and following the manufacturer's instructions. Samples were screened using an Olympus DP10 microscope (model BX50F, wavelength of 425 nm) and classified as positive if fluorescence

was observed in promastigote cytoplasm or membrane at a serum dilution of 1:80 or higher. According to LeishVet (Solano-Gallego et al. 2011) and the Canine Leishmaniosis Working Group (CLWG) guidelines (Paltrinieri et al. 2010), IFAT is a gold standard test for canine leishmaniosis and to evaluate possible relapses.

To test for the presence of *Leishmania* DNA, total genomic DNA was extracted from 200 µl of peripheral blood using the DNeasy® Blood and Tissue kit (QIAGEN®, Germany) according to the manufacturer's instructions. DNA amplification by qPCR was done in a total volume of 20 µl, comprising 10 µl of TaqMan® Gene Expression Master Mix (Applied Biosystems™, USA), 2 µl of ultra-pure water (Merck Millipore™ KGaA, Germany), 300 nM of forward and reverse primers for each set as well as 250 nM for each probe (Table 14) and 2 µl of target DNA. Reactions were carried out using the 7300 Real-Time PCR thermal cycler (Applied Biosystems™), with the following cycling conditions: 10 min at 95 °C for AmpliTaq® Gold activation, followed by a total of 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The positive control was constructed by cloning PCR fragments generated by the same primers into a pGEM®-T Easy Vector (Promega, USA), according to the manufacturer's instructions. Ligated fragments were transformed into JM109 competent cells and plasmid DNA was prepared using the QIAprep® Spin Miniprep Kit (QIAGEN®). The insert was sequenced using primers pUC/M13 (Promega) to ensure transformation stability. To exclude the presence of *Leishmania* amastigotes, lymph node and bone marrow slides were stained with Giemsa and observed by optical microscopy (Microscope Olympus CX31, using a 1000x magnification).

Table 14. Primers and TaqMan probes used for hemoparasite screening

Target	Oligo	Oligonucleotide sequence (5'→3')	Product size (bp)	Reference
Leishmania (Kinetoplast)	Frw	GGAAGGTGTCGTAAATTCTGGAA	124	(Helhazar et al. 2013)
	Rev	CGGGATTTCTGCACCCATT		
	Probe[1]	AATTCCAAACTTTTCTGGTCCTCCGGGTAG		
Ehrlichia and *Anaplasma* (16S rRNA)	Frw	ACCTATAGAAGAAGTCCCGGCAA	100	(Gal et al. 2008)
	Rev	ACCTACGTGCCCTTTACGCCC		
	Probe[1]	GCAGCCGCGGTAATACGGAGGGGGC		
Babesia (18S rRNA)	Frw	ACCCATCAGCTTGACGGTAGGGT	97	(Jefferies et al. 2003)
	Rev	AGCCGTCTCTCAGGCTCCCT		
	Probe[2]	ACCGAGGCAGCAACGGGTAACGGGGA		
Rickettsia (OmpA)	Frw	AACCGCAGCGATAATGCTGAGTAGT	130	(Kidd et al. 2008)
	Rev	CCCTGCAGAAGTTATCTCATTCCAA		
	Probe[2]	AGCGGGGCACTCGGTGTTGCTGCA		

Presence of parasites in dog blood was evaluated by qPCR. Product base pair (bp) for each pathogen and primer references are also indicated. Frw - forward primer; Rev - reverse primer; [1]probe labelled with 6-FAM at the 5'-end and quenched with TAMRA at the 3'-end; [2]probe labelled with JOE at the 5'-end and quenched with TAMRA at the 3'-end.

2.3.5. CVBD exclusion

Detection of antibodies against *Babesia*, *Anaplasma*, *Ehrlichia* and *Rickettsia* was performed using commercial diagnostic tests (Megacor® MegaScreen, Austria, FLUOBABESIA *canis* - cut off 1:32; FLUOANAPLASMA *phagocytophilum* - cut off 1:50; FLUOEHRLICHIA *canis* - cut off 1:50; FLUORICKETTSIA *conorii* - cut off 1:40). The absence of *Babesia*, *Anaplasma*, *Ehrlichia*, and *Rickettsia* DNA was also evaluated by qPCR (Table 14) as previously described. To exclude the presence of *Dirofilaria immitis* microfilaria, blood samples were evaluated by Knott technique and parasite antigens were assessed by Witness® *Dirofilaria* kit (Zoetis, Portugal) according to the manufacturer's instructions.

2.3.6. Cell isolation

Dog peripheral blood was re-suspended in PBS (1:1 v/v), overlaid onto a 1:2 Histopaque®-1077 solution (Sigma-Aldrich, Germany) and centrifuged at 400 *g* for 30 min at 18 °C. Mononuclear cells were harvested and washed in cold PBS (300 *g*, 10 min, 4 °C), re-suspended in PBS, and the total volume adjusted to 2×10^7 cells.ml^{-1}. Lymph node and bone marrow aspirates were centrifuged at 400 *g* (4 °C) for 5 and 15 min, respectively, and re-suspended in 100 µl, with the total volume also adjusted for 2×10^7 cells.ml^{-1}. Then, 200 µl of peripheral blood mononuclear cells (PBMCs) and 100 µl of lymph node and bone marrow cell suspensions were centrifuged at 400 *g* (4 °C) for 5 min, re-suspended in 600 µl of RLT Buffer (QIAGEN®) supplemented with β-mercaptoethanol and stored at -80 °C until further use.

2.3.7. mRNA extraction and reverse transcription

Total RNA extracted from PBMCs, lymph node, and bone marrow cells, using RNeasy® Mini Kit (QIAGEN®) and QIAshredder® spin columns (QIAGEN®) was treated with DNase I Amplification Grade (Invitrogen™, USA) according to the manufacturer's instructions. For cDNA synthesis, 1 µg of purified RNA, presenting a 260/280 absorbance ratio ranging between 1.9 and 2.1 was denatured at 65 °C for 5 min and reverse transcribed at 37 °C for 60 min in a 30 µl final reaction mixture containing 6 µl of 5× M-MLV RT Buffer (Promega), 200 U/µl SCRIPT Reverse Transcriptase enzyme (Jena Bioscience, Germany), 500 µl dNTP Mix (Jena Bioscience), 1 µl of Oligo(dT)18 primers (Thermo Fisher Scientific Inc.™, EU), and 40 U/µl RiboLock RNase Inhibitor (Thermo Fisher Scientific Inc.™). cDNA samples were then heated at 95 °C for 10 min for enzyme inactivation and stored at -20 °C until further use.

2.3.8. Cytokine gene expression

To evaluate the effect of treatment in pro-inflammatory, anti-inflammatory and regulatory cytokines, the accumulation of mRNA encoding for IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α, TGF-β and IFN-γ was assessed by qPCR in PBMC, lymph node and bone marrow cell. cDNA amplification was conducted in a 20 µl final reaction mixture containing 10 µl of SYBR® Green PCR Master Mix (Applied Biosystems™), 80 nM of forward and reverse primers for each cytokine and for housekeeping gene β-actin (Table 15), 4 µl of ultra-pure water (Merck Millipore™ KGaA) and 2 µl of canine cDNA. Each sample amplification was performed in triplicate, using the following conditions: 10 min at 95 °C for AmpliTaq® Gold activation followed by a total of 40 cycles (thermal profile for each cycle: 15 s at 95 °C, 1 min at 60 °C). An extra dissociation step was added to confirm the specificity of amplification by melting point analysis, and absence of nonspecific products. External cDNA standards for all target cytokines and internal control used in every reaction were constructed as previously described. The concentration of standards was determined by measuring the OD at 260 nm followed by calculation of the corresponding copy number, and serial dilutions of resulting clones were used as standard curves, each containing a known amount of input copy number (Rodrigues et al. 2006; Barbosa et al. 2011).

Table 15. Primers used for quantification of cytokine mRNA expression by qPCR

Target	Oligo	Oligonucleotide sequence (5'→3')	Product size (bp)	Reference
IL-2	Frw Rev	GCATCGCACTGACGCTTGTA TTGCTCCATCTGTTGCTCTGTT	86	(Peters et al. 2005)
IL-4	Frw Rev	CATCCTCACAGCGAGAAACG CCTTATCGCTTGTGTTCTTTGGA	83	(Huang et al. 2008)
IL-5	Frw Rev	GCCTATGTTTCTGCCTTTGC GGTTCCCATCGCCTATCA	106	(Menezes-Souza et al. 2011)
IL-10	Frw Rev	CAAGCCCTGTCGGAGATGAT CTTGATGTCTGGGTCGTGGTT	78	(Yu et al. 2010)
IL-12p40	Frw Rev	CAGCAGAGAGGGTCAGAGTGG ACGACCTCGATGGGTAGGC	109	(Peters et al. 2005)
TNF-α	Frw Rev	AATCATCTTCTCGAACCCCAAGT GGAGCTGCCCCTCAGCTT	74	(Sauter et al. 2005)
TGF-β	Frw Rev	CAGAATGGCTGTCCTTTGATGTC AGGCGAAAGCCCTCGACTT	79	(Huang et al. 2008)
IFN-γ	Frw Rev	TCAACCCCTTCTCGCCACT GCTGCCTACTTGGTCCCTGA	113	(Menezes-Souza et al. 2011)
β-actin	Frw Rev	ACGGAGCGTGGCTACAGC TCCTTGATGTCACGCACGA	62	(Sauter et al. 2005)

bp - base pair; Frw - forward primer; Rev - reverse primer

Copy numbers of target genes were normalized to the housekeeping gene β-actin, therefore correcting for minor variations in mRNA isolation and reverse transcription. Final results were expressed as the copy number of each cytokine per 1000 copies of the housekeeping gene. Amplification efficiencies were greater than 90%.

2.3.9. Data analysis

An exploratory multivariate statistical analysis, specifically the Principal Component Analysis (PCA), was performed per tissue, on all datasets, in order to identify principal components accounting for the majority of the variation and graphically assess the separation between the healthy control, sick (Tp0) and treated dogs (Tp1, Tp2 and Tp3). This statistical analysis was performed using JMP version 14.3.0 (SAS Institute). Likewise, a K-Means Cluster analysis was also used to complement the previous PCA analysis and confirm grouping separation. In order to reduce the number of irrelevant or redundant variables and present a more robust model, a feature selection method was employed. Using the Predictor Screening tool from JMP the individual contribution of each variable was obtained, and the selected features were considered in the final models.

Statistical analysis between treatment groups was performed using GraphPad Prism software package version 8.0.1. Data normality was assessed using the Kolmogorov-Smirnoff test. Wilcoxon signed rank test was used to compare hematological and biochemical results in each dog treatment group between Tp0 and Tp3, with differences being considered significant when $p < 0.05$. The non-parametric Kruskal-Wallis Test (one-way ANOVA on ranks) with Dunn's post hoc test was used to evaluate differences in mRNA levels between treatment groups and the CG. The Repeated Measures ANOVA Test with Tukey's post hoc test was used to compare dogs at different time-points.

2.4. Results

2.4.1. Both treatment protocols lead to dog's clinical remission

Blood smears of dogs from MT+A, MG+A and Control Group were all negative for CVBD causing agents. The dogs presented negative serology for *Babesia*, *Anaplasma*, *Ehrlichia* and *Rickettsia*, and were negative for DNA detection of these parasites. Dogs were also negative in rapid immune migration for *D. immitis* antigen and microfilaria were absent in Knott technique. Clinical signs observed in sick dogs at the beginning of the study (Tp0) included loss of body weight (Fig 20A and B), local/generalized lymphadenopathy,

decreased/increased appetite, lethargy, mucous membrane pallor, polyuria/polydipsia, cutaneous alopecia, onychogryphosis (Fig. 20D), hyperkeratosis, exfoliative-dermatitis, and erosive-ulcerative dermatitis (Fig. 20C). Other clinical signs, such as epistaxis, lameness and masticatory muscle myositis were also observed. No clinical signs were detected in dogs of the Control Group. Sick dogs showed also changes in hematological and biochemical parameters, including a mild decrease of hemoglobin values, mild erythropenia, lower hematocrit values, thrombocytopenia (Table 16), mild renal azotemia (Table 17), hyperglobulinemia with increased alpha 2 and gamma globulin fractions, and decreased values of alpha 1 and albumin/globulin ratio (Table 18, Fig. 21). Dogs of group MT+A presented higher BUN values and an accentuated AST and ALT while dogs of the MG+A group exhibited BUN normal values and a slight increase in ALT and AST values (Table 17, Fig. 21).

Table 16. Hemogram values exhibited by dogs of MT+A and MG+A groups

	MT+A Group (n=6)				Tp0 vs	MG+A Group (n=6)				Tp0 vs	Control Group	Reference
Hemogram	Tp0	Tp1	Tp2	Tp3	Tp3	Tp0	Tp1	Tp2	Tp3	Tp3	(n=5)	Interval
RBC (×10^6/µl)	5.49±1.32	5.30±1.85	5.71±1.07	5.87±0.91	-	5.22±0.55	5.54±1.18	6.36±0.65	6.82±0.68	*	7.21±1.05	5.5-8.5
Hemoglobin (g/dl)	12.32±2.95	11.83±3.55	13.08±2.50	12.66±3.24	-	11.24±2.15	12.33±2.59	14.25±1.73	15.50±1.26	*	16.26±2.41	12-18
Hematocrit (%)	37.85±10.89	35.45±12.20	38.98±7.60	37.44±9.51	-	34.80±6.09	37.38±9.93	42.45±5.86	45.27±3.63	*	51.40±7.29	37-55
MCV (µm^3)	68.48±4.13	67.15±2.95	68.24±2.80	68.72±2.13	-	66.38±7.04	67.12±9.17	66.63±4.71	66.53±3.46	-	71.40±1.69	60-74
MCH (pg)	22.60±1.53	22.67±1.35	22.92±0.89	23.26±0.54	-	21.02±1.94	22.30±1.48	22.42±1.07	22.75±1.25	-	22.54±0.65	19.5-24.5
MCHC (g/dl)	33.10±2.95	33.83±2.65	33.70±2.42	33.84±0.78	-	32.32±1.60	33.60±3.51	33.72±1.49	34.22±1.28	-	31.62±1.63	31-36
RDW (%)	13.16±0.94	13.78±0.95	13.88±1.09	13.46±1.44	-	13.43±0.99	14.45±2.35	13.08±1.07	13.00±0.97	-	12.34±0.54	12-18
Leukocytes (×10³/µl)	8.17±2.98	7.83±2.38	9.22±2.13	7.76±3.82	-	7.50±2.47	8.62±3.83	9.03±2.90	9.55±3.42	-	10.24±3.18	6-17
Lymphocytes (×10³/µl)	1.67±0.51	2.23±0.86	3.18±1.64	2.96±2.29	-	1.56±0.85	2.12±1.11	2.37±0.81	2.22±0.87	-	2.80±0.70	1-4.8
Monocytes (×10³/µl)	0.66±0.25	0.48±0.19	0.48±0.22	0.40±0.23	-	0.68±0.24	0.67±0.50	0.50±0.26	0.47±0.23	-	0.48±0.20	0.2-2
Neutrophils (×10³/µl)	5.60±2.27	4.38±1.35	4.92±1.41	3.86±1.45	-	5.11±1.45	5.40±2.71	5.77±2.40	6.43±2.95	-	5.96±2.14	3-11.8
Eosinophils (×10³/µl)	0.27±0.28	0.70±0.57	0.58±0.40	0.52±0.64	-	0.14±0.21	0.42±0.23	0.35±0.19	0.38±0.21	*	0.98±0.40	0.1-1.3
Basophils (×10³/µl)	0.02±0.04	0.03±0.05	0.04±0.05	0.00±0.00	-	0.02±0.04	0.03±0.05	0.07±0.08	0.03±0.05	-	0.06±0.05	0-0.5
Platelets (×10³/µl)	280.67±133.4	233.83±130.8	254.00±150.8	235.00±43.62	-	212.80±133.5	246.50±125.1	227.17±60.38	222.50±58.32	-	217±25.84	200-500
MPV (µm^3)	11.82±2.94	12.82±2.88	11.14±2.27	11.82±2.28	-	14.73±3.14	11.82±2.70	11.33±2.56	10.73±1.82	-	10.38±1.28	5-15
Procalcitonin (%)	0.30±0.14	0.28±0.10	0.26±0.13	0.26±0.09	-	0.28±0.15	0.23±0.15	0.27±0.08	0.25±0.10	-	0.22±0.04	0.2-0.5
PDW (%)	65.04±11.24	64.33±9.75	66.12±11.74	64.62±10.11	-	74.08±3.95	57.98±21.28	70.58±8.48	73.87±5.91	-	59.06±5.03	40.6-65.2

At diagnosis time (Tp0), and one (Tp1), two (Tp2) and three (Tp3) months after the beginning of the treatment. Blood samples of sick (n=12) and healthy dogs (control group [CG], n=5) were used to evaluate hemogram parameters. Reference values are also included. Wilcoxon signed rank text was used to compare between Tp0 and Tp3 in each treatment group. * $p < 0.05$; MCH - Mean Corpuscular Hemoglobin; MCHC - Mean Corpuscular Hemoglobin Concentration; MCV - Mean Corpuscular Volume; MPV - Mean Platelet Volume; PDW - Platelet Distribution Width; RBC - Red Blood Cells; RDW - Red cell Distribution Width.

Three dogs of group MT+A also showed creatinine values inferior to 1.4 mg/dL and mild proteinuria, presenting a urine protein:creatinine ratio (UPC) of 0.6. Control group dogs exhibited normal hematological and biochemical parameters, serum proteins and urinalysis values. Lymph

node and bone marrow smears of dogs from both MT+A and MG+A groups presented amastigote forms inside macrophages associated with lymphoid hyperplasia. Dogs from both groups showed anti-*Leishmania* antibody titers ranging between 1:80 and 1:320.

Table 17. Biochemical parameters and urinalysis results exhibited by dogs of MT+A and MG+A groups

	MT+A Group (n=6)				Tp0 vs	MG+A Group (n=6)				Tp0 vs	Control Group	Reference
Biochemical parameters	Tp0	Tp1	Tp2	Tp3	Tp3	Tp0	Tp1	Tp2	Tp3	Tp3	(n=5)	Interval
BUN (mg/dl)	60.35±22.86	45.27±35.79	33.23±8.49	25.25±7.04	*	26.03±5.09	31.92±3.84	34.50±7.11	34.60±6.82	*	36.33±3.64	15-40
Creatinine (mg/dl)	1.20±0.88	1.08±0.68	1.19±1.04	0.88±0.62	-	0.55±0.08	0.64±0.12	0.87±0.26	0.82±0.27	-	0.92±0.24	0.4-1.4
Total bilirubin (mg/dl)	0.05±0.02	0.05±0.01	0.06±0.02	0.06±0.04	-	0.04±0.00	0.04±0.00	0.05±0.02	0.04±0.00	-	0.06±0.02	0.04-0.4
Direct bilirubin (mg/dl)	0.03±0.01	0.03±0.01	0.04±0.02	0.02±0.01	-	0.02±0.01	0.02±0.01	0.03±0.01	0.02±0.01	-	0.04±0.01	0-0.3
Indirect bilirubin (mg/dl)	0.02±0.01	0.02±0.01	0.02±0.01	0.04±0.04	-	0.03±0.01	0.02±0.01	0.02±0.01	0.02±0.01	-	0.02±0.02	0-0.3
AST (U/l)	62.80±29.48	45.83±12.12	46.17±11.74	42.60±17.62	-	49.25±2.87	42.40±13.79	58.50±9.07	37.83±17.36	-	40±4.19	10-40
ALT (U/l)	93.83±76.94	86.00±42.70	78.33±42.16	65.20±40.53	-	44.17±36.34	29.33±16.19	30.33±11.71	37.67±16.11	-	40.25±7.46	10-70
Alkaline phosphatase (U/l)	146.1±166.4	131.78±161.7	146.48±243.8	30.05±15.95	*	49.18±15.34	38.95±13.42	35.30±7.10	35.28±12.70	*	41.45±29.56	20-200
Sodium (mmol/l)	145.60±4.67	148.50±8.76	146.50±3.02	143.60±3.21	-	142.25±4.86	146.67±2.34	146.67±2.42	148.50±3.56	-	146.75±2.22	140-151
Potassium (mmol/l)	4.86±0.79	4.70±0.89	4.68±0.49	4.54±0.44	-	4.62±0.34	4.59±0.39	4.50±0.27	4.40±0.26	-	5.07±0.53	3.4-5.4
Chloride (mmol/l)	112.80±1.92	102.67±13.94	113.50±4.93	92.78±51.56	-	108.00±4.08	108.17±9.35	104.17±7.17	111.67±6.02	-	113.00±6.48	105-120
Calcium (mg/dl)	9.99±0.51	9.54±0.35	9.48±0.72	9.08±0.93	-	9.92±0.42	9.93±0.30	9.89±0.32	10.14±0.36	-	9.50±1.57	9.5-12
Inorganic phosphorus (mg/dl)	4.96±0.68	5.90±2.87	4.35±1.60	3.58±0.92	-	3.95±0.59	3.73±0.67	3.35±1.03	3.58±1.25	-	4.60±0.79	2.1-5
Biliary acids (µmol/l)	3.07±2.11	3.22±2.94	3.18±3.34	3.36±2.83	-	1.40±0.25	3.54±3.44	2.19±1.28	1.72±1.11	-	2.47±1.34	1-10
Urinalysis												
Creatinine (mg/dl)	<1.4	<1.4	<1.4	<1.4	-	<1.4	<1.4	<1.4	<1.4	-	< 1.4	< 1.4
UPC	<0.2-0.6	<0.2-0.5	<0.2	<0.2	-	<0.2 – 0.4	<0.2-0.4	<0.2	<0.2	-	< 0.2	< 0.2

At diagnosis time (Tp0), and one (Tp1), two (Tp2) and three (Tp3) months after the beginning of the treatment. Blood and urine samples of sick (n=12) and healthy dogs (control group [CG], n=5) were used to evaluate biochemical parameters and urinalysis. Reference values are also included. Wilcoxon signed rank text was used to compare between Tp0 and Tp3 in each dog group. * $p < 0.05$; ALT - Alanine aminotransferase; AST - Aspartate aminotransferase; BUN - Blood Urea Nitrogen; UPC - Urine Protein Creatinine Ratio.

No antileishmanial antibodies were detected in dogs from the Control Group (Table 19). One month after treatment (Tp1) dogs of MG+A exhibited higher vivacity and energy than dogs from MT+A. Three months after treatment onset (Tp3), both groups exhibited a successful recovery, showing remission of all clinical signs. Dogs from the MT+A group presented a significant recovery ($p < 0.05$) of BUN values to normal levels. AST and ALT quickly recovered to normal values in dogs of group MG+A (Table 17). Although presenting higher AST and ALT values, combined treatment of miltefosine and allopurinol promoted the decrease of AST and ALT in dogs from the MT+A group, albeit slower, with urinalysis values returning to normal.

Table 18. Serum proteins of dogs of MT+A and MG+A groups

	MT+A Group (n=6)				Tp0 vs Tp3	MG+A Group (n=6)				Tp0 vs Tp3	Control Group (n=5)	Reference Interval
Proteinogram	Tp0	Tp1	Tp2	Tp3		Tp0	Tp1	Tp2	Tp3			
Total protein (g/dl)	9.58±1.55	7.70±0.60	7.56±1.18	7.60±1.30	*	8.43±1.46	7.66±0.89	7.92±1.30	6.85±0.56	-	6.28±0.59	5.5-7.5
Albumin (g/dl)	2.46±0.84	2.48±0.53	2.72±0.24	2.56±0.58	-	2.14±0.50	2.50±0.40	3.23±1.01	3.05±0.36	-	3.03±0.40	2.26-4.3
Alpha 1 (g/dl)	0.22±0.04	0.20±0.00	0.20±0.00	0.18±0.04	-	0.20±0.00	0.22±0.04	0.27±0.08	0.25±0.05	-	0.28±0.05	0.1-0.31
Alpha 2 (g/dl)	1.40±0.25	1.32±0.40	1.30±0.35	1.22±0.23	-	1.46±0.23	1.40±0.10	1.47±0.35	1.20±0.11	-	0.95±0.06	0.5-1.1
Beta (g/dl)	1.78±0.29	1.46±0.09	1.64±0.26	1.72±0.42	-	1.58±0.33	1.76±0.13	1.73±0.14	1.32±0.31	-	1.38±0.29	0.93-2
Gama (g/dl)	3.32±2.36	2.26±1.43	1.70±1.44	1.92±1.22	-	2.74±1.52	1.82±0.91	1.28±0.26	1.10±0.43	-	0.65±0.19	0.3-1
Albumin: globulin ratio (%)	0.44±0.29	0.50±0.19	0.60±0.17	0.56±0.30	-	0.38±0.13	0.50±0.19	0.68±0.17	0.80±0.13	*	0.95±0.13	0.6-1.1

At diagnosis time (Tp0), and one (Tp1), two (Tp2) and three (Tp3) months after the beginning of the treatment. Blood samples sick (n=12) and healthy dogs (control group [CG], n=5) were used to evaluate serum proteins. Reference values are also included. Wilcoxon signed rank text was used to compare between Tp0 and Tp3 in each dog group. * $p < 0.05$.

Dogs in MG+A group exhibited a normalization of the albumin globulin ratio two months after the beginning of treatment (Tp2) and one month later (Tp3) total protein and gamma globulin were within reference values. However, in dogs of the MT+A group the total protein and gamma globulin remained high and alpha 2 globulin normalized three months after the beginning of the treatment (Tp3) (Table 18, Fig. 21). Three months after treatment onset (Tp3), MG+A dogs were negative for anti-*Leishmania* antibodies and, with the exception of one dog that had a titer of 1:320 group MT+A dogs were also negative. When re-evaluated six months after the initial diagnosis this positive dog was negative for antileishmanial antibodies (Table 19). Furthermore, amastigote forms were no longer observed in lymph node and bone marrow smears of dogs from both groups.

Table 19. Anti-*Leishmania* antibody titers

***Leishmania* antibody titer**	**Tp0**	**Tp1**	**Tp2**	**Tp3**	**Tp6**
Group 1 (n=6)	1:80 - 1:320	< 1:80 - 1:320	< 1:80 - 1:160	< 1:80	<1:80
Group 2 (n=6)	1:80 - 1:320	< 1:80 - 1:320	≤ 1:80	< 1:80-1:320	<1:80
Control Group (n=5)	< 1:80	< 1:80	< 1:80	<1:80	<1:80

At diagnosis time (Tp0), and one (Tp1), two (Tp2), three (Tp3) and six (Tp6) months after the beginning of treatment, peripheral blood of sick (n=12) and control dogs (control group [CG], n=5) were collected and used to evaluated anti-*Leishmania* antibody titers by IFAT. A cut-off of 1:80 was used.

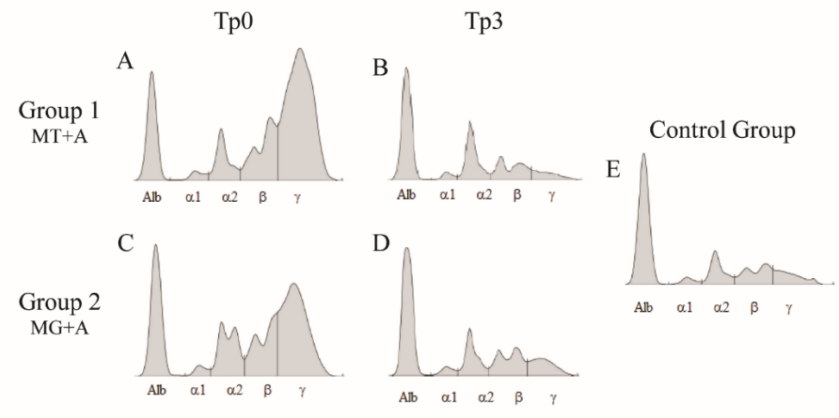

Figure 21. Serum protein electrophoresis of sick and treated dogs
Representative proteinograms of sick (Tp0; (A) - MT+A; (B) - MG+A), treated (Tp3; (C) - MT+A; (D) - MG+A) and clinically healthy dogs (E) are shown. Alb - Albumin; α1 - α1-globulin; α2 - α2-globulin; β - β-globulin; γ - γ-globulin.

2.4.2. Principal Component and Cluster analysis enable the distinction between healthy and sick dogs

Principal component analysis in PBMCs confirmed that healthy and sick dogs could be distinguished based on their expression of IFN-γ, IL-2, IL-4, IL-5, IL-12 and TGF-β along with IFAT results, with these features explaining 65.5% of the distribution (Fig. 22A). In lymph node, PCA was also able to distinguish healthy and sick dogs based on the expression of IFN-γ, IL-2 and IL-10 along with IFAT results, with 63.4% of the distribution being explained by these variables (Fig. 22C). For bone marrow the expression of IFN-γ, IL-4, IL-5 and IL-12 along with IFAT results, enabled the distinction between healthy and sick dogs, with these features explaining 72.3% of the distribution (Fig. 22E). These results are also supported by cluster analysis (Figs. 22B, D and F), with the formation of two separate groups. Dogs from both treatment groups could not be distinguished based on the selected features, but the transition from the sick dog's cluster towards the healthy dog cluster along the time-points can be observed in PBMC, lymph node and bone marrow.

2.4.3. *Leishmania* infection shapes dogs' cytokine profile

Sick dogs (MT+A and MG+A) showed a significant accumulation of IFN-γ mRNA in cells of PBMC (p_{MT+A} = 0.0057; p_{MG+A} = 0.0425) (Fig. 23J), lymph node (p_{MT+A} = 0.001; p_{MG+A} = 0.0028) (Fig. 23K) and bone marrow (p_{MT+A} = 0.0097; p_{MG+A} = 0.0267) (Fig. 23L) when compared with clinically healthy dogs (CG). Bone marrow cells of dogs of MT+A showed a significant upregulation of IL-12 (p = 0.0059) (Fig. 23F) in comparison to control dogs. On the other hand, lymph node cells of sick dogs evidenced a significant reduction in IL-2 mRNA (p_{MT+A} = 0.0365; p_{MG+A} = 0.0068) (Fig. 23B). Dogs of MG+A group also showed a significant

downregulation of IL-2 gene expression in PBMC (p = 0.0193) (Fig. 23A) and TNF-α in lymph node cells (p = 0.0186) (Fig. 23H). While dogs of MT+A group showed a significant upregulation of TNF-α gene expression in bone marrow cells (p = 0.0413) (Fig. 23I). No significant differences were found in gene expression of IL-12 by PBMC and lymph node cells, IL-2 by bone marrow cells and TNF-α by PBMC when compared to clinically healthy dogs.

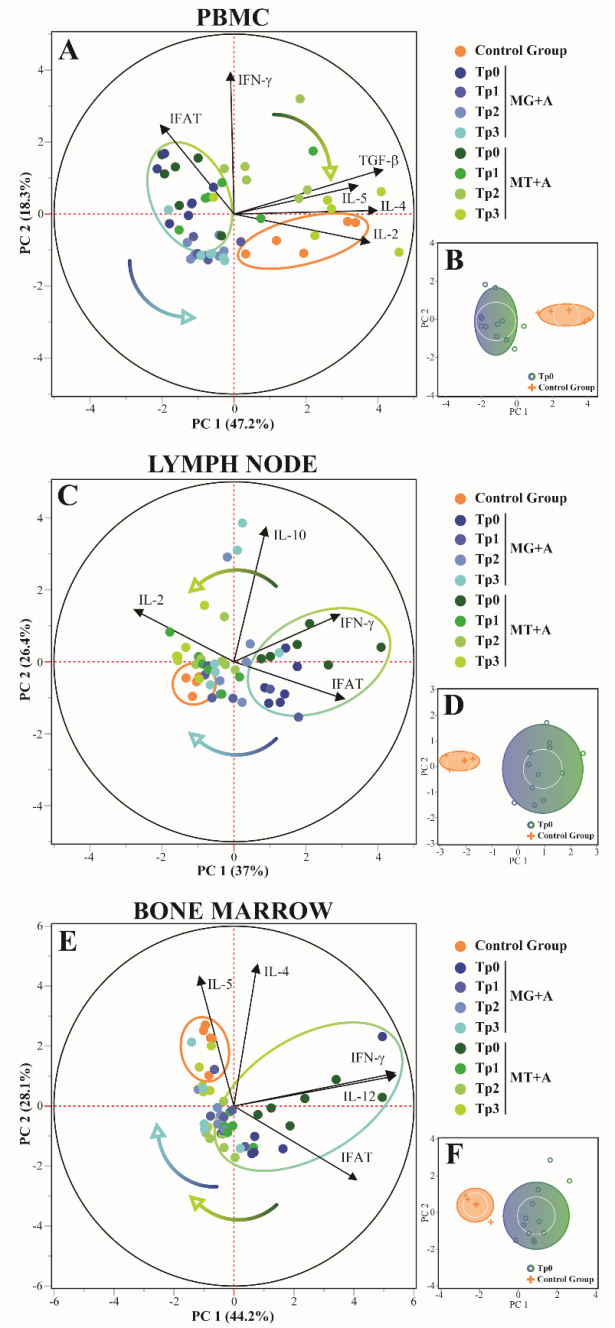

Figure 22. Principal Component and Cluster Analysis of cytokine expression in PBMC, lymph node and bone marrow

Principal component analysis was used to identify the first two principal components which explain 65.5%, 63.4% and 72.3% for each respective tissue, of the variation in the dataset. (A, C, E) - Biplot of score and loading plots showing the variables which load on the respective principal components. Control, MG+A and MT+A groups are presented by different colored dots along all time-points, with the control group and the sick dogs (Tp0) delimited by their respective halo. Colored arrows show the transition of treated dogs over time. (B, D, F) - Cluster analysis confirming the separation of healthy and sick dogs (Tp0) using the selected variables. PC – Principal Component.

PBMC (p_{MT+A} = 0.0662; p_{MG+A} = 0.0032) (Fig. 24A) and bone marrow (p_{MG+A} = 0.0138) (Fig. 24C) of sick dogs evidenced a significant down regulation of IL-4 gene expression in comparison to the CG. In lymph node cells, no significant differences were observed in the IL-4 gene expression. PBMC of sick dogs from MT+A group (p = 0.0031) (Fig. 24D) and bone marrow cells of dogs of group MG+A (p = 0.0082) (Fig. 24F) showed a statistically significant downregulation of IL-5 gene expression. Additionally, lymph node cells of MT+A showed a significant accumulation of IL-5 mRNA (p = 0.0235) (Fig. 24E).

A significant IL-10 downregulation in PBMC of MG+A (p = 0.0153) (Fig. 25A) and an upregulation in lymph node cells of sick dogs (p_{MT+A} = 0.0041, p_{MG+A} = 0.0112) (Fig. 25B). No significant differences in IL-10 gene expression were observed in bone marrow cells of sick dogs when compared with control dogs.

A significant reduction in the accumulation of TGF-β mRNA was observed in PBMC (p_{MG+A} = 0.0112) (Fig. 25D) and lymph node cells (p_{MT+A} = 0.0425; p_{MG+A} = 0.0057) (Fig. 25E) of sick dogs in relation to the CG. Bone marrow cells of MG+A group (Fig. 25F) also showed a significant TGF-β downregulation (p = 0.0186).

Although there were differences between sick dogs, these results seem to indicate that *Leishmania* infection can shape the dogs' immune response by inducing IFN-γ upregulation while others pro-inflammatory (IL-2), anti-inflammatory (IL-4) and regulatory (TGF-β) cytokines were downregulated. Additionally, the modulation of TNF-α, a key player in macrophage activation, IL-5, which is involved in the differentiation of activated B lymphocytes into Ig-secreting plasma cells, and IL-10, a cytokine associated with immune regulation, seems to be tissue specific.

2.4.4. Increased gene expression of pro-inflammatory cytokines persists after treatment with miltefosine in combination with allopurinol

Gene expression of cytokines that were modified by infection was further evaluated along all time-points and studied tissues. In dogs treated with MT+A, bone marrow (Fig. 23F) cells evidenced an IL-12 gene expression similar to CG one month after the beginning of treatment and throughout the study, pointing towards normalization. IFN-γ gene expression was still up-regulated in PBMC (p_{Tp1} = 0.0027, p_{Tp2} = 0.0023, p_{Tp3} = 0.0013) (Fig. 23J), lymph node (p_{Tp2} = 0.0061) (Fig. 23K) and bone marrow (p_{Tp1} = 0.0057, p_{Tp2} = 0.0061, p_{Tp3} = 0.0047) (Fig. 23L) cells during the observation period. In lymph node cells, there was a slight increase in IFN-γ mRNA accumulation at Tp2 when compared to Tp1 (p_{Tp1} = 0.0032). Nevertheless, a tendency to normalization was observed in bone marrow.

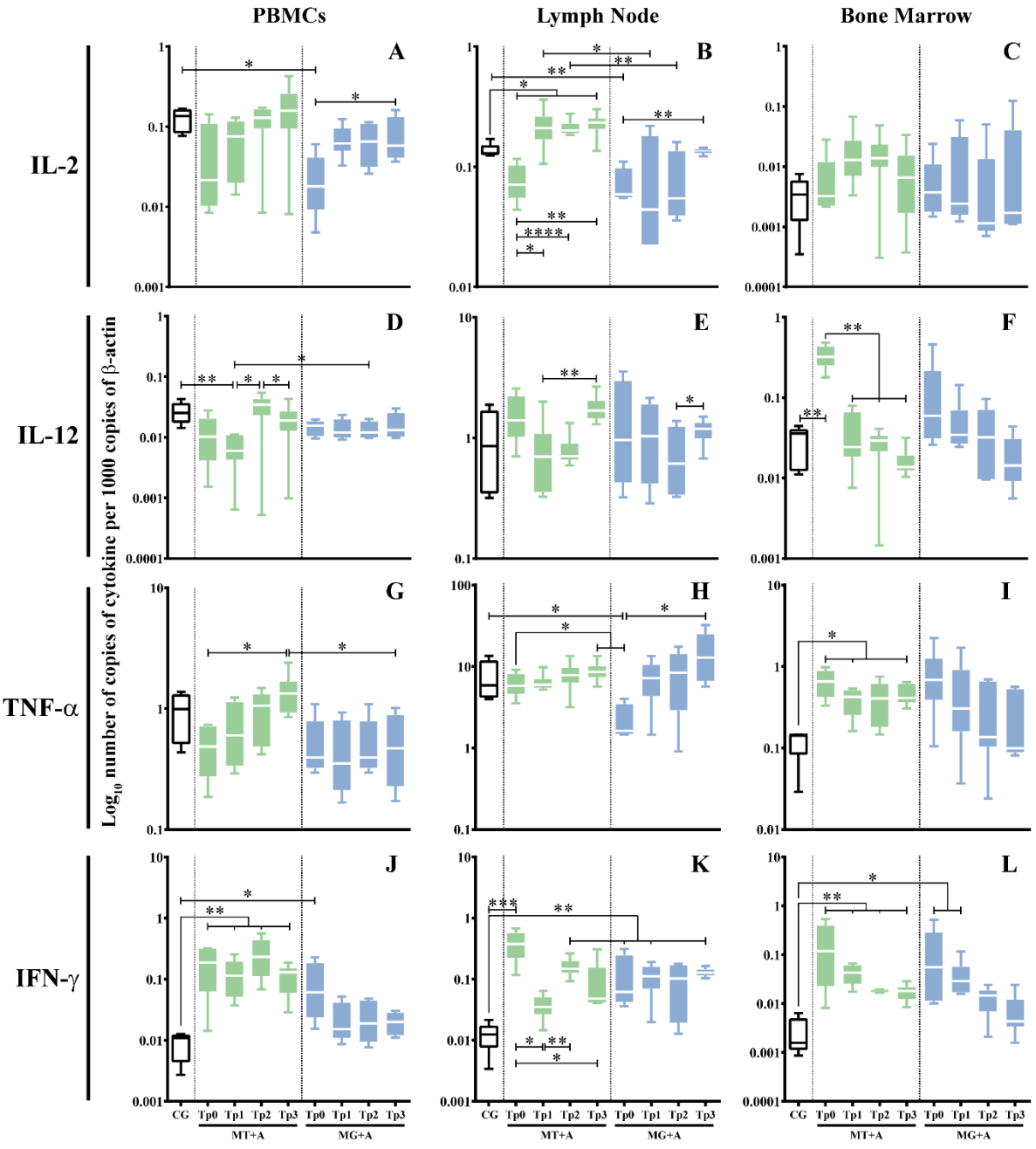

Figure 23. Pro-inflammatory cytokine gene expression in dogs treated with either MT+A or MG+A protocol along all time-points

IL-2 (A, B, C), IL-12 (D, E, F), TNF-α (G, H, I) and IFN-γ (J, K, L) mRNA in PBMC (A, D, G, J), lymph node (B, E, H, K) and bone marrow (C, F, I, L) cells of dogs from MT+A, MG+A and Control Group (CG) was evaluated by qPCR. Results of 17 dogs and three replicates per sample are represented by box and whisker plot, median, minimum and maximum values. The non-parametric Kruskal-Wallis Test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatments groups and the CG. The Repeated Measures ANOVA Test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. * (p<0.05), ** (p<0.01), *** (p<0.001) and **** (p<0.0001) indicate statistical significance.

A significant high amount of IL-2 mRNA was found in lymph node cells at Tp3 (p = 0.0143) when compared to the CG (Fig. 23B). In bone marrow cells, TNF-α gene expression persisted elevated (p_{Tp1} = 0.0303, p_{Tp3} = 0.0481) throughout all time-points (Fig. 23I).

In PBMC, IL-4 gene expression recovered by Tp2 when compared with CG (Fig. 24A), while in bone marrow a low accumulation of IL-4 mRNA (Fig. 24C) was observed (p_{Tp1} = 0.0365, p_{Tp2} = 0.0420) throughout the study. Even so, at TP3 there was a slight upregulation of IL-4 gene expression, revealing a tendency to revert to normal values. Although a fluctuation of IL-5 gene expression was observed (Fig. 24D), at Tp3 it reverts to values compared with CG. In lymph node cells, although IL-5 gene expression maintained increased at Tp1 (Fig. 24E) when compared with CG (p = 0.0124), at Tp3 a trend to reduction in IL-5 mRNA accumulation points towards normalization.

IL-10 and TGF-β gene expression revealed a tendency to recuperation to normal values. Namely, IL-10 mRNA accumulation in lymph node (Fig. 25B) was significantly decreased when compared to Tp0 (p_{Tp1} = 0.0338, p_{Tp2} = 0.0144, p_{Tp3} = 0.0409), along with a significant increase of TGF-β mRNA accumulation in PBMC (p < 0.0001) at Tp3 (Fig. 25D) similar to CG and in lymph node at Tp2 (p = 0.0112) when compared with Tp0 (Fig. 25E).
Despite the generalized tendency of treated dogs to achieve normal levels, the upregulation of pro-inflammatory cytokines (IFN-γ and IL-2) together with the trend to the normalization of anti-inflammatory (IL-4 and IL-5) and regulatory cytokines (IL-10 and TGF-β) point towards a persistent inflammatory immune response during the three months of treatment.

2.4.5. Upregulation of IFN-γ gene expression persists after treatment with meglumine antimoniate combined therapy

Dogs treated with MG+A evidenced a normalization of IFN-γ gene expression in PBMC (Fig. 23J). IFN-γ gene expression remained significantly higher (p_{Tp1} = 0.0029, p_{Tp3} = 0.0018) in lymph node cells in comparison to CG (Fig. 23K). On the contrary, bone marrow cells (Fig. 23L) showed a progressive decrease of IFN-γ mRNA. At Tp1 (p = 0.0425) the values were significantly increased when compared to the CG. However, by Tp2 and Tp3, IFN-γ gene expression lowered towards levels comparable to the CG. On the other hand, IL-2 (Fig. 23B) and TNF-α (Fig. 23H) gene expression in lymph node cells was similar to the CG. IL-2 at Tp1 in PBMC (Fig. 23A) presented values similar to control dogs, with Tp3 having significant difference to Tp0 (p = 0.0425). The same was verified in lymph node, with IL-2 recovering to amounts comparable to CG by Tp3 (p = 0.0098). TNF-α (Fig. 23H) in lymph node recovered to values similar to CG showing a significant difference when compared to Tp0 (p = 0.0451).

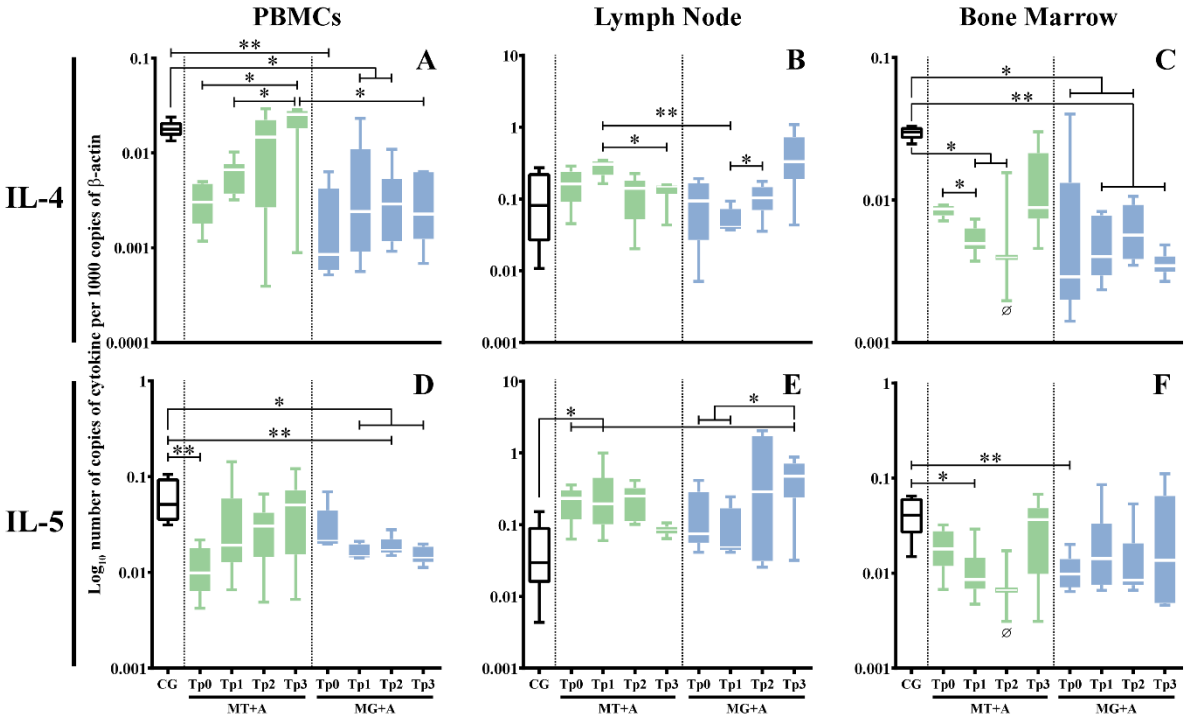

Figure 24. Anti-inflammatory cytokine gene expression in dogs treated with either MT+A or MG+A protocol along all time-points

IL-4 (A, B, C) and IL-5 (D, E, F) mRNA in PBMC (A, D), lymph node (B, E) and bone marrow (C, F) cells of dogs from MT+A, MG+A and Control Group (CG) was evaluated by qPCR. Results of 17 dogs and three replicates per sample are represented by box and whisker plot, median, minimum and maximum values. The non-parametric Kruskal-Wallis Test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatments groups and the CG. The Repeated Measures ANOVA Test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. * (p<0.05) and ** (p<0.01) indicate statistical significance. Ø shows mRNA expression values of only three dogs.

During treatment follow-up, IL-4 gene expression remained downregulated in PBMC (p_{Tp1} = 0.0219, p_{Tp2} = 0.0297) (Fig. 24A) and in bone marrow cells (p_{Tp1} = 0.0068, p_{Tp2} = 0.0229, p_{Tp3} = 0.0013) (Fig. 24C) when compared with the CG. In PBMC, IL-5 gene expression was also downregulated (p_{Tp1} = 0.0199, p_{Tp2} = 0.0071, p_{Tp3} = 0.0343) (Fig. 24D). Despite a slight reduction in the accumulation of IL-5 mRNA in bone at Tp2, it was noticed a tendency to normalization (Fig. 24F).

During treatment, accumulation of IL-10 (p_{Tp1} = 0.0076, p_{Tp2} = 0.0101, p_{Tp3} = 0.0108) (Fig. 25A) and TGF-β (p_{Tp1} = 0.0192, p_{Tp2} = 0.0235, p_{Tp3} = 0.0473) (Fig. 25D) mRNA was highly reduced in PBMC when compared to the CG. However, in lymph node cells, IL-10 (Fig. 25B) and TGF-β (Fig. 25E) gene expression was similar to control dogs. Nonetheless, at Tp3, a slight increase of TGF-β mRNA in lymph node was observed when compared to Tp2 (p = 0.0285). Despite bone marrow cells showed a normalization of TGF-β gene expression (Fig. 25F), two months after treatment (Tp3) a significant decrease of TGF-β mRNA accumulation (p = 0.0453) was observed when compared to healthy dogs. *Leishmania* infected dogs treated

with meglumine antimoniate in combination with allopurinol (MG+A) evidence a generalized tendency to achieve normal cytokine levels in the *Leishmania* host tissues evaluated in the current study. However, the persistent upregulation of IFN-γ gene expression associated with downregulation of IL-4, IL-5, IL-10 and TGF-β gene expression indicates the possible predominance of an inflammatory immune response. On the other hand, the slight increase of TGF-β at Tp3 in the lymph node can point towards the local activation of a regulatory immune response.

2.4.6. The activity of miltefosine and meglumine antimoniate combined therapies can influence cytokine gene expression

To estimate the influence of the drugs in cytokine generation, the cytokines that were not significantly altered by *Leishmania* infection were analyzed by comparing gene expression of sick (Tp0) and treated dogs (Tp1-Tp3).

After the first month of treatment (Tp1) with miltefosine in association with allopurinol (MT+A), PBMC evidenced a downregulation of IL-12 ($p = 0.0025$) (Fig. 23D) and IL-10 (Fig. 25A), and a slight upregulation of IL-2 (Fig. 23A) and TNF-α (Fig. 23G). During the second- and third-month IL-2, TNF-α and IL-10 showed a progressive upregulation, with IL-12 having an accentuated increase at Tp2 ($p = 0.0411$) and a slight decrease by Tp3 ($p = 0.0233$). Regarding lymph node cells , it was observed a considerable upregulation of IL-12 (Fig. 23E) after Tp1 ($p = 0.0062$), along with a slight overexpression of TNF-α (Fig. 23H) at Tp2 and Tp3 ($p = 0.0138$) time-points and a considerable gene expression of IL-4 (Fig. 24B) at Tp1 followed by downregulation by Tp2 and Tp3 ($p = 0.0410$). In bone marrow, the treatment caused accumulation of IL-2 (Fig. 23C) mRNA that persisted until Tp2, along with an increase of TGF-β (Fig. 25F) that peaked at Tp3 ($p = 0.0106$). IL-5 (Fig. 24F) mRNA levels showed a downregulation by Tp1 ($p = 0.0343$) that persisted until Tp2. A progressive downregulation of IL-10 (Fig. 25C) was evident from Tp1 to Tp3 ($p = 0.0452$).

PBMC of dogs treated with MG+A showed a progressive downregulation of IL-12 (Fig. 23D) from Tp0 to Tp2, followed by an increase at Tp3, and a progressive TNF-α (Fig. 23G) increase from Tp0 that reached maximum values by Tp3. Lymph node cells presented IL-12 (Fig. 23E) mRNA levels increased by Tp3 ($p = 0.0484$), exhibiting a slight and transitory downregulation of IL-4 (Fig. 24B) levels at Tp1 ($p = 0.0293$) followed by a progressive upregulation that peaked at Tp3. IL-5 (Fig. 24E) was slightly downregulated at Tp1 but showed an accentuate increase when meglumine antimoniate was discontinued (Tp2), with a slight decrease by Tp3 ($p = 0.0344$). Regarding bone marrow cells, a continuous decrease in IL-12 and TNF-α mRNA accumulation was noticed from Tp1 to Tp3. However, IL-2 gene expression

presented an irregular pattern, suffering a downregulation at Tp2. When compared with Tp0, IL-10 presented a progressive downregulation until Tp3.

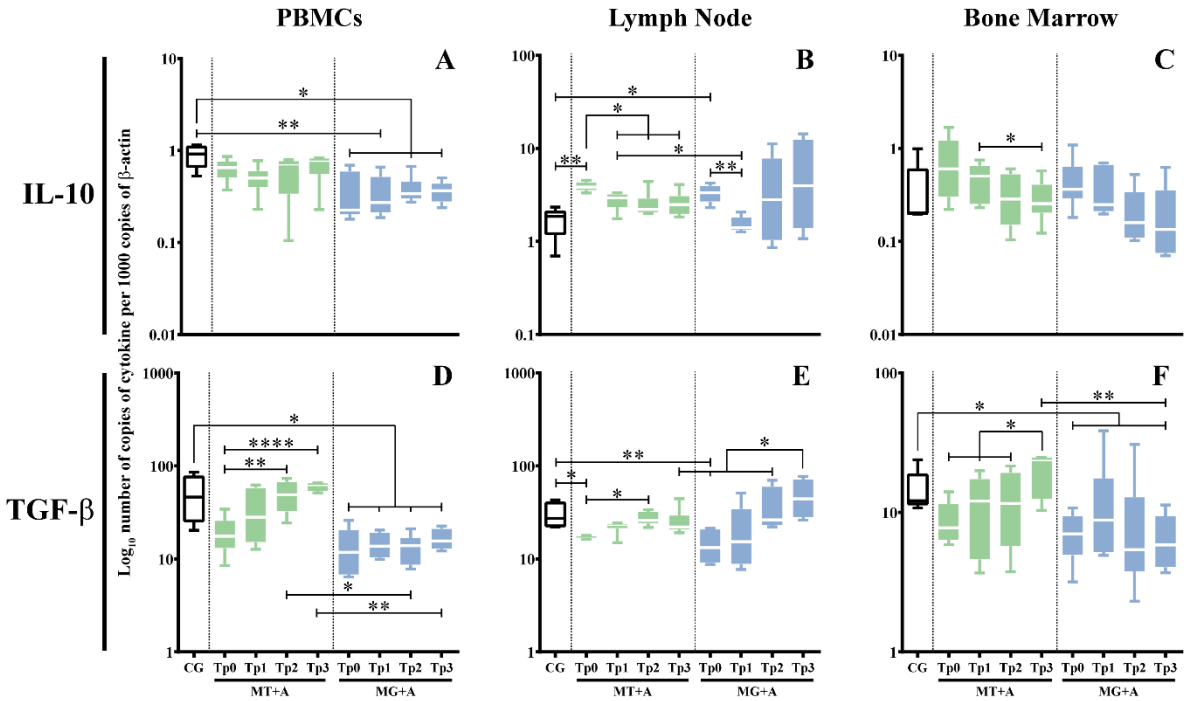

Figure 25. Regulatory cytokine gene expression in dogs treated with either MT+A or MG+A protocol along all time-points
IL-10 (A, B, C) and TGF-β (D, E, F) mRNA in PBMC (A, D), lymph node (B, E) and bone marrow (C, F) cells of dogs from MT+A, MG+A and Control Group (CG) was evaluated by qPCR. Results of 17 dogs and three replicates per sample are represented by box and whisker plot, median, minimum and maximum values. The non-parametric Kruskal-Wallis Test (one-way ANOVA on ranks) with Dunn's post hoc test was used for statistical comparisons between treatments groups and the CG. The Repeated Measures ANOVA Test with Tukey's post hoc test was used for statistical comparisons inside each treatment group. * (p<0.05), ** (p<0.01) and **** (p<0.0001) indicate statistical significance.

These findings indicate that MG+A directs the overexpression of cytokines in blood and lymph node and is possible that allopurinol plays a key role in enhancing cytokine generation. In the bone marrow, the drugs seem to downregulate cytokine gene expression. MT+A also seems to enhance cytokine gene expression. However, when miltefosine was discontinued, IL-4 in lymph node and IL-10 in the bone marrow became downregulated.

2.5. Discussion

Progression of *L. infantum* infection is mainly dependent on the competence of the dog's immune system, which is related to inherent characteristics such as genetic background. Thus, the spectrum of clinical manifestations can range from subclinical infection to severe disease. During active disease, dog's immune response has been mainly characterized by a

marked humoral immune response and specific immunosuppression of T lymphocyte proliferation (Carvalho et al. 1989). Despite being the major domestic reservoir of *L. infantum*, dogs have also intrinsic value, more notably a recognized social and affective role. Therefore, the use of therapies that can ensure a successful CanL treatment is highly required.

Several studies have pointed out the efficacy and faster recovery rate of dogs treated with meglumine antimoniate in combination with allopurinol (Denerolle and Bourdoiseau 1999; Miró et al. 2009; Torres et al. 2011; Manna et al. 2015). Regarding the progress of hematological, biochemical and urinary parameters, it is worth to emphasize that both combined therapies used in the current study were able to recover erythrocytes, hemoglobin, hematocrit and UCP normal values, while leukocytes, neutrophils, creatinine and albumin were within the reference intervals during the three months of treatment. Dogs evidencing less clinicopathological alterations, that were selected to be treated with meglumine antimoniate in combination with allopurinol, presented a fast recovery of hematological, biochemical and urinary parameters. Dogs showing more clinicopathological alterations, and which were treated with miltefosine in combination with allopurinol, took longer to reach normalization of those parameters.

Three months after CanL diagnosis (Tp3), both combined therapies were successful in promoting remission of clinical signs, recovering of hematological and biochemical normal values in all dogs and in restraining parasite infection since amastigotes were not found in the bone marrow and lymph node smears. Anti-parasite antibodies also diminished to non-significant titers in most of the dogs, with only one dog treated with MG+A taking more time to become negative (> 3 months).

During CanL, *L. infantum* parasites are hosted in several organs of the reticuloendothelial system, having a widespread influence on the host's immune system. As previously reported (Quinnell et al. 2001; Alves et al. 2009; Barbosa et al. 2011), in CanL, IFN-γ gene expression is increased in parasite-host tissue prior to any treatment. Also, in the current study PBMC, lymph node and bone marrow cells evidenced a pronounced generation of IFN-γ. Although such immune response is widely verified in many other studies, it also raised the question if this Th1 immune response is positively correlated with parasite control. Previous studies in experimentally infected hamsters and in humans suffering from visceral leishmaniosis have shown high parasite loads in Th1 environments, indicating an IFN-γ inability to confer protection (Kenney et al. 1998; Melby et al. 1998). Thus, the main consensus indicates that sick dogs express high levels of IFN-γ in *Leishmania*-target tissues, possibly directing a Th1 immune response against persistent infection.

The most studied tissue regarding cytokine expression during CanL is the peripheral blood, which in animals presenting clinical signs is characterized as having suppression of T

cell mediated immunity and production of high levels of specific antibodies (Pinelli, van der Kaaij, et al. 1999), as a consequence of a predominantly Th2 response with production of anti-inflammatory cytokines, such as IL-4 and IL-5 (Mosmann and Moore 1991). In the present study, with the exception of high IFN-γ gene expression, peripheral blood IL-2, TGF-β, IL-4 and IL-5 of non-treated dogs were decreased, suggesting that *Leishmania* caused an overall lymphocyte deactivation, leading to unbalance of pro- and anti-inflammatory immune mediators. Still, taking into consideration that the peripheral blood is not the tissue of election for *L. infantum* replication and persistence (Peters and Sacks 2006; Maia and Campino 2012), along with possible natural genetic variability between dogs, it may be the reason why there is so much divergence between studies regarding cytokine expression in this tissue.

Despite most of CanL studies being focused in only one tissue, usually the peripheral blood, more and more studies consider that every single tissue affected by this parasite presents its own immune response (Gomes-Pereira et al. 2004; Alves et al. 2009; Alexandre-Pires et al. 2010; Barbosa et al. 2011). IL-10 is a key regulatory cytokine that prevents excessive pathology. This cytokine can negatively regulate innate and adaptive immune responses by impairing the production of pro-inflammatory (e.g. IL-12, IL-2, IFN-γ and TNF-α) and anti-inflammatory (IL-4 and IL-5) cytokines, restraining T cell activity in lymph nodes and limiting tissue inflammation. In CanL, the lymph node is reported as having a predominantly Th1 immune response (Barbosa et al. 2011). Besides this, a true consensus has not been established, with studies showing higher expression of Th1 cytokines, like IFN-γ and TNF-α (Garden et al. 2011), in pre-scapular lymph nodes of dogs without external clinical signs and lower parasite burden, pointing towards a possible role of these cytokines in controlling parasite replication. In contrast, dogs presenting clinical signs showed no expression of IL-4 and IL-12, but high levels of immunosuppressor cytokines like IL-10 and TGF-β (Garden et al. 2011), posing a role in disease progression. In the current study, the lymph node of dogs with CanL seems to evidence a mixed Th1/Treg immune response with low IL-2, but high IL-12 and IFN-γ, along with down expression of TGF-β but over expression of IL-10, pointing towards a balance between the differentiation of IFN-γ mediated inflammatory response and a regulatory immune response that could favor parasite persistence.

Considering the cytokine expression in bone marrow of dogs with CanL, to our best knowledge, there are only a few documented studies (Quinnell et al. 2001; Alves et al. 2009; Barbosa et al. 2011), which report this tissue as a predominantly Th1 environment that tends to develop high parasite loads, characterized by an increased expression of IFN-γ and TNF-α and low to no detection of IL-10, along with lower expression of IL-4 (Quinnell et al. 2001; Alves et al. 2009). In the current study, bone marrow cells of sick dogs also evidence IFN-γ overexpression and low expression of IL-4, IL-5 and TGF-β pointing to a predominantly pro-

inflammatory immune response. Furthermore, the PCA and cluster analysis reinforce that each tissue presents a distinct cytokine pattern of response to infection, confirming previous reports (Barbosa et al. 2011).Furthermore, infection level also seems to influence local cytokine gene expression, namely TNF-α, that points towards a diminished generation of this cytokine in lymph node cells of dogs presenting less clinicopathological signs (MG+A), and overexpression in bone marrow cells of dogs with severe clinicopathological signs (MT+A). TNF-α together with IFN-γ induce the upregulation of inducible nitric oxide synthase (iNOS) by macrophages, directing L-arginine oxidation and nitric oxide (NO) production (Nathan and Hibbs 1991). NO is a powerful oxidative molecule that mediates parasite killing. Thus, the hypothesis that TNF-α can be a biomarker of CanL severity needs to be further investigated. Furthermore, IL-5, a cytokine linked to growth and differentiation of B cells, evidenced to be over-expressed in lymph node cells of dogs presenting more clinicopathological signs (MT+A). These findings point to a higher B cell activation in lymph node. The over expression of IL-12 in bone marrow cells of dogs exhibiting more clinicopathological signs (MT+A), a signaling pathway cytokine that prime naïve T cells to differentiate into Th1 cells, supports the possible establishment of a Th1 cell population.

By analyzing the peripheral blood, popliteal lymph node and bone marrow along the course of two of the most used CanL protocol treatments, the current study shows evidence of a higher IFN-γ generation during the three months of follow up of dogs treated with MT+A. Furthermore, lymph node cells also exhibited a TNF-α overexpression, suggesting that there are conditions for macrophage activation and parasite inactivation, and increased generation of IL-2, indicating a possible lymphocyte proliferation. These findings indicate that miltefosine associated therapy does not promote reduction of pro-inflammatory immune response, but, induces the normalization of anti-inflammatory IL-4 and IL-5 and of immune-suppressor TGF-β in mononuclear blood cells, of immune-suppressor IL-10 in lymph node and of IL-5, TGF-β and pro-inflammatory IL-12 in bone marrow.

MG+A lead to the normalization of the pro-inflammatory immune response, restoring IFN-γ and IL-2 expression levels in blood cells, IL-2, IL-12 and TNF-α in lymph node and IFN-γ in the bone marrow. Although showing some instability, IL-5 tends to normal values in the bone marrow. Treatment also seems to induce the normalization of immunosuppressor cytokines in the lymph node. However, the continuous overexpression of IFN-γ in lymph node cells points towards the maintenance of a local inflammatory response despite the drug activity in promoting the remission of clinical signs, and the rise of IFN-γ gene expression in mononuclear blood cells one-month post-treatment suggests the predomination of a Th1 immune response. On the other hand, IL-4 and IL-5 stay downregulated in mononuclear blood cells as well as IL-10 and TGF-β indicating the inhibition of Th2 and Treg immune response

even during dogs' clinical improvement. In bone marrow as well, treatment did not induce the normalization of IL-4 gene expression.

The effect of drug therapies used in the current study in cytokine gene expression was investigated in the cytokines that were not significantly affected by parasite infection (Tp0). Although combined therapies seem to have similar outcomes, it was not possible to find a distinctive pattern, exhibiting cytokine, and tissue dependent effects. The drug activity possibly empowered by free parasite antigens seems to favor mainly cytokine generation.

The current study enables a close overview of the effect of the two most used antileishmanial therapies, miltefosine and meglumine antimoniate in association with allopurinol, in reversing CanL progression on naturally infected dogs, including clinical signs remission, normalization of hematological, biochemical and urinary parameters, and IFAT seroconversion. Both combined therapies are effective in CanL treatment, favoring clinical recovery of all dogs and the overexpression of pro-inflammatory cytokines, pointing towards the persistence of inflammatory immune environments that can direct parasite inactivation at least during the initial three months of treatment. The current study also demonstrates that anti-inflammatory and regulatory cytokines do not seem to play a key role in CanL immune response. Furthermore, the combined therapies also appear to play a direct role in cytokine generation. These are relevant findings, since both are two of the most used protocols in the treatment of this zoonotic parasitosis, the evolution of the cell-mediated immune response generated while under these specific treatments should be further studied. With the recent implementation of miltefosine for CanL treatment in Brazil, an extremely endemic country for canine and human leishmaniosis, it becomes a subject of ensuring the best for the reinforcement of Public Health protection.

Authors' contributions

GS, CM, MS and IF conceived and designed the study. MS, CM, MP, JG, JC, AB, AR, JM, IF collected samples. MS, LG and IF processed samples and did subsequent microscopic, molecular and serological tests. MS and CM conducted the experiments. MS, GS, IF and MB analyzed the data. MS and GS conducted statistical analysis. MS, GS and IF drafted the manuscript. GS, IF, GA, MB, AD, LT, AR, MB and DS made in depth reviews of the manuscript. All authors read and approved the final manuscript.

Funding

Funding for this work was provided by the Portuguese Foundation for Science and Technology (FCT) (PTDC/CVT/118566/2010), the Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), Faculdade de Medicina Veterinária, Universidade de Lisboa,

through project UID/CVT/00276/2019 (Funded by FCT) and Global Health and Tropical Medicine (GHTM) through project UID/Multi/04413/2013 (Funded by FCT). Marcos Santos (SFRH/BD/101467/2014) held a PhD scholarship from the Portuguese Foundation for Science and Technology (FCT).

Acknowledgements

The authors would like to thank the cooperation and all the help provided by the veterinarians and staff of the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon (FMV-ULisboa), along with MSc Telmo Pina Nunes for the statistical counseling and a special acknowledge to the owners and their respective dogs for their contribution to this study.

CHAPTER III:

Immunophenotyping of peripheral blood, lymph node, and bone marrow T lymphocytes during canine leishmaniosis and the impact of antileishmanial chemotherapy

Marcos F. Santos, Graça Alexandre-Pires, Maria A. Pereira, Lídia Gomes, Armanda V. Rodrigues, Alexandra Basso, Ana Reisinho, José Meireles, Gabriela M. Santos-Gomes, Isabel P. da Fonseca (2020).

In press in Frontiers in Veterinary Science. doi: 10.3389/fvets.2020.00375

3.1. Abstract

Dogs are a major reservoir of *Leishmania infantum*, etiological agent of canine leishmaniosis (CanL) a zoonotic visceral disease of worldwide concern. Therapeutic protocols based on antileishmanial drugs are commonly used to treat sick dogs and improve their clinical condition. To better understand the impact of *Leishmania* infection and antileishmanial drugs on the dog's immune response, this study investigates the profile of $CD4^+$ and $CD8^+$ T cell subsets in peripheral blood, lymph node and bone marrow of sick dogs and after two different CanL treatments. Two CanL groups of six dogs each were treated with either miltefosine or meglumine antimoniate combined with allopurinol. Another group of ten clinically healthy dogs was used as control. Upon diagnosis and during the following 3 months of treatment, peripheral blood, popliteal lymph node, and bone marrow mononuclear cells were collected, labeled for surface markers CD45, CD3, CD4, CD8, CD25, and intracellular nuclear factor FoxP3, and T lymphocyte subpopulations were immunophenotyped by flow cytometry. CanL dogs presented an overall increased frequency of $CD8^+$ and $CD4^+CD8^+$ double-positive T cells in all tissues and a decreased frequency of $CD4^+$ T cells in the blood. Furthermore, there was a higher frequency of $CD8^+$ T cells expressing $CD25^+FoxP3^+$ in the blood and bone marrow. During treatment, these subsets recovered to levels similar those of healthy dogs. Nevertheless, antileishmanial therapy caused an increase of $CD4^+CD25^+FoxP3^+$ T cells in all tissues, associated with the decrease of $CD8^+CD25^-FoxP3^-$ T cell percentages. These findings may support previous studies that indicate that *L. infantum* manipulates the dog's immune system to avoid the development of a protective response, ensuring the parasite's survival and the conditions that allow the completion of Leishmania life cycle. Both treatments used appear to have an effect on the dog's immune response, proving to be effective in promoting the normalization of T cell subsets.

Keywords: Antileishmanial therapy; Bone Marrow; Canine leishmaniosis; Effector T cells; Flow Cytometry; Lymph node; Peripheral blood mononuclear cells; Treg cells.

3.2. Introduction

Leishmaniosis is considered a neglected tropical disease (WHO 2019a) that affects humans and domestic and sylvatic animals. Parasites of the genus *Leishmania* are obligatory intracellular protozoa and the etiological agent of this parasitic disease (Novo et al. 2016). The main host cell for *Leishmania* parasites is the macrophage, which the parasite is able to manipulate and prevent activation by various mechanisms and, thus, avoid their intracellular death and perpetuate the infection (van Zandbergen et al. 2004; Cecílio et al. 2014; Martínez-López et al. 2018). Canine leishmaniosis (CanL), endemic in about 50 countries and two major regions, South America and the Mediterranean basin, is caused by *Leishmania infantum* (Baneth et al. 2008). Dogs affected by this disease can present a wide variety of specific and unspecific clinical signs (Solano-Gallego et al. 2009; LeishVet Guidelines 2018). CanL conventional treatments improve the clinical condition of dogs and reduce the parasite burden (Nogueira et al. 2019). Although when therapy is discontinued, relapses are common (João et al. 2006; Ikeda-Garcia et al. 2007; Manna et al. 2009), indicating that treatment does not promote parasite clearance in all cases. Thus, it is important to improve the efficacy of the treatment protocols applied to CanL to promote the clinical cure of the dog, ensure parasite clearance and prevent further transmission. According to the most recent guidelines (LeishVet Guidelines 2018), the recommended CanL treatment protocols combine allopurinol with either meglumine antimoniate or miltefosine. Meglumine antimoniate is a pentavalent antimonial considered a multifactorial drug whose effects are still unclear. However, some authors have referred the promotion of *Leishmania* DNA damage by oxidative stress and influence on macrophage microbicidal activity (Frézard et al. 2009; Mcgwire and Satoskar 2014; Moreira et al. 2017). Pentavalent antimonials, which belong to the same family of meglumine antimoniate, such as sodium antimony gluconate, have been shown to interfere with the host's immune system by activating macrophages to release interleukin 12 (IL-12), leading to the subsequent production of interferon-γ (IFN-γ) by other immune cells, that induce the phosphorylation of extracellular signal-regulated kinase 1 (ERK-1) and ERK-2, driving the production of reactive oxygen species (ROS) (Basu et al. 2006). Moreover, they also appear to induce the expression of class I molecules of the major histocompatibility complex (MHC), stimulating $CD8^+$ T cells that lead to apoptosis of infected cells (Haldar et al. 2011; Passero et al. 2018). Although these drugs have proved antileishmanial activity *in vitro* and *in vivo*, pentavalent antimonials have failed to treat visceral leishmaniosis in human patients who are also infected with HIV or receiving immunosuppressive therapy (Haldar et al. 2011), indicating that a complete cure is dependent on T cell-mediated responses (Murray et al. 1989; Murray et al. 1991). Miltefosine is an alkylphosphocholine compound able to induce apoptosis by mechanisms still not entirely

clear, although the specific disturbance of the lipid content on the parasite's membrane and the modulation of macrophage activity are the most consensual modes of action (Pérez-Victoria et al. 2006; Sundar and Olliaro 2007; Bianciardi et al. 2009; Dorlo et al. 2012; Passero et al. 2018). Several studies have reported the immunomodulatory properties of miltefosine, with *in vitro* studies showing the induction of the release of tumor necrosis factor α (TNF-α)and nitric oxide (NO) by peritoneal macrophages of BALB/c mice (Zeisig et al. 1995) and enhancement of IFN-γ receptors, thus restoring responsiveness to this cytokine in macrophages infected by *L. donovani* and promoting an IL-12 dependent Th1 response (Wadhone et al. 2009). Also, in healthy human peripheral blood cells, it was found that miltefosine was able to increase the production of IFN-γ, acting as a co-stimulator of the IL-2-mediated T cell activation process, together with increased expression of CD25, showing the possible immunomodulatory activity of miltefosine (Vehmeyer et al. 1991). Allopurinol, a purine analog of adenosine nucleotide, blocks RNA synthesis, inhibiting *Leishmania* growth (Denerolle and Bourdoiseau 1999; Page 2008). To date, meglumine antimoniate or miltefosine in combination with allopurinol are both considered first-line treatments in Europe (Solano-Gallego et al. 2009; LeishVet Guidelines 2018). Recently, in Brazil, miltefosine therapy was approved for CanL treatment (Ribeiro et al. 2018). Taking into account the emergence of a greater number of reports on drug resistance, whether it be in humans or dogs (Pérez-Victoria et al. 2006; Frézard et al. 2009; Haldar et al. 2011; Yasur-Landau et al. 2016), it is crucial to deepen the understanding of the mode of action of the most used antileishmanial therapies.

In dogs, disease outcome is mainly determined by the cell-mediated immune response, with T cells playing a key role in cytokine release, which interacts with infected macrophages, influencing macrophage activation and subsequent killing of internalized parasites. According to the cytokine environment, naïve $CD4^+$ T lymphocytes can differentiate into a protective subset (Th1) or a Th2 cell subset, which favors the progress of infection (Pinelli et al. 1994). A protective Th1 immune response is characterized by a high production of pro-inflammatory cytokines as is the case of IFN-γ, TNF-α and IL-2. These cytokines stimulate the cytotoxic activity of $CD8^+$ T cells and activate macrophage respiratory burst, leading to the synthesis of ROS and induce NO production, which can cause major damage to the parasite membrane, leading to the death of the parasite (Liew and O'Donnell 1993; Pinelli et al. 1994; Santos-Gomes et al. 2002). On the other hand, a Th2 response directs the release of anti-inflammatory cytokines and stimulates the humoral immune response, favoring the establishment of infection and disease exacerbation (Baneth et al. 2008; Solano-Gallego et al. 2009). Previous works on symptomatic dogs with CanL have demonstrated that the lack of adequate cell-mediated immune response might be associated with decreased levels of $CD4^+$ T cells and high antibody titers (Bourdoiseau, Bonnefont, Magnol, et al. 1997; Moreno et al. 1999; Guarga

et al. 2000; Alvar et al. 2004). *In vitro* studies of cytotoxic $CD8^+$ T cells from asymptomatic dogs demonstrated a role in resistance to CanL by enhancing IFN-γ production and causing the lysis of infected macrophages (Pinelli et al. 1995).

A critical role of immune regulation has been attributed to a sub-group of cells denominated regulatory T (Treg) cells, which seem to be recruited to the sites of *Leishmania* infection, enabling parasite survival and ensuring the transmission cycle (Belkaid et al. 2002; Mendez et al. 2004). Experimental studies of cutaneous leishmaniosis performed in *L. major*-infected mice showed that Treg cells are essential for the development and maintenance of persistent cutaneous disease (Belkaid et al. 2002). The fast increase of $CD4^+CD25^+$ Treg cells at the sites of *L. major* infection suppressed parasite-eliminating immune mechanisms (Mendez et al. 2004). Accumulation of IL-10-producing Treg cells observed in the bone marrow of patients with *L. donovani* visceral leishmaniosis can cause immunosuppression, prevent the release of pro-inflammatory cytokines, like IFN-γ, avoid macrophage activation and be associated with unresponsiveness to treatment (Rai et al. 2012). Another study showed increased $CD4^+CD25^+$ Treg cells exhibiting high levels of Forkhead box Protein 3 (FoxP3) gene expression along with transformation growth factor β (TGF-β) in spleen and draining lymph nodes of BALB/c mice infected with *L. infantum* (Rodrigues et al. 2009). This cell subpopulation contributes to immunosuppression and control of parasite-mediated-immunopathology during infection. Treg cell subsets that constitutively express CD25 and synthesize IL-10 and TGF-β drive the suppression of cell-mediated immune responses (Allos et al. 2019). These cells are considered potent suppressors of the activation of $CD8^+$ T cells (Piccirillo and Shevach 2001). Nevertheless, another study showed a reduced percentage of $CD3^+CD4^+FoxP3^+$ Treg cells in dogs infected with *L. infantum*, independently of antibody titer (Cortese et al. 2013). Although $CD8^+$ T suppressor cells have been identified, their mode of action and purpose are not fully understood (Shevach 2006). Some studies have shown that resting $CD4^+$ lymphocytes are resistant to $CD8^+CD25^+FoxP3^+$ Treg cells, which indicates that the initiation of cell-mediated immune response is not likely to be affected by $CD8^+$ Treg cells. In contrast, $CD8^+$ Treg cells can play a critical role in suppressing ongoing $CD4^+$ T cell responses (Hu et al. 2012). Besides, the activity of $CD4^+CD25^+FoxP3^+$ Treg cells appears to be mediated through the release of immune-suppressive cytokines and by cell contact-dependent mechanisms (Hu et al. 2012). With regard to leishmaniosis, few studies focus on Treg cells, and less are those that have analyzed the $CD8^+$ Treg cell fraction. Tiwananthagorn et al. (2012) reported that in the liver of *L. donovani*-infected mice, $CD4^+FoxP3^+$ Treg cells, but not $CD8^+FoxP3^+$ T cells, are essential for the increased susceptibility to *Leishmania* infection and high IL-10 production.

T cells expressing both CD4 and CD8 have been identified in peripheral blood and secondary lymphoid organs of several species, such as pigs, monkeys, humans, chickens, rats, mice, and dogs (Zuckermann and Husmann 1996; Akari et al. 1997; Mizuki et al. 1998; Zuckermann 1999; Kenny et al. 2000; Hillemeyer et al. 2002; Alexandre-Pires et al. 2010). These $CD4^+CD8^+$ double-positive cells appear to constitute memory $CD4^+$ helper T cells that, upon activation, develop the ability to express the CD8α chain and, in cases such as pigs, produce high levels of IFN-γ in response to stimulation with viral antigens (Zuckermann and Husmann 1996). This subpopulation has been identified as being increased in chronic diseases, such as cancer, autoimmune diseases, and viral infections (Matsui et al. 1989; Bagot et al. 1998; Kitchen et al. 2004; Desfrançois et al. 2010; Talker et al. 2015). Several studies have also reported the presence of CD25 and FoxP3 in DP T cells of dogs, revealing a possible regulatory activity among this subpopulation (Rothe et al. 2017; Rabiger et al. 2019).

Thus, the current study aims to evaluate the kinetics of $CD4^+$ and $CD8^+$ T cell subsets in tissues that commonly harbor *Leishmania* parasites in both sick and treated dogs. Sick dogs (CanL) were treated by two of the most used protocols for CanL during a 3-month period, and peripheral blood, lymph node, and bone marrow T cells were immunophenotyped.

3.3. Materials and Methods

3.3.1. Dog selection

Twenty-three household dogs living in the endemic area of the Metropolitan Region of Lisbon (Portugal) were diagnosed with CanL at the clinical stage I/II, according to the LeishVet Consensus Guidelines (Solano-Gallego et al. 2011), and at stage C, following the Canine Leishmaniasis Working Group Guidelines (Paltrinieri et al. 2010). Twelve of these sick dogs fulfilled the minimum requirements to enter the study (Fig. 26), which included having at least 1.5 years of age, weighing more than 5 kg, not having been vaccinated for leishmaniosis, being negative for circulating pathogens potentially responsible of canine vector-borne diseases (CVBDs), and have not undergone any treatment in the last 8 months that could interfere with the immune response (such as corticosteroids, antibiotics, or immunomodulators). The present study also included a control group of 10 clinically healthy dogs that were negative for *Leishmania* antibodies and other CVBDs and not vaccinated for leishmaniosis. All dog owners gave written consent after being informed about the objectives of the study and every procedure. The selected animals included 15 males and 7 females of various breeds, with ages ranging between 2 and 9 years and weight between 7.6 and 32.1 kg. Clinical examination

and sample collection were done by veterinarians at the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon.

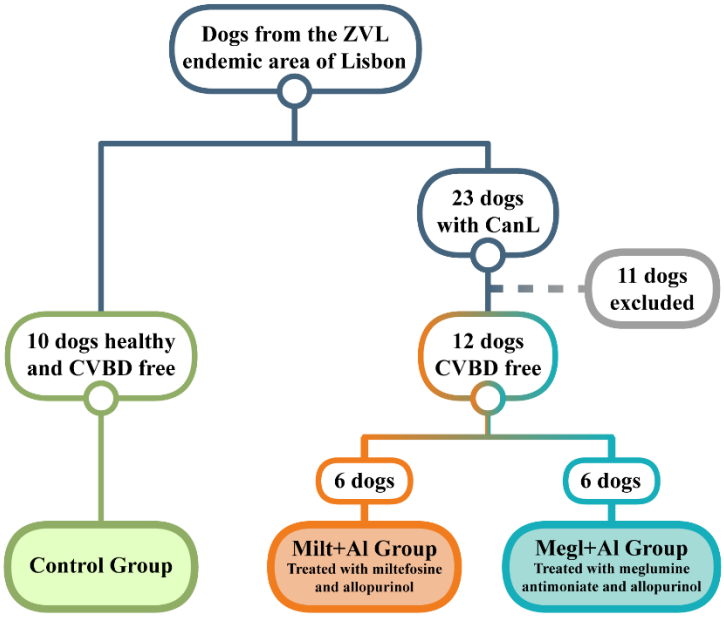

Figure 26. Dog selection diagram used in the current study
From a population of dogs living in an endemic area of zoonotic visceral leishmaniosis (ZVL), two groups clinically diagnosed with canine leishmaniosis (CanL) were established and treated with either miltefosine in combination with allopurinol (Milt+Al) or meglumine antimoniate in association with allopurinol (Megl+Al). A group of clinically healthy dogs and free of any canine vector-borne disease (CVBD) was also selected as the control group.

As previously described by our group (Santos et al. 2019), dogs diagnosed with CanL that presented biochemical parameters such as increased blood urea nitrogen (BUN), creatinine, and/or alanine aminotransferase (ALT), aspartate aminotransferase (AST), and urine protein-to-creatinine (UPC) ratio between 0.2 and 0.6, which point to the possibility of developing hepatic and renal lesions, were treated with miltefosine [Milteforan®, Virbac S.A, France; 2 mg/kg *per os*, *semel in die* (SID) for 4 weeks] combined with allopurinol [Zyloric®, Laboratórios Vitória, Portugal; 10 mg/kg, *per os*, *bis in die* (BID) for at least 6 months], and correspond to Group Milt+Al. Dogs that exhibited changes in serum proteins and UPC ratios between 0.2 and 0.4 were treated with meglumine antimoniate (Glucantime®, Merial Portuguesa, Portugal; 100 mg/kg SID for 4 weeks) combined with allopurinol (10 mg/kg, *per os*, BID for at least 6 months) and were included in Group Megl+Al. To prevent new infections during the study and *Leishmania* transmission, deltamethrin-impregnated collars were applied to all dogs.

3.3.2. Experimental design

To investigate the effect of *Leishmania* infection and antileishmanial treatments in helper, cytotoxic, and regulatory T cell subsets, peripheral blood, popliteal lymph node, and bone marrow mononuclear cells were isolated from sick dogs (CanL) before the beginning of treatment (M0) and monthly after treatment (M1, M2, and M3). These cells were immunophenotyped by evaluating the surface expression of CD45, CD3, CD4, CD8, and CD25 and the intracellular expression of FoxP3. To reduce the number of animals used in this study and to ensure any ethical concern for animal discomfort and well-being, the amount of sample collection and its periodicity were reduced to a minimum. Furthermore, peripheral blood, popliteal lymph node, and bone marrow samples were collected from sick dogs before the onset of treatment (M0) to establish the baseline levels of cell populations, avoiding the need of an additional group of untreated sick dogs. Peripheral blood, popliteal lymph node and bone marrow samples were also collected from clinically healthy dogs [control group (CG)]. The present study followed the directive 86/609/EEC of the Council of the European Union and was approved by the Ethics and Animal Welfare Committee of the Faculty of Veterinary Medicine, University of Lisbon.

3.3.3. Isolation of peripheral blood, lymph node, and bone marrow mononuclear cells

Peripheral blood mononuclear cells were obtained through density gradient centrifugation (Histopaque®-1077 solution, Sigma-Aldrich, Germany). Dog peripheral blood was resuspended in PBS (1:1 v/v), overlaid on half of that total volume in Histopaque®-1077 solution and centrifuged 400 × *g* for 30 min at 18 °C. Peripheral blood mononuclear cells were then harvested at the interface of PBS and Histopaque® and washed twice in cold PBS (300 × *g*, 10 min, 4 °C). Whenever red blood cells were still visible in the pellet, a step of lysis was done by adding 5 ml of RBC Lysis Buffer (eBioscience, USA) for 5 min and stopping the reaction with 10 ml of PBS, followed by a centrifugation at 300 × *g* (4 °C) for 10 min. The pellet was then resuspended in Flow Cytometry Staining Buffer (FCSB) (eBioscience), and the total volume adjusted for 2×10^7 cells ml^{-1}. Lymph node and bone marrow aspirates were centrifuged at 400 × *g* (4 °C) for 5 and 15 min, respectively, and resuspended in FCSB with the total volume also adjusted for 2×10^7 cells ml^{-1}. These samples were then kept on ice until antibody labeling.

3.3.4. Flow Cytometry

To characterize regulatory and effector T cell subpopulations, a multicolor panel was designed for flow cytometry analysis, and each fluorochrome-conjugated antibody was titrated for optimal staining (Table 20).

Table 20. Flow cytometer setup, fluorochrome panel and labelling

Instrument: Beckman Coulter Cyan ADP						
Laser lines	405 nm	488 nm			642 nm	
Emission filters	450/50	530/40	575/25	680/30	665/20	750LP
Fluorochrome	eFluor® 450	FITC	PE	PerCP/Cy5.5	APC	Alexa Fluor® 700
Biomarker	CD45	CD3	CD25	FoxP3	CD4	CD8
Brightness	■■	■■■	■■■■■	■■■	■■■■■	■■
Antibody	rat anti-dog	mouse anti-dog	mouse anti-dog	anti-mouse/rat	rat anti-dog	rat anti-dog
Clone	YKIX716.13	CA17.2A12	P4A10	FJK-16s	YKIX302.9	YCATE55.9
Company	e-Biosciences	AbD Serotec	e-Biosciences	e-Biosciences	e-Biosciences	AbD Serotec
Volume	5 µL per test (1:20)	8 µL per test (1:12.5)	5 µL per test (1:20)	5 µL per test (1:20)	5 µL per test (1:20)	10 µL per test (1:50)

The green-shaded squares indicate the level of brightness for each corresponding fluorochrome, from dim (1 square) to the brightest (5 squares).

Cell suspensions (50 µl) were incubated with the following monoclonal antibodies (30 minutes at 4°C in the dark): rat anti-dog CD45 (clone YKIX716.13, eBiosciences Inc.), mouse anti-dog CD3 (clone CA17.2A12, AbD Serotec, UK), anti-dog CD4 (clone YKIX302.9, eBiosciences Inc.), rat anti-dog CD8 (clone YCATE55.9, AbD Serotec) and mouse anti-dog CD25 (clone P4A10, eBiosciences Inc.) (Table 21).

Table 21. Fluorochrome compensation panel graph by sample type and tissue

Sample type	**Marker**						**Blood**	**Lymph Node**	**Bone Marrow**
	CD45	CD3	CD4	CD8	CD25	FoxP3			
Unstained							**X**	**X**	**X**
Single-stained	■						**X**		
		■					**X**		
			■				**X**		
				■			**X**		
					■		**X**		
						■	**X**	**X**	**X**
FMO-CD25	■	■	■	■		■	**X**	**X**	**X**
FMO-FoxP3	■	■	■	■	■		**X**	**X**	**X**
All	■	■	■	■	■	■	**X**	**X**	**X**

The green-shaded slots indicate the antibody label used in each sample type, while the crosses indicate which samples were analyzed in each tissue.

Then, cells were washed twice with 1 ml of FCSB and centrifuged at 400 × *g* (4°C) for 5 min. Afterward, 1 ml of FoxP3/Transcription Factor Fixation/Permeabilization Working

Solution (eBioscience Inc.) was added, and cells were incubated overnight at 4°C in the dark. Next, 500 µl of 1× Permeabilization Buffer (eBioscience Inc.) was added, and cells were centrifuged at 400 × *g* (4°C) for 5 min, followed by two washes at 400 × *g* (4°C) for 5 min with 1 ml of 1× Permeabilization Buffer and a last washing step with 500 µl of FCSB. Cells were resuspended in a total of 100 µl of FCSB and incubated for 15 min at 4°C in the dark. Intracellular staining with anti-mouse/rat FoxP3 (clone FJK-16s, eBioscience Inc.) monoclonal antibody was done by incubating for at least 30 min (4°C) in the dark, followed by two washes with 1× Permeabilization Buffer at 400 × *g* (4°C) for 5 min. For flow cytometry acquisition (three-laser equipped CyAn ADP apparatus, Beckman Coulter, using the Summit v4.3, Dako Colorado Inc. software), cells were resuspended in a final volume of 300 µl of FCSB. For each sample, a minimum of 20,000 gated events were acquired, and data analysis was performed using FlowJo version 10.0.7 (Tree Star, CA). To define the best gating strategy to be applied (Fig. 27), compensation was done with unstained, single-stained, and "fluorescence minus one" (FMO) samples (Table 21).

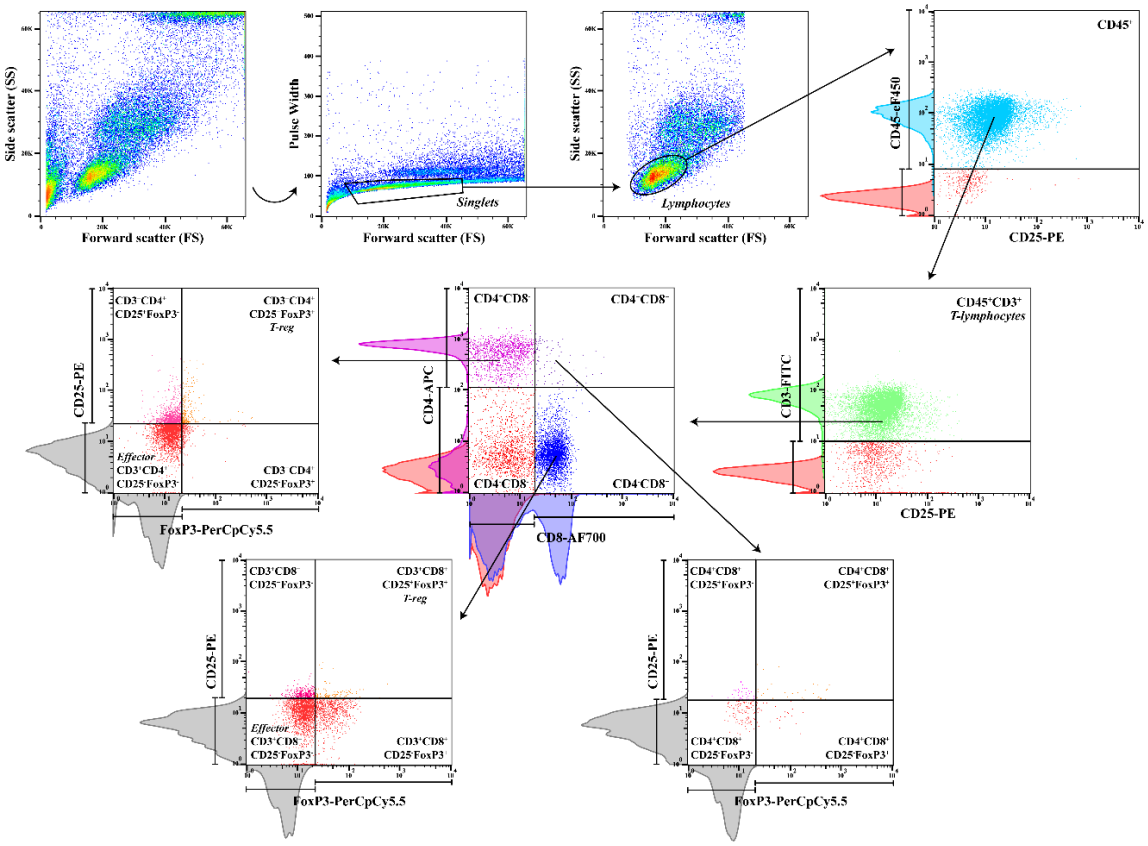

Figure 27. Gating strategy
Peripheral blood sequential gating strategy for a panel of six antibodies to identify the different cell subpopulations after doublet exclusion. CD45, a pan-leukocyte marker, and CD3, a T-lymphocytes specific marker, were used to define the T-lymphocyte population, with posterior separation of $CD4^+$ and $CD8^+$ cells, $CD4^+CD8^+$ double-positive T cells, and subsequent regulatory $CD25^+FoxP3^+$ and effector $CD25^-FoxP3^-$ cells. Red histograms from unstained control samples and colored histograms from single-stained control samples were used to define the sequential gating, along with gray histograms from fluorescence minus one (FMO) controls to gate for rare cells ($CD25^+FoxP3^+$).

A recent study (Burel et al. 2019) showed relevant proof that the doublet discrimination usually made in flow cytometry analysis, with the reasoning that they constitute experimental artifacts, may hide cell-to-cell contact, in particular, T cell-monocyte association that is not disrupted during sample processing. Thus, in the current study, a simple approach was used to compare the absolute count of doublets in healthy, sick and treated dogs following the gating strategy shown in Figure 28A.

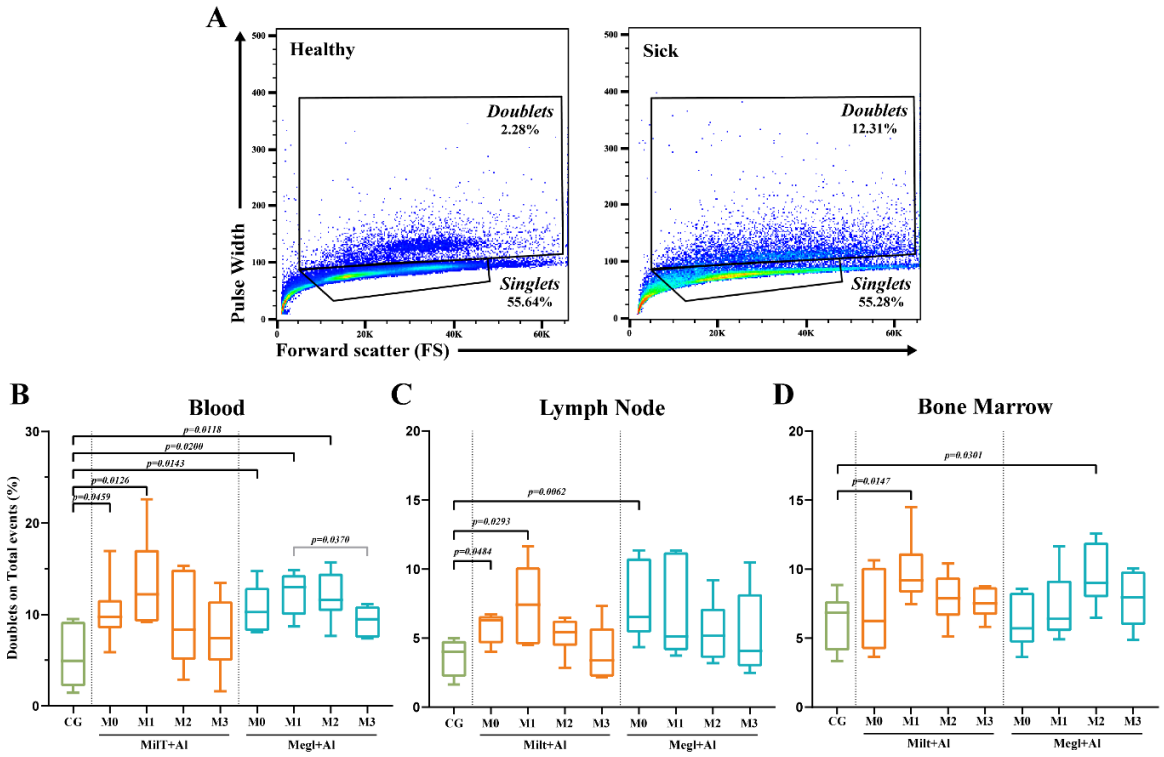

Figure 28. Doublet analysis
(A) Gating strategy example in the blood of a healthy [control group (CG)] and a sick dog (M0). Percentage of doublets gated on total events for blood **(B)**, lymph node **(C)**, and bone marrow **(D)** before and after the beginning of treatment. Results of 22 dogs are represented by box and whisker plots and median, minimum, and maximum values. The non-parametric Kruskal-Wallis test (one-way ANOVA on ranks) with Dunn's *post hoc* test was used for statistical comparisons inside each treatment groups and the control group (CG). The repeated measures ANOVA test with Tukey's *post hoc* test was used for statistical comparisons inside each treatment group. *p*-values are indicated in every statistically significant comparison.

3.3.5. Statistical analysis

Statistical analysis between control, infected, and treated groups was performed using GraphPad Prism software package (version 8.0.1, GraphPad Software Inc.). The Kolmogorov-Smirnoff test was used to assess data normality. The non-parametric Kruskal-Wallis Test (one-way ANOVA on ranks) with Dunn's *post hoc* test was used to evaluate differences in cell subset levels between sick, treated, and control groups. Lastly, the repeated measures ANOVA Test with Tukey's *post hoc* test was used to compare dogs between the several months M0, M1, M2, and M3.

3.4. Results

3.4.1. Canine leishmaniosis promotes a high number of cell doublets that reach healthy values during treatment

A significant increase of events in the doublets gate in both blood ($p_{Milt+Al}$ = 0.0459; $p_{Megl+Al}$ = 0.0143) (Fig. 28B) and lymph node ($p_{Milt+Al}$ = 0.048; $p_{Megl+Al}$ = 0.0062) (Fig. 28C) was observed in sick dogs (M0) when compared with the control group. One month after Milt+Al treatment (M1), blood (p = 0.0126), lymph node (p = 0.0293), and bone marrow (p = 0.0147) presented a significantly high frequency of doublets. Although, during treatment, doublets return to frequencies close to those of the control group. In dogs treated with Megl+Al, peripheral blood exhibited significant high percentages of doublets in the first (p_{M1} = 0.02) and second (p_{M2} = 0.0108) months of treatment. On the other hand, the bone marrow presented only a transient increase of doublets 2 months (p_{M2} = 0.0301) after the beginning of the treatment (Fig. 28D).

3.4.2. Canine leishmaniosis chemotherapy causes an imbalance of T lymphocyte population

Peripheral blood (Fig. 29A) and lymph node (Fig. 29B) of dogs with active leishmaniosis (M0) presented T lymphocyte ($CD45^+CD3^+$) levels similar to clinically healthy dogs. However, the subsequent administration of either treatment resulted in lymphocyte frequency reduction. Dogs under Megl+Al therapy showed a significant reduction of the percentage of blood T cell population ($CD45^+CD3^+$ cells) after 2 (p_{M2} = 0.0239) and 3 (p_{M3} = 0.0046) months of treatment.

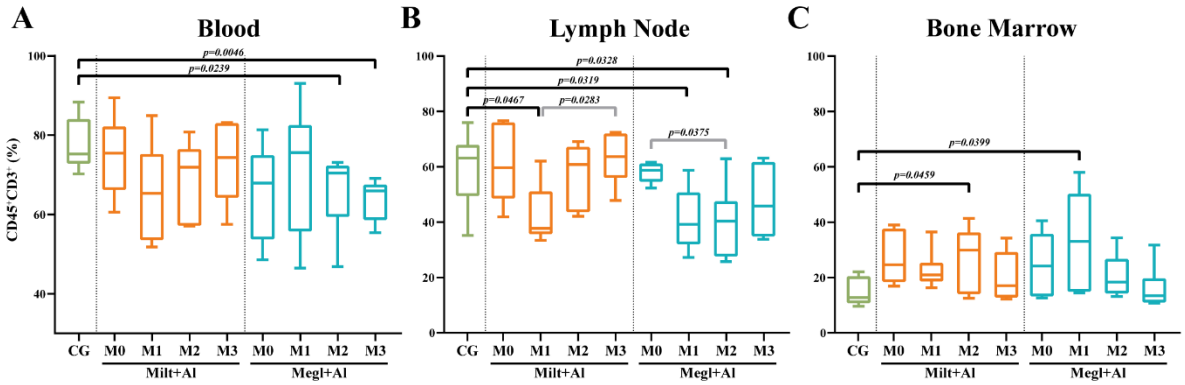

Figure 29. Frequency of lymphocytes ($CD45^+CD3^+$) in blood (A), lymph node (B), and bone marrow (C) of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3)
Results of 22 dogs are represented by box and whisker plots and median, minimum, and maximum values. The non-parametric Kruskal-Wallis test (one-way ANOVA on ranks) with Dunn's *post hoc* test was used for statistical comparisons between treatment groups and the CG) The repeated measures ANOVA test with Tukey's *post hoc* test was used for statistical comparisons inside each treatment group. *p*-values are indicated in every statistically significant comparison.

However, in the lymph node, a significant frequency reduction of the T cell population was observed at 1 (p_{M1} = 0.0319) and 2 (p_{M2} = 0.0328) months with this therapy. Furthermore, bone marrow T cells (Fig. 29C) frequency significantly increased after the first month of treatment with MegI+Al (p_{M1} = 0.0399), reaching values similar to clinically healthy dogs by the second month (M2). One month after the beginning of treatment with Milt+Al, a transient reduction of lymph node T cells (p_{M1} = 0.0467) was observed. The bone marrow, in turn, showed a transient higher frequency of T cells (p_{M2} = 0.0459) 2 months after treatment, recovering to levels identical to those of control dogs in the third month (M3).

3.4.3. Anti-leishmanial therapy favors the predominance of CD4⁺ T cells over CD8⁺ T cells

According to several authors, the CD4⁺/CD8⁺ T cell ratio acquired by flow cytometry analysis can be considered a simple and fast way to assess cell-mediated immune response (Paltrinieri et al. 2010; Papadogiannakis et al. 2010). When compared with healthy dogs, blood (p_{M0} = 0.0177) (Fig. 30A) and lymph node (p_{M0} = 0.0246) (Fig. 30B) cells of sick dogs presented a significant decrease of the CD4/CD8 ratio to values close to 1, pointing to similar frequencies of CD8⁺ and CD4⁺ T cells. During treatment, this ratio progressed toward values closer to 2, indicating the predomination of CD4⁺ T cells. On the other hand, the bone marrow CD4⁺/CD8⁺ T cell ratio (Fig. 30C) of sick dogs was similar to that of healthy dogs, with ratios ranging between 0.5 and 1. These values point towards a variation between a slight predomination of CD8⁺ T cells and an identical frequency of both T cell subsets.

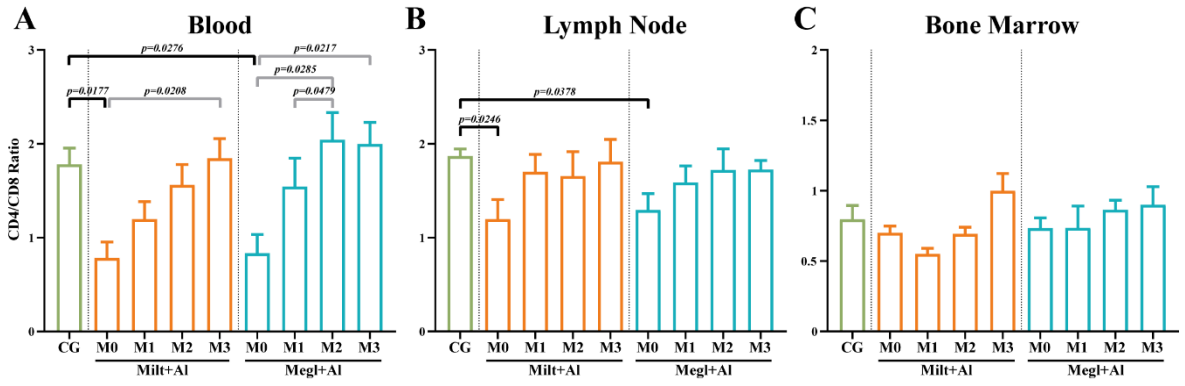

Figure 30. CD4/CD8 ratio in blood (A), lymph node (B), and bone marrow (C) of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3)
Results of 22 dogs are represented by mean values ± SEM. The non-parametric Kruskal-Wallis test (one-way ANOVA on ranks) with Dunn's *post hoc* test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's *post hoc* test was used for statistical comparisons inside each treatment group. *p*-values are indicated in every statistically significant comparison.

3.4.4. Canine leishmaniosis increases $CD4^+CD8^+$ double-positive T cells frequency in peripheral blood, lymph node, and bone marrow

Sick dogs (M0) showed increased frequencies of $CD4^+CD8^+$ DP T cells in the blood (Fig. 31A) ($p_{Milt+Al}$ = 0.0182; $p_{Megl+Al}$ = 0.0015), lymph node (Fig. 31B) ($p_{Milt+Al}$ = 0.0234; $p_{Megl+Al}$ = 0.0318), and bone marrow (Fig. 31C) ($p_{Milt+Al}$ = 0.005; $p_{Megl+Al}$ = 0.006) when compared to healthy dogs. The administration of either treatment protocol resulted in a maintenance of these high frequencies of $CD4^+CD8^+$ DP T cells in all tissues during the first month of treatment (M1), progressively normalizing by the following month (M2), with the exception of lymph node of dogs treated with the Megl+Al protocol that recovered 1 month after treatment.

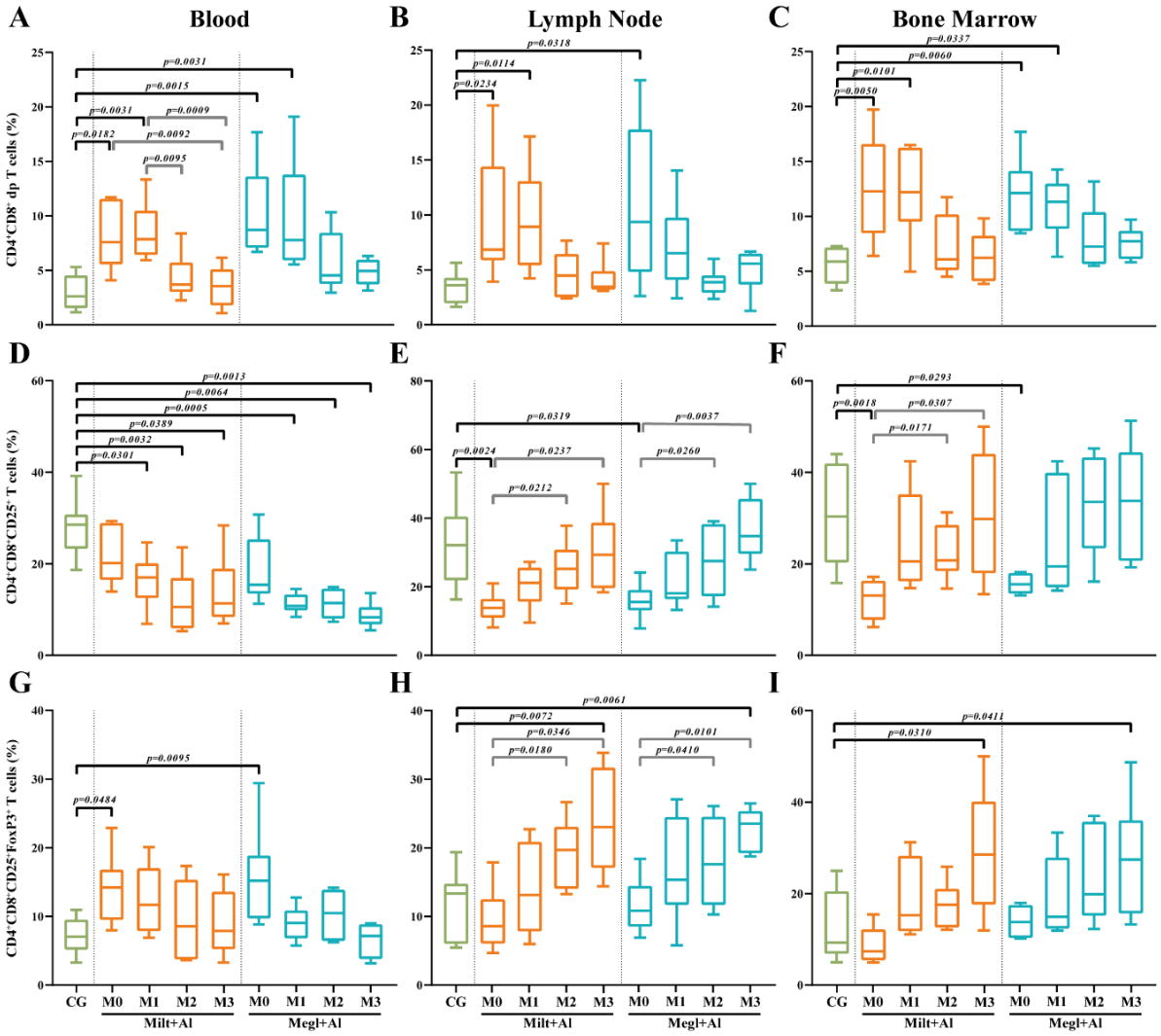

Figure 31. Frequency of $CD4^+CD8^+$ double-positive (DP) T cells
Frequency of DP T cells (A, B and C) expressing CD25 (D, E and F) and CD25 and FoxP3 (G, H and I) were evaluated in the peripheral blood (A, D and G), lymph node (B, E and H), and bone marrow (C, F and I) of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3). Results of 22 dogs are represented by box and whisker plots and median, minimum and maximum values. The non-parametric Kruskal-Wallis test (one-way ANOVA on ranks) with Dunn's *post hoc* test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's *post hoc* test was used for statistical comparisons inside each treatment group. p-values are indicated in every statistically significant comparison.

3.4.5. $CD4^+CD8^+$ double-positive T cells expressing regulatory phenotype decrease in peripheral blood of sick dogs and increase in the lymph node and bone marrow after treatment

Lymph node (Fig. 31E) and bone marrow (Fig. 31F) of sick dogs showed a significant frequency reduction of DP T cells expressing CD25 molecules (lymph node: $p_{Milt+Al} = 0.0024$; $p_{Megl+Al} = 0.0319$ / bone marrow: $p_{Milt+Al} = 0.0018$; $p_{Megl+Al} = 0.0293$), which recovered to values similar to clinically healthy dogs during treatment. However, in peripheral blood treatment caused a significant decrease of this T cell subset (Fig. 31D). In turn, the percentage of $CD25^+FoxP3^+$ DP T cells in the blood of sick dogs (Fig. 31G) was higher than in healthy dogs ($p_{Milt+Al} = 0.0484$; $p_{Megl+Al} = 0.0095$), while being similar to the control group in the lymph node (Fig. 31H) and bone marrow (Fig. 31I). Treated dogs presented a normalization of the frequencies in blood after 1 month of treatment, while showing a progressive increase in this subpopulation, reaching higher frequencies than the control group, in the lymph node ($p_{Milt+Al} = 0.0072$; $p_{Megl+Al} = 0.0061$) and bone marrow ($p_{Milt+Al} = 0.0310$; $p_{Megl+Al} = 0.0411$) in the third month.

3.4.6. *Leishmania* infection results in the increase of blood $CD8^+$T cells frequencies with $CD25^+FoxP3^+$ phenotype

Blood of sick dogs (M0) exhibited a significant decrease in the frequency of the $CD4^+$ T cell subset ($p_{Milt+Al} = 0.0253$; $p_{Megl+Al} = 0.0467$) (Fig. 32A) along with a high requency of the $CD8^+$ T cell subset ($p_{Milt+Al} = 0.0018$; $p_{Megl+Al} = 0.0052$) (Fig. 32B). Both treatments were able to recover normality for the $CD4^+$ and $CD8^+$ T cell fractions. However, dogs under the Megl+Al protocol recovered to values similar to those of clinically healthy dogs during the first month of treatment (M1), faster than the group treated with Milt+Al that only recovered after the second month (M2). The frequency of blood T cells with $CD4^+CD25^+$ phenotype showed some fluctuation, mainly during Megl+Al treatment (Fig. 32C), although with no statistical differences when compared with clinically healthy dogs. However, a significant increase in the frequency of the $CD8^+CD25^+$ T cell subset ($p_{Milt+Al} = 0.0071$; $p_{Megl+Al} = 0.0246$) was observed in sick dogs (M0) when compared with that of the control group (Fig. 32D). This cell subset returned to normal values immediately after the beginning of both treatments (M1). $CD4^+CD25^+FoxP3^+$ T ($p_{Milt+Al} = 0.0411$; $p_{Megl+Al} = 0.0310$) and $CD8^+CD25^+FoxP3^+$ ($p_{Milt+Al} = 0.0118$; $p_{Megl+Al} = 0.0052$) T cell subsets of sick dogs (M0) presented higher frequencies than the control group (Fig. 32E and 32F). After administration of both treatments, an increase in the frequency of the $CD4^+CD25^+FoxP3^+$ T cell subset was observed ($p_{Milt+Al(M1)} = 0.0092$; $p_{Megl+Al(M2)} = 0.0029$), with the values returning to healthy levels at M2 and M3, for the Milt+Al and Megl+Al groups,

respectively. Likewise, the $CD8^+CD25^+FoxP3^+$ T cell subset recovered to values comparable to those of control dogs after 3 months for both treatment protocols.

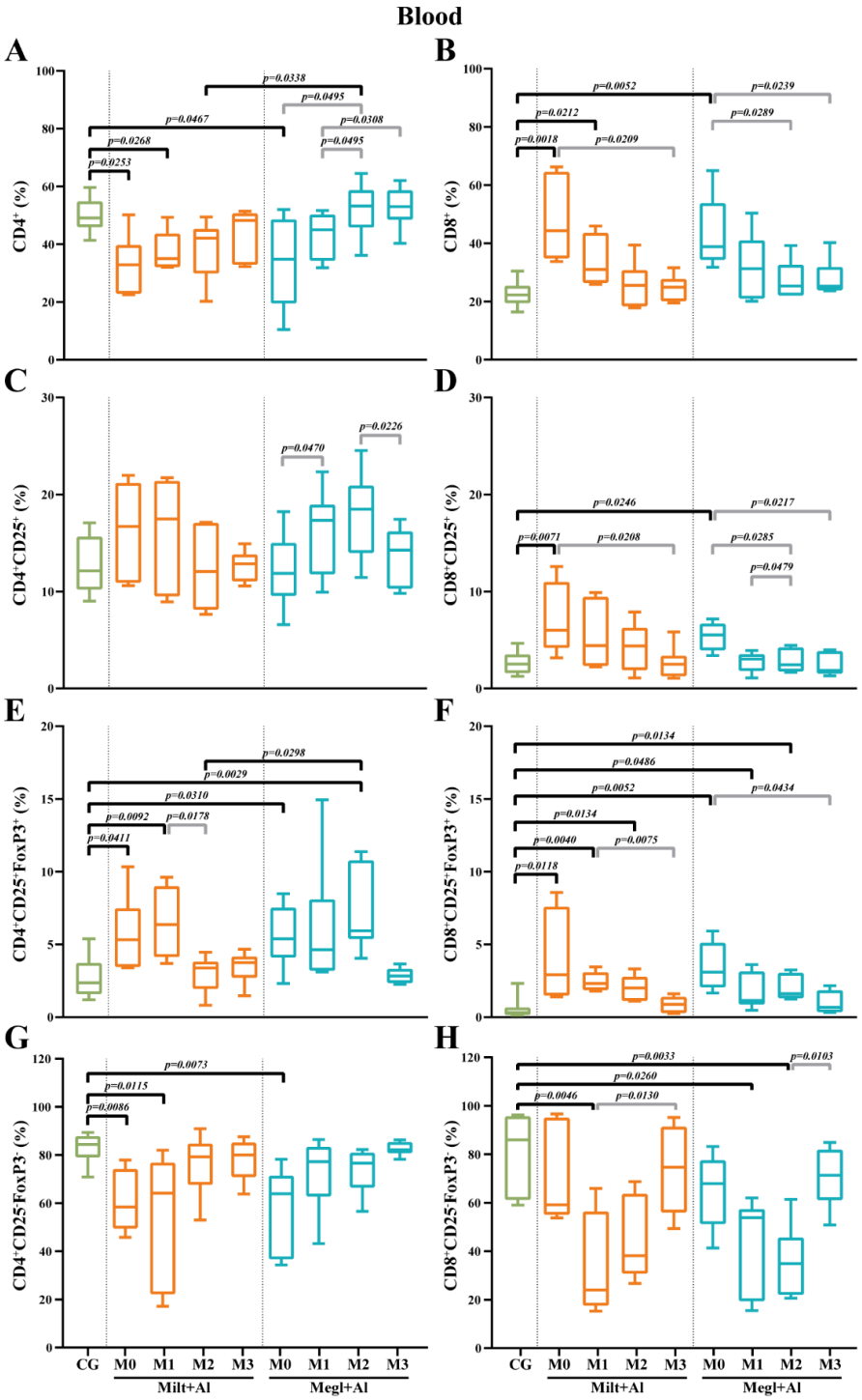

Figure 32. Frequency of $CD4^+$ (A), $CD8^+$ (B), regulatory ($CD25^+FoxP3^+$) (C, D, E and F), and effector ($CD4^+CD25^-FoxP3^-$/$CD8^+CD25^-FoxP3^-$) (G and H) T lymphocytes in the blood of healthy [control group (CG)], sick (M0) and treated dogs (M1, M2, and M3)

Results of 22 dogs are represented by box and whisker plots and median, minimum and maximum values. The non-parametric Kruskal-Wallis test (one-way ANOVA on ranks) with Dunn's *post hoc* test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's *post hoc* test was used for statistical comparisons inside each treatment group. *p*-values are indicated in every statistically significant comparison.

Effector T cell subsets of sick dogs (M0) presented different patterns. $CD4^+CD25^-FoxP3^-$ T cells were significantly lower than those of the control group ($p_{Milt+Al} = 0.0086$; $p_{Megl+Al} = 0.0073$). However, dogs recovered to healthy values 1 month after the beginning of treatment with Megl+Al (M1) and after 2 months of Milt+Al therapy (M2) (Fig. 32G). On the other hand, $CD8^+CD25^-FoxP3^-$ T cells of sick dogs were similar to those of healthy dogs, but subsequent treatments led to a significant reduction in cell frequency ($p_{Milt+Al(M1)} = 0.0046$; $p_{Megl+Al(M1)} = 0.026$), with the Megl+Al group recovering to normal frequencies by the third month (M3) and the Milt+Al group after the second month (M2) (Fig. 32H).

3.4.7. CanL promotes the increase of lymph node $CD8^+$ T cells frequencies, and treatment leads to imbalance of effector and regulatory T cell subsets

In the lymph node of sick dogs, the frequency of $CD4^+$ T cells was similar to that of healthy dogs (Fig. 33A), but the $CD8^+$ T cell fraction presented a higher percentage ($p_{Milt+Al} = 0.0052$; $p_{Megl+Al} = 0.0120$) (Fig. 33B). Furthermore, treatment administration caused a reduction of the $CD8^+$ T cell frequencies to values similar to control dogs. Three months after the onset of treatment with Megl+Al, the $CD4^+$ T cell fraction was significantly diminished ($p = 0.0389$) when compared with clinically healthy dogs.

In sick dogs, the level of $CD4^+$ (Fig. 33C) and $CD8^+$ (Fig. 33D) T cells with $CD25^+$ phenotype was similar to healthy dogs. However, both treatments protocols led to a transient increase of the $CD4^+CD25^+$ T cell subset frequencies after 1 month of Milt+Al treatment ($p_{M1} = 0.0463$) and 2 months of Megl+Al ($p_{M2} = 0.0471$). The $CD8^+CD25^+$ T cell subpopulation of dogs under the Milt+Al protocol showed a significant increase 2 ($p_{M2} = 0.0200$) and 3 ($p_{M3} = 0.0071$) months after the beginning of treatment (Fig. 33D).

Likewise, sick dogs showed similar frequencies of $CD4^+CD25^+FoxP3^+$ and $CD8^+CD25^+FoxP3^+$ T cells compared to healthy dogs. Moreover, after treatment, these dogs exhibited a significant increase in the frequency of the $CD4^+CD25^+FoxP3^+$ T cell subset (Fig. 33E). In dogs treated with Milt+Al, a peak of the frequency of $CD4^+$ Treg cells was observed two months ($p_{M2} = 0.0182$) after the beginning of treatment. One and 2 months after administration, Megl+Al also promoted a $CD4^+$ Treg frequency increase ($p_{M1} = 0.0172$; $p_{M2} = 0.0098$), that subsequently reverted to normal values. Moreover, Milt+Al caused a significant increase in the frequency of $CD8^+CD25^+FoxP3^+$ T cells ($p_{M1} = 0.0027$; $p_{M2} = 0.0071$; $p_{M3} = 0.0145$), while the Megl+Al protocol only resulted in a transient increase of this subpopulation 1 month after treatment ($p_{M1} = 0.0399$) (Fig. 33F).

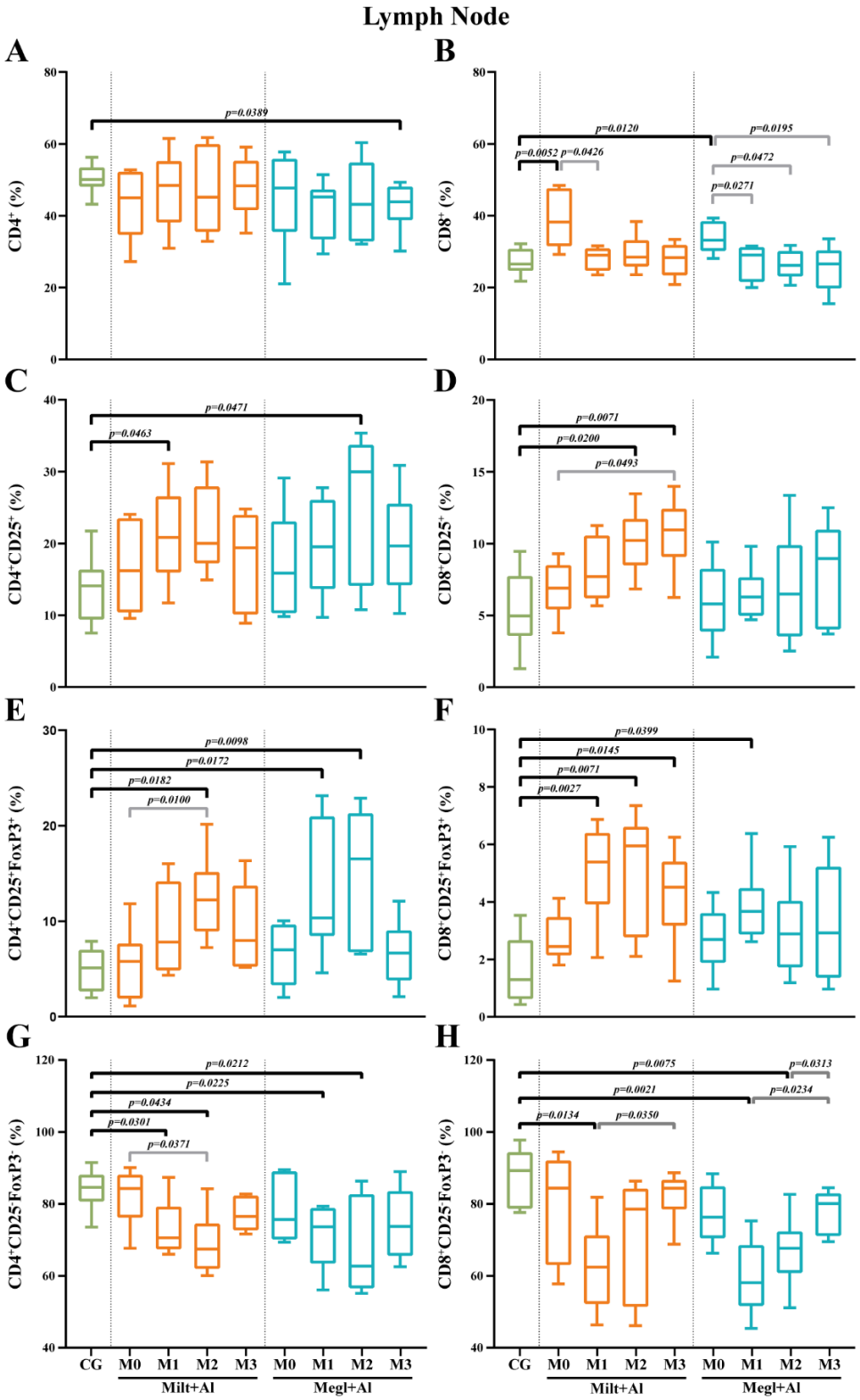

Figure 33. Frequency of $CD4^+$ (A), $CD8^+$ (B), regulatory ($CD25^+FoxP3^+$) (C, D, E and F), and effector ($CD4^+CD25^-FoxP3^-$/$CD8^+CD25^-FoxP3^-$) (G and H) T lymphocytes in the lymph node of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3)

Results of 22 dogs are represented by box and whisker plots and median, minimum and maximum values. The non-parametric Kruskal-Wallis test (one-way ANOVA on ranks) with Dunn's *post hoc* test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's *post hoc* test was used for statistical comparisons inside each treatment group. *p*-values are indicated in every statistically significant comparison.

Effector T cell subsets in the lymph node of sick dogs were similar to those of healthy dogs. After treatment administration, $CD4^+CD25^-FoxP3^-$ T cell frequencies showed a progressive reduction during the first and second month with both the Milt+Al (p_{M1} = 0.0301; p_{M2} = 0.0434) and the Megl+Al protocol (p_{M1} = 0.0225; p_{M2} = 0.0212) (Fig. 33G). $CD8^+CD25^-FoxP3^-$ T cell frequencies also presented a significant reduction after drug administration ($p_{Milt+Al}$ = 0.0134; $p_{Megl+Al}$ = 0.0021), with the Milt+Al-treated dogs recovering cell frequency levels by the second month (M2) and the Megl+Al-treated dogs by the third month (M3) (Fig. 33H).

3.4.8. *Leishmania* infection causes the increase of bone marrow $CD8^+$ T cell frequencies with $CD25^+FoxP3^+$ phenotype

In the bone marrow of sick dogs, the number of $CD4^+$ T cells (Fig. 34A) was similar to clinically healthy dogs. The administration of Milt+Al did not cause significant alterations in the $CD4^+$ T cell fraction, while dogs under the Megl+Al protocol exhibited a transient frequency increase (p = 0.0134) 2 months after the onset of treatment. Meanwhile, a prominent increase of the frequency of $CD8^+$ T cells was observed in sick dogs ($p_{Milt+Al}$ = 0.0293; $p_{Megl+Al}$ = 0.0495) (Fig. 34B). This high frequency of $CD8^+$ T cells in the bone marrow persisted during both treatments (Milt+Al: p_{M1} = 0.0367; p_{M2} = 0.0310) (Megl+Al: p_{M1} = 0.0463; p_{M2} = 0.0411), returning to values similar to control dogs by the third month (M3).

Regarding the $CD4^+CD25^+$ T cell subpopulation (Fig. 34C), no considerable differences were observed in the bone marrow of sick dogs when compared with that of clinically healthy dogs. Moreover, dogs treated with Megl+Al evidenced a transient decrease of the frequency of $CD8^+CD25^+$ T cells by month 2 (p_{M2} = 0.0367) that quickly recovered (Fig. 34D).

In the bone marrow of sick dogs, the frequency of Treg cells ($CD4^+CD25^+FoxP3^+$) was similar to that of control dogs (Fig. 34E). Nevertheless, an increase of the frequency of this cell subset was observed 1 month ($p_{Milt+Al}$ = 0.0484; $p_{Megl+Al}$ = 0.0484) after either treatment, followed by normalization. Similar to peripheral blood, the $CD8^+CD25^+FoxP3^+$ T cell subset frequencies (Fig. 34F) of sick dogs was significantly higher ($p_{Milt+Al}$ = 0.0086; $p_{Megl+Al}$ = 0.0389). Both treatments led to a reduction of cell frequencies to values similar to those of the control group.

$CD4^+CD25^-FoxP3^-$ T cells frequencies of sick dogs were significantly lower in comparison with those of healthy dogs ($p_{Milt+Al}$ = 0.0232; $p_{Megl+Al}$ = 0.0016) (Fig. 34G). However, the Megl+Al group recovered to values close to those of healthy dogs 1 month earlier than the Milt+Al group. The frequencies of $CD8^+CD25^-FoxP3^-$ T cells of sick dogs, on the other hand, were similar to those of healthy dogs, with the administration of either treatment leading to a

significant decrease 1 ($p_{Milt+Al}$ = 0.0439; $p_{Megl+Al}$ = 0.0147) and 2 months ($p_{Milt+Al}$ = 0.0095; $p_{Megl+Al}$ = 0.0484) after the beginning of treatment (Fig. 34H).

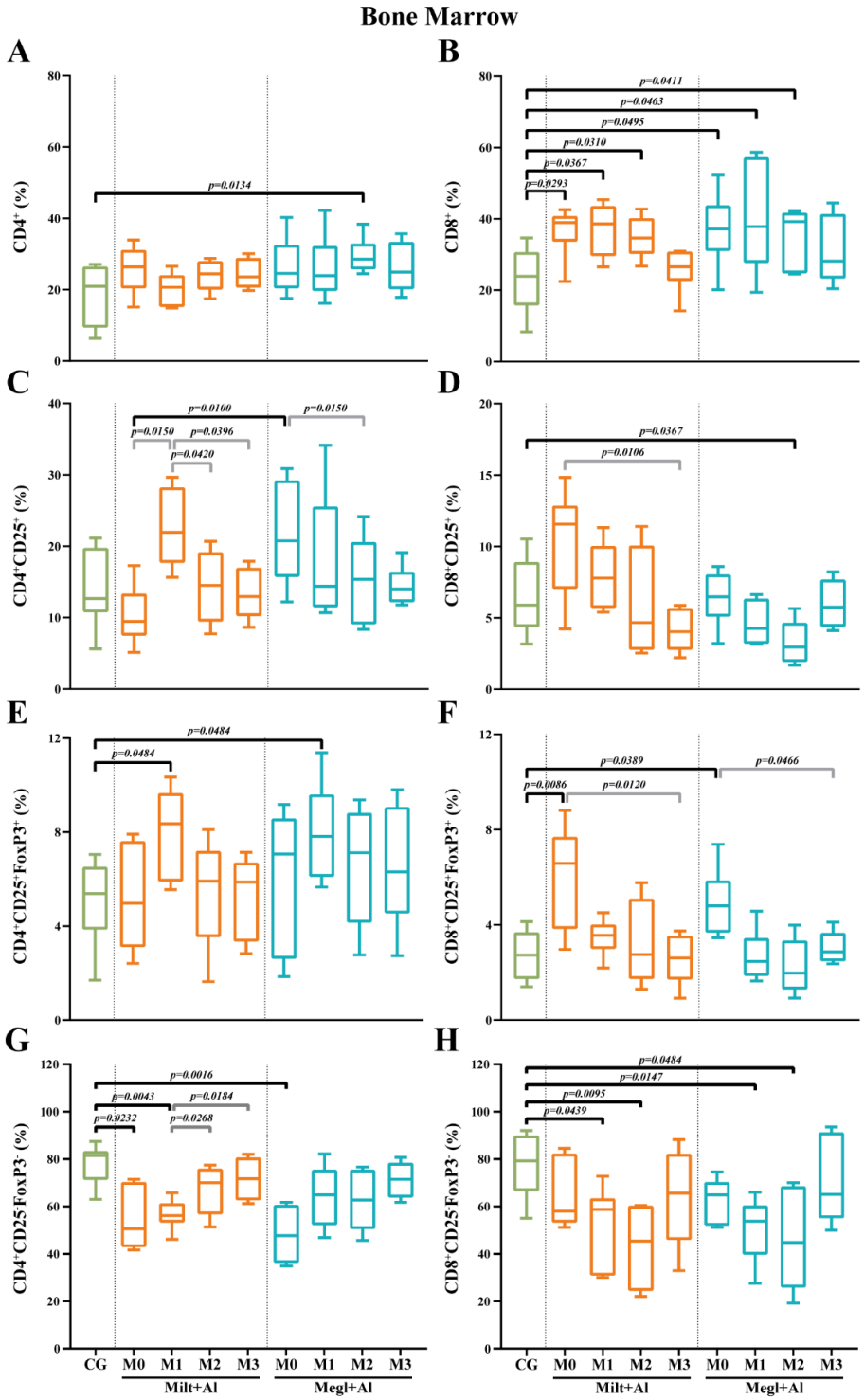

Figure 34. Frequency of CD4$^+$ (A), CD8$^+$ (B), regulatory (CD25$^+$FoxP3$^+$) (C, D, E and F), and effector (CD4$^+$CD25$^-$FoxP3$^-$/CD8$^+$CD25$^-$FoxP3$^-$) (G and H) T lymphocytes in bone marrow of healthy [control group (CG)], sick (M0), and treated dogs (M1, M2, and M3)

Results of 22 dogs are represented by box and whisker plots and median, minimum and maximum values. The non-parametric Kruskal-Wallis test (one-way ANOVA on ranks) with Dunn's *post hoc* test was used for statistical comparisons between treatment groups and the CG. The repeated measures ANOVA test with Tukey's *post hoc* test was used for statistical comparisons inside each treatment group. *p*-values are indicated in every statistically significant comparison.

3.5. Discussion

CanL treatment has an inherent connection with the ability of the dog's immune system to develop a competent cellular immune response against *L. infantum*. Thus, comprehending the cellular immune response and the dynamics of T cell subsets in dogs naturally infected with *Leishmania*, especially in organs that usually harbor these parasites, is of utmost relevance not only for the treatment and management of CanL but also as guidelines for the development of prophylactic and therapeutic tools. A better knowledge of the effect of antileishmanial therapy on the cellular immune response of dogs can facilitate the development of strategies to reduce the transmission of the parasite and, consequently, lead to a decrease in the incidence of zoonotic visceral leishmaniosis. Therefore, in the current study, T cell subpopulations of dogs naturally infected with *L. infantum* were phenotypically characterized before treatment and during the influence of antileishmanial drugs.

In the current study, it was found that sick dogs have increased doublet frequencies in peripheral blood and lymph node, decreasing to values similar to clinically healthy dogs after treatment. As was proposed by Burel et al. (2019), these changes in the doublet levels associated with CanL and during the first months of treatment may reflect a possible cell-to-cell interaction between T lymphocytes and antigen-presenting cells. It is also possible that the doublets could increase as a result of interaction of Treg:lymphocyte, as Treg cells, which seem to be increased in CanL, appear to exert immune suppression by mechanisms dependent on cell contact (Lee et al. 2018). In the present work, it was not possible to delve deeper into these interactions since this was a secondary objective of the study. In this sense, not enough events were collected in the doublet region to obtain meaningful information on further subpopulations. This way, further detailed studies are needed to corroborate this hypothesis, with the correlation between CanL and the level of doublets being able to be used as a possible marker of disease to monitor treatment success and predict potential relapses (Burel et al. 2019).

Several authors have correlated symptomatic dogs with decreased levels of $CD4^+$ T cells and CD4/CD8 ratios in peripheral blood (Bourdoiseau, Bonnefont, Magnol, et al. 1997; Cortese et al. 2015), along with high antibody titers. Other authors verified that higher infectivity to sand flies by naturally infected dogs was associated with lower proportions of $CD4^+$ T cells in the blood (Guarga et al. 2000). Furthermore, it has also been shown that the administration to dogs infected with *Leishmania* of antileishmanial drugs, such as amphotericin B and meglumine antimoniate, promoted the increase of the percentage and the absolute cell count of $CD4^+$ T cells in the blood, respectively (Bourdoiseau, Bonnefont, Magnol, et al. 1997; Moreno et al. 1999). On the other hand, other treatment protocols, such as allopurinol in

monotherapy, although able to improve the number of circulating $CD4^+$ T cells in the blood, were not able to restore values to those within the normal range (Papadogiannakis et al. 2010). Thus, the findings obtained in the current study are in line with previous reports. Sick dogs presented low CD4/CD8 ratios in peripheral blood and lymph node, recovering to values equal to the healthy group after the administration of both treatments. Following our results, and according to several authors (Bourdoiseau, Bonnefont, Magnol, et al. 1997; Moreno et al. 1999; Guarga et al. 2000; Alvar et al. 2004; Papadogiannakis et al. 2010), the CD4/CD8 ratio can be a useful indicator of the immunological condition of sick dogs and a possible tool with prognostic value. Some authors also describe a decline of the percentage of $CD3^+$ lymphocytes in the peripheral blood of CanL symptomatic dogs, as a direct consequence of the reduction in the frequency of $CD4^+$ T cells (Moreno et al. 1999; Alexandre-Pires et al. 2010). Other authors, on the contrary, have reported a significant increase of $CD3^+$ T cells in sick dogs, especially in dogs severely affected (Miranda et al. 2007). Nevertheless, the administration of antileishmanial therapy in both situations restored $CD3^+$ lymphocytes within normal values (Moreno et al. 1999; Miranda et al. 2007; Alexandre-Pires et al. 2010). Moreover, the results of the present study point to a dual effect of antileishmanial therapy on bone marrow and lymph node. Both treatments led to a reduction in the frequency of lymph node T cells ($CD45^+CD3^+$) along with an increase in bone marrow. Interestingly, only meglumine antimoniate in association with allopurinol resulted in a decrease of the frequency of blood T cells.

Protective immunity against CanL is usually considered to be dependent on a Th1 immune response (Baneth et al. 2008). The predominance of IFN-γ producing $CD4^+$ T cells is crucial for macrophage activation in order to kill internalized *Leishmania* through the production of NO and ROS (Novais et al. 2018; Jawed et al. 2019). A reduction of the $CD4^+$ T cell population is usually associated with the inability to control the infection, allowing the survival and replication of *Leishmania* parasites in macrophages, which can subsequently lead to increased infectibility to sand flies (Guarga et al. 2000). Murine studies have shown that *Leishmania* parasites negatively interfere with the ability of IFN-γ to induce the expression of MHC-II mRNA, leading to parasitized macrophages with a low expression of MHC class II molecules (Reiner et al. 1988). Thus, due to their reduced capacity as antigen-presenting cells, these macrophages are therefore unable to provide co-stimulatory signals to $CD4^+$ T cells (Saha et al. 1995; Pinelli, Rutten, et al. 1999), which, in turn, are not stimulated, do not proliferate, and do not produce IFN-γ.

Although the complete role of $CD8^+$ T cells in CanL is still debated, there are studies of leishmaniosis in humans and mice showing a functional duality. $CD8^+$ T cells can either play a protective role by releasing IFN-γ, or they can be pathogenic to the host, causing excessive

inflammation at the site of infection (Novais et al. 2018) as a result of cytotoxic activity, which can exacerbate disease progression (Novais and Scott 2015). Following the results of previous reports (Bourdoiseau, Bonnefont, Magnol, et al. 1997; Moreno et al. 1999; Giunchetti et al. 2008), the sick dogs included in the current study also showed an increased frequency of $CD8^+$ T cells in the blood, lymph node, and bone marrow, along with significantly decreased levels of $CD4^+$ T cells in the blood. These findings suggest that $CD8^+$ T cells are at the forefront of the fight against *Leishmania* infection, especially in tissues that commonly harbor *Leishmania* parasites. Nonetheless, antileishmanial therapy led to the recovery of the T cell population in all tissues. And whether due to the direct action of the antileishmanial drugs or the availability of free antigens as a consequence of *Leishmania*'s death caused by therapy, a shift of T cell population occurs, leading to a rapid reduction in the frequency of $CD8^+$ T cells in the blood and lymph node.

Regulatory T cells are generally considered to be a subset of $CD4^+$ T cells, which express the non-constitutive IL-2R-α chain (CD25) and the transcriptional factor FoxP3 (Sakaguchi et al. 1995; Ramsdell 2003). The main function of these cells is to suppress excessive or misguided immune responses and prevent autoimmune diseases (Furtado et al. 2002; Jawed et al. 2019). Few are Treg studies done in CanL, which account for the lack of overall information on these subpopulations (Hosein et al. 2017). In dogs experimentally infected with *L. infantum*, FoxP3 RNA was increased in the skin and liver, but in the lymph node, the authors verified a decrease associated with disease progression (Hosein et al. 2015). Figueiredo et al. (2014) referred that CanL enhanced FoxP3 expression in the jejunum and colon. However, the skin of *L. chagasi* (syn. *L. infantum*)-infected dogs revealed lower levels of FoxP3 expression (Menezes-Souza et al. 2011). Another study found no correlation between TGF-β or IL-10 producing $CD4^+$ Treg cells in the blood and spleen and the parasitic load of naturally infected dogs (Silva et al. 2014).

In the present study, sick dogs showed increased frequencies of blood $CD4^+$ Treg cell associated with decreased percentages of $CD4^+$ ($CD25^-FoxP3^-$) effector T cells, signaling a lack of adequate cellular immune response, which can prolong the presence of the parasite, facilitating parasite transmission. Antileishmanial therapy allowed the normalization of blood $CD4^+$ Treg and effector T cell subsets, especially in dogs under the meglumine plus allopurinol protocol, restoring the action of $CD4^+$ effector T cells.

Curiously, and following the obtained results, CanL does not seem to cause significant changes in $CD4^+$ Treg cells and $CD4^+$ effector T cell subsets of lymph nodes. Similarly, in a study with mice infected with *L. infantum*, a high frequency of $CD4^+CD25^+$ T cells expressing FoxP3 was found in the lymph nodes in the first weeks of infection, followed by a decrease in the subsequent chronic phase of the disease (Rodrigues et al. 2009), supporting the observed

results in the present study. In addition, the administration of CanL drugs caused a transient disturbance in Treg cells and effector T cell subsets. By directing the reduction in the frequency of effector T cells associated with the increase of the Treg cell subset, therapy appears to promote the development of a suppressive immune response located in the dog's lymph node. Despite this, 3 months after the start of treatment, the values normalize. Therefore, it is possible that miltefosine and meglumine antimoniate, which were administered to sick dogs only during the first 4 weeks of treatment, are primarily responsible for the development of a suppressive immune response that can limit inflammation.

In patients with visceral leishmaniosis caused by *L. donovani*, the bone marrow revealed an increase of Treg cells ($CD4^+CD25^+FoxP3^+$) that outnumbered effector T cells ($CD4^+CD25^+FoxP3^-$) (Rai et al. 2012). These Treg cells were shown to be a source of IL-10 and persisted in patients even after successful chemotherapy with sodium antimony gluconate. In the current study, both treatments induced a quick increase in the frequency of the $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^-FoxP3^-$ T cell subsets in the bone marrow of dogs, but for a short period of time, normalizing by the third month of observation. In this case, the findings support the hypothesis that the increase in the frequency of $CD4^+$ Treg cells can be a possible consequence of miltefosine and meglumine antimonial drugs.

In CanL, as in other diseases in which the immune system is deeply involved, the presence and action of $CD8^+$ Treg cells are still a matter of discussion. In a study of human visceral leishmaniosis, the authors proposed that IL-10 produced by $CD8^+$ T cells could lead to a downregulation of cytokine production, in particular pro-inflammatory cytokines like TNF-α and IFN-γ, blocking this way the antileishmanial macrophage activity (Peruhype-Magalhães et al. 2006). Subsequent studies have shown the presence of a subset of $CD8^+$ Treg cells that can inhibit the $CD4^+$ T cell-mediated immune response by inducing apoptosis of activated $CD4^+$ T cells (Chen et al. 2013). This way, the increased frequency of the $CD8^+CD25^+FoxP3^+$ T cell subset in the blood and bone marrow of sick dogs showed in the current study could represent a complementary mechanism of immune regulation that may favor parasite survival (Novais and Scott 2015). Treatment of CanL with miltefosine or meglumine antimoniate in combination with allopurinol directs blood $CD8^+$ Treg cells to progressively return to normal values. These antileishmanial drugs seem to cause a shift in blood and bone marrow lymphocytes by reducing the increased frequency of the $CD8^+CD25^+FoxP3^+$ T cell subset and reduced effector $CD8^+$ ($CD25^-FoxP3^-$) T cells to restrain the local inflammatory immune response and cytotoxicity in order to lessen possible tissue damage.

$CD4^+CD8^+$ DP T cells have been identified in dogs with and without CanL (Alexandre-Pires et al. 2010; Bismarck et al. 2012; McGill et al. 2018). In the current study, the frequency of $CD4^+CD8^+$ DP T cell subsets was revealed to be increased in peripheral blood, lymph node,

and bone marrow of dogs with CanL. Considering the chronic profile of CanL, these findings are in line with previous studies (Matsui et al. 1989; Bagot et al. 1998; Kitchen et al. 2004; Desfrançois et al. 2010; Talker et al. 2015) that have established a link between increased DP T cells and chronic diseases. Furthermore, DP T cells have also been associated with increase production of IFN-γ in pigs (Zuckermann and Husmann 1996), similar to previous results in dogs with CanL (Santos et al. 2019). Moreover, the presence of $CD4^+CD8^+CD25^+FoxP3^+$ T cell subset in the peripheral blood of sick dogs reveals a possible regulatory activity, as proposed by other authors (Rothe et al. 2017), while the lymph node and bone marrow presented decreased percentages of CD25, reflecting a possible cytotoxic role (Rabiger et al. 2019) resulting from the infection with *L. infantum*. In turn, in the present study, the administration of either treatment led to a change in both profiles, with DP T cells in the blood losing the regulatory phenotype, possibly in order to fight the infection, while the lymph node and bone marrow apparently switching to a regulatory profile to nullify possible excessive cytotoxic damage. In any case, since the role of these $CD4^+CD8^+$ DP T cells are not yet fully understood in vivo, further in-depth studies are still needed in these subpopulations in order to elucidate their modes of action.

The immune response to *Leishmania*, in humans, mice, or dogs, seems to be far complex and influenced by several types of immune cells and different immune mediators, establishing an elaborate network. Either way, there seems to be a consensus that *Leishmania* parasites lead to differentiation of specific cell immunophenotypes in different tissues. CanL in this study led to an increased frequency of $CD8^+$ T cells in all tissues, along with increased $CD4^+CD8^+$ DP T cell frequencies, resulting in a predominant pro-inflammatory profile. $CD8^+$ Treg cells frequencies were also significantly increased in the blood and bone marrow, showing a possible action on immune responses mediated by $CD4^+$ T cells, which can lead to parasite tolerance and disease progression. In the present work, the administration of either treatment protocol led to an overall recovery of the T cell subpopulations by the end of observation, reflecting the clinical improvement of the dogs (Santos et al., 2019). Nonetheless, it should be noted that both protocols resulted in an increase of $CD4^+$ Treg cell frequencies in all tissues, possibly in order to significantly reduce the frequency of $CD8^+CD25^-FoxP3^-$ T cells present and to control the local inflammatory immune responses. Lastly, with respect to the effectiveness of either treatment, despite not being the scope of this work, the recovery of many subpopulations was achieved more quickly with the Megl+Al protocol than with the Mil+Al protocol, which is in agreement with previous results (Santos et al. 2019).

Monitoring T cell subsets by using specific biomarkers and analyzing the effectiveness of CanL treatments allows a better understanding of the interplay between the parasite and the dog's immune response, which should improve patient management, lead to the

development of more efficient and less toxic chemotherapies and encourage the use of prophylactic measures that favor the reduction of zoonotic visceral leishmaniosis.

Authors' contributions

GS, IF, and MS conceived and designed the study. AB, AR, IF, JM, MP, and MS collected samples. IF, LG, and MS processed samples and did subsequent microscopic, serological and molecular tests. GA and MS conducted the experiments. GA, GS, IF, and MS analyzed the data. GS and MS conducted statistical analysis. GS, IF, and MS drafted the manuscript. AVR, GA, GS, and IF made in-depth reviews of the manuscript. All authors read and approved the final manuscript.

Funding

Funding for this work was provided by the Portuguese Foundation for Science and Technology (FCT) (PTDC/CVT/118566/2010), the Centro de Investigação Interdisciplinar em Sanidade Animal (CIISA), Faculdade de Medicina Veterinária, Universidade de Lisboa, through project UIDP/CVT/00276/2020 (funded by FCT) and Global Health and Tropical Medicine (GHTM) through project UID/Multi/04413/2013 and PTDC/CVT-CVT/28908/2017 (funded by FCT). Marcos Santos held a PhD scholarship (SFRH/BD/101467/2014) from the Portuguese Foundation for Science and Technology (FCT).

Acknowledgements

The authors would like to thank the cooperation and all the help provided by the veterinarians and staff of the Teaching Hospital of the Faculty of Veterinary Medicine, University of Lisbon (FMV-ULisboa), along with MSc Telmo Pina Nunes for the statistical counseling and a special acknowledgment to all the owners and their respective dogs for their contribution to this study. Lastly, we would also like to thank the team from the Unit of Imaging and Cytometry at Instituto Gulbenkian de Ciência (IGC), in particular MSc Cláudia Andrade, MSc Cláudia Bispo and PhD Rui Gardner for their support in the design of the flow cytometry experiments and fluorochrome panel.

CHAPTER IV:
Leishmaniosis: new insights in a changing world

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In press in Advances in Animal Health, Medicine and Production - State-of-the-art research at CIISA, Springer Nature.

4.1. Abstract

Canine leishmaniosis caused by *Leishmania infantum* is a zoonotic disease of serious veterinary concern in the Mediterranean basin. In Portugal has been reported in dogs, cats and synanthropic rodents. Epidemiological changes and new hosts may contribute to increase zoonotic risk. A better knowledge on immune response, treatment and diagnosis are at the forefront of research on this disease. Host immune response is multifactorial, reflecting the organ specificity. Macrophages (MØ) are the definitive host cells, although neutrophils (PMN) are the first cells to encounter parasites soon after inoculation in the dermis. The PMN-parasite interaction decreases parasite viability, but PMN-MØ interaction induces nitric oxide production and release of neutrophil extracellular traps that contain parasites, controlling dog infection at early stages. Liver resident Kupffer cells (KC) efficiently phagocyte *Leishmania* by establishing an intimate contact with circulating blood. The impact of meglumine antimoniate (MG) over infected canine KC was investigated. The effect of different treatment protocols in dog's immune response was assessed. MG and miltefosine treatments plus allopurinol restore lymphokine gene expression, pointing through a drug-induced reduction of anti-inflammatory and regulatory cytokines. Furthermore, increasing feline leishmaniosis and the inconsistent results of therapeutic protocols led the team to evaluate their safety and effectiveness in cat.

Keywords: *Leishmania*; leishmaniosis; host-immune-response; zoonosis; treatment.

4.2. What is canine leishmaniosis (CanL)?

CanL is a chronic and multisystemic disease caused by the intracellular protozoan parasite *Leishmania infantum* transmitted by Phlebotomine sand flies. A wide range of nonspecific clinical signs is displayed with diverse intensities and symptoms which can affect any organ and be influenced by several factors (Santos-Gomes and Pereira da Fonseca 2008). These include parasite strain and virulence, host genetic background, age, gender, breed, coexistent infections, immune competence and nutrition status (Miró et al. 2008). CanL clinical diagnosis can be complicated with 50% of the infected dogs not presenting clinical signs for several years. Other dogs present acute clinical signs and pathological abnormalities with severe disease and progression to death (Solano-Gallego et al. 2009). Whereas, some dogs exhibit clinical signs within 3 months to numerous years post-infection or even naturally progress to cure (Koutinas and Koutinas 2014).

In dogs with CanL we can identify animals whose lesions are limited to only one lymph node or cases with generalized lymphadenopathy. On the other hand, the lymph nodes most

affected are the superficial ones, identifying more lesions in the mandibular, cervical, prescapular, axillary and popliteal region. In the early stage of the disease, the lymph nodes present lymphadenomegaly, although they never reach the lymph node size with high-grade malignant lymphoma. On palpation they are painless, with smooth surface, not adherent and of increased consistency. At cut, they are swollen and show a ferruginous coloration due to the accumulation of hematic pigment hemosiderin. With the evolution of the disease, regression of adenopathy occurs. On histopathological examination and in the initial phase of the disease, we can observe follicular lymphoid hyperplasia, with enlargement of the lymphoid follicles due to the presence of abundant B-type blast lymphoid cells (centroblasts) in the germinal centers. In the paracortical zones and medullary cords, a proliferative reaction of macrophages (MØ) is observed, whose cytoplasm is full of *Leishmania* amastigotes, there is an increase in the number of plasmocytes and a decrease in the number of mature lymphocytes. In an advanced or chronic phase, the phenomena of lymphocytolysis at the level of germinal centers (with hyalinosis) and intense plasmacytosis at the medullary level are associated with connective tissue hyperplasia and sometimes sclerosis (Alexandre-Pires et al. 2010).

Bone marrow changes may be focal and have a more fluid consistency than normal as well as a uniform red color. There are no alterations in marrow adipose tissue (Rebêlo 1988). Histopathological examination shows granulomas rich in epithelioid MØ, granulocytes and T lymphocytes together with parasites internalized by MØ. There is a marked hyperplasia of the plasma cells that can reach 50% of the myelogram cells. Plasma cells are well differentiated, and no atypia is present, and their presence is linked to polyclonal hypergammaglobulinemia. Several deposits of hemosiderin may also be seen. Usually, involutive and non-regenerative myelopathy develops with depletion of the erythroblastic, leukoblastic and megakaryoblastic cell series (Bourdeau 1988).

Although, the mechanisms that are involved in *Leishmania* resistance or susceptibility in dogs are not known, and a wide range of immune responses and clinical presentations have been reported in CanL: two extreme immune responses have been described associated with disease susceptibility or resistance. Disease susceptibility is generally related to aggravated humoral non-protective immune response and reduced cell mediated immune response characterized by mixed Th1 and Th2 cytokines production, leading altogether to symptomatology and clinicopathological abnormalities (Alvar et al. 2004). On the other hand, disease resistance is associated with $CD4^+$ T cell protective immunity mediated by the production of interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α, which will be responsible for MØ anti-*Leishmania* activity. During infection MØ constitute antigen presenting cells (APC) by processing the foreign antigens that can bind to class II molecules of the major histocompatibility complex (MHC-II) (Kaye et al. 1994). These are subsequently

recognized by the T cell receptors (TCR) (Kaye et al., 1994) which can become tolerant or differentiate into effector cells (Geppert et al. 1990).

A study using MØ and lymphocytes derived from dogs of different sexes, breeds and ages has reported an increased expression of MHC-II in MØ infected with *L. infantum* promastigotes or when cultured with *L. infantum* antigens and in the presence of lymphocytes (Diaz et al. 2012). These findings suggest that the parasite's antigen presentation by MØ in addition to MHC-II expression can be maximized by lymphocytes (Diaz et al. 2012).

Additionally, the activation of T lymphocytes by MHC-II-restricted antigens can induce the production of IFN-γ which can stimulate MHC expression, foreign antigen processing and the presentation of both MHC-I and MHC-II restricted antigens. Other studies reported, unchanged surface MHC-I or MHC-II expression upon infection of MØ derived from beagle dogs with *L. infantum* (Pinelli, Rutten, et al. 1999) or up-regulation of MHC-II levels and decrease on APC function in *L. donovani* infection (Kaye et al. 1994). Furthermore, loss of T cell activity and inactivation of MØ oxidative pathways were associated with lack of co-stimulatory expression and a reduced release of nitric oxide (NO) both in the presence of *L. infantum* parasites or respective antigens (Diaz et al. 2012). Thus, suggesting a regulation of host immune response by promastigote stage specific molecules without the parasite being present (Diaz et al. 2012). In this sense, it is possible that promastigote stage specific molecules are responsible for the suppression of host immune response with consequent *Leishmania* survival, replication and dispersion. Thereby, the identification of the parasite molecules that interfere with the normal activation of the dog immune system and related pathways are critical in the clarification of *Leishmania* survival mechanisms within the host. This information would also greatly contribute to the determination of new targets for vaccine and therapy design.

4.3. What about new *Leishmania* vertebrate hosts?

The epidemiology of leishmaniosis has been changing with the increasing number of studies focusing in new vertebrate hosts. All the new information about wildlife as possible reservoir hosts of *Leishmania* spp. can possibly contribute to the knowledge of the true zoonotic risk of leishmaniosis. While dogs are considered the main reservoir of *Leishmania infantum* infection in endemic areas in Europe, with apparent prevalence rates ranging from 5% to 30%, the existence of other wild vertebrate reservoirs might be a possible cause of the deficient success of control measures. Different studies, mostly in Spain, Italy and France have been done in an increasing number of species, undoubtedly due to the increased wildlife monitoring programs that enable the identification of infected host species, especially carnivores, but also due to the use of more specific and sensitive molecular techniques.

Serologic or direct evidence of *L. infantum* infection in animals from the Canidae, Felidae, Mustelidae, Viverridae and Herpestidae families have been reported in Europe. More recently, *L. infantum* infected lagomorphs and rodents have also been detected in Europe (Millán et al. 2014).

In Europe, the presence of *Leishmania* spp. in red foxes (*Vulpes vulpes*) have for long been studied, but other Canidae have also been detected with the infection, such as the grey wolf (*Canis lupus*) (Beck et al., 2008) and the Golden jackal (*Canis aureus*) (Ćirović et al. 2014). The red fox (*V. vulpes*) due to its taxonomic relationship with the dog, and because it is the most abundant wild carnivore in Europe has been considered an important host. *Leishmania* spp. infected-foxes were detected in the Arrábida region, Southern Portugal, reaching a prevalence rate of 5.63%, which is probably sufficient to maintain endemicity. Although some foxes did not show clinical signs, it was possible to isolate the parasite. In Portugal, isoenzymatic studies showed that parasites isolated from foxes were identical to other strains isolated from man and dogs (Abranches et al. 1983). Serological and molecular studies in free-ranging red foxes from other European countries also detected a considerable number of infected animals. In Liguria, Italy, serology using immunofluorescence assay and enzyme linked immunosorbent assay (ELISA), detected a prevalence of 18% in 50 animals (Mancianti et al. 1994). In Guadalajara, Spain, a survey of leishmaniosis and other parasites in 67 foxes revealed a prevalence of 74% *Leishmania* infection using molecular methods (Criado-Fornelio et al. 2000). Wolves have also been studied in southern Europe with positive results for *Leishmania* infection. In Asturias, Spain, a region considered non-endemic to *L. infantum*, 102 wolves were studied by molecular methods to detect *Leishmania* DNA. An average prevalence of 33% for wolves was reported, with a widespread presence of the parasite in the region and an apparent increase in its prevalence in wolves during the last decade (Oleaga et al. 2018). In another study from Central Portugal and Central and Northern Spain, captive wolves were tested using ELISA and a molecular test and, positive animals were also detected (Sastre et al. 2008). The population of Eurasian golden jackal (*C. aureus*) from Southeastern Europe, Asia, the Middle East and the Caucasus is increasing and spreading quickly, and some studies have revealed their potential role as carriers of zoonotic diseases and this species should be taken under consideration when applying surveillance monitoring schemes. Studies from Serbia tested golden jackal for *Leishmania* species by real-time PCR and detected a prevalence of 6.9% in a total of 216 samples collected (Ćirović et al. 2014). Expanding populations of jackals can play a significant role in spreading different diseases including *L. infantum*. Some studies confirm that once established, the populations of Eurasian golden jackals constitute natural reservoirs for many canine vector-borne diseases, analogous to the role of the coyotes in North America (Mitková et al. 2017). Wild Canidae are extremely useful as sentinel species for the detection and field studies of

Leishmania and confirms the value of wildlife sanitary surveillance programs for the detection and monitoring of zoonotic diseases (Oleaga et al. 2018).

Feline leishmaniosis caused by *L. infantum* is frequently reported in endemic areas and is becoming an emerging feline disease. This is due not only to the increased level of feline medical care, but also to the availability of more sensitive diagnostic tools that contributed to increased number of detected cases in cats (Cantacessi et al. 2015). *L. infantum* has been detected in cats in several southern European countries such as Portugal, Spain, Italy, France, Greece and Cyprus but also in other parts of the world. Recently *L. tropica* and *L. major* were confirmed in cats in Turkey (Paşa et al. 2015). Prevalence, molecular and serologic studies show a lower prevalence in cats compared to dogs and also the diagnosis of clinical cases in cats is rare (Pennisi et al. 2015). Travelling and rehoming cats can result in the detection of clinical cases in non-endemic areas (Rüfenacht et al. 2005). Wild Felidae species from Europe have also been screened with detection of a positive wildcat (*Felis silvestris*) (del Río et al. 2014) and one Iberian lynx (*Lynx pardinus*) (Sobrino et al. 2008). Some species, such as the Iberian lynx are of high conservation value and this infection could have a serious impact on their morbidity and mortality.

Small carnivore species from Mustelidae and Viverridae families have also been detected as positive for *Leishmania* infection. In Mallorca, Spain there was the first report of infection by *L. infantum* in the pine marten (*Martes martes*) (Millán et al. 2011). Stone marten (*M. foina*) and European badger (*Meles meles*) were also detected infected in Spain but none of those three species had visible lesions. Viverridae carnivores such as the common genet (*Genetta genetta*) (del Río et al. 2014) and Herpestidae such as the Egyptian mongoose (*Herpestes ichneumon*) (Sobrino et al. 2008) have also been detected as seropositive. While some populations of such carnivores are decreasing in number, other populations such as the Egyptian mongoose are increasing and these animals, if confirmed as reservoir hosts, might contribute to the epidemiology of leishmaniosis.

The natural infection of *L. infantum* in rodents such as mice (*Mus musculus*) and rats (*Rattus norvegicus*) have been recently identified for the first time in Portugal using molecular methods (Helhazar et al. 2013) but other species such as Black rats (*Rattus rattus*) were detected as positive in Italy (Zanet et al. 2014). Further studies are needed to clarify if these animals have an important role as reservoirs in the parasite life cycle since rats and mice are extremely prolific animals and have a life expectancy that maintains the parasite availability for phlebotomine vectors thus increasing the risk for humans and domestic animals (Helhazar et al. 2013). These studies show the need for efficient rodent control measures to prevent transmission of *Leishmania* parasites.

The Iberian hare (*Lepus granatensis*) has recently been recognized as the origin of a leishmaniosis outbreak in humans in Spain and xenodiagnosis showed that this species is also

able to infect sand flies (Molina et al. 2012). Retrospective studies had shown a high prevalence in this species but also on European hare (*L. europaeus*) from six regions of Spain (Ruiz-Fons et al. 2013). A few molecular and serologic studies in the European rabbit (*Oryctolagus cuniculus*) showed prevalence's from 0.6% to 45.7%, depending on the method (Chitimia et al. 2011).

The role of wildlife in the epidemiology of leishmaniosis is increasingly being studied, particularly the comparison of parasite isolates from different mammal families, humans and dogs. Other vertebrate taxonomic groups will also be included, for instance in transmission studies. Some vertebrate species should be included in surveillance programs as sentinel animals while endangered species with protected status should be monitored for different infections, including leishmaniosis and other that are invasive or considered as pests should be included in population control programs.

4.4. How does the host innate immune response work?

Leishmania promastigotes are deposited in the dermis of the mammalian host through the bite of a sand-fly vector. The local innate immune response constitutes the first line of defense against *Leishmania* parasites. Polymorphonuclear neutrophils (PMN) are the most abundant circulating leukocytes and the first cells to reach the inoculation site, actively guided by chemotactic factors. *In vivo* studies showed that the inoculation of *Leishmania* parasites in hamsters (Wilson et al. 1987), mice (Thalhofer et al. 2011) and dogs (Santos-Gomes et al. 2000) through needle injection induces a rapid dermal infiltration of PMN. Two-photon intra-vital microscopy studies carried out in C57BL/6 mice-*L. major* infected through sand-fly bite confirmed that PMN are the first cells to infiltrate the dermis (Peters et al. 2008).

Although tissue damage following sand-fly bites or needle injection in the absence of parasites induced PMN recruitment (Peters et al. 2008), the contribution of parasite-derived signals in PMN recruitment was studied. *In vitro* studies showed that viable *L. major*, *L. aethiopica* and *L. donovani* promastigotes release chemoattractant factors that induce the migration of human PMN (van Zandbergen et al. 2002). Viable *L. infantum* promastigotes and culture supernatants also induce a strong chemotaxis of canine PMN (Pereira et al. 2017), indicating that the parasite has the ability to modulate leukocyte recruitment at the early phase of infection.

As described in *L. donovani*-human PMN (Pearson and Steigbigel 1981), the attachment between *L. infantum* promastigotes and canine PMN is non-random. Indeed, promastigotes preferentially adhere to PMN by the flagellum tip (anterior pole) (Pereira et al. 2017) (Fig. 35A and B), which probably reflects the concentration of the main adhesion molecules (gp63 and LPG) in specific areas (adhesiotopes) of the parasite membrane (Rittig

and Bogdan 2000). The attachment via the flagellum tip promotes the protrusion of symmetrical pseudopods that maintain the directional entry of the parasite into the PMN (symmetrical phagocytosis), favoring parasite killing (Hsiao et al. 2011). PMN rapidly internalize the parasite at inoculation sites and at visceral organs, becoming the predominant parasitized cells over the first few hours following *L. donovani* and *L. infantum* infection (Wilson et al. 1987; Thalhofer et al. 2011). Experimental *L. infantum* infection showed that 3 to 4 h after dermal injection, promastigotes had already been internalized by canine PMN, proving the early involvement of these cells in CanL (Santos-Gomes et al. 2000). *In vitro* studies revealed that about one third of canine and C57BL/6 mice PMN had internalized the parasite within 3 h (Marques et al. 2015; Pereira et al. 2017).

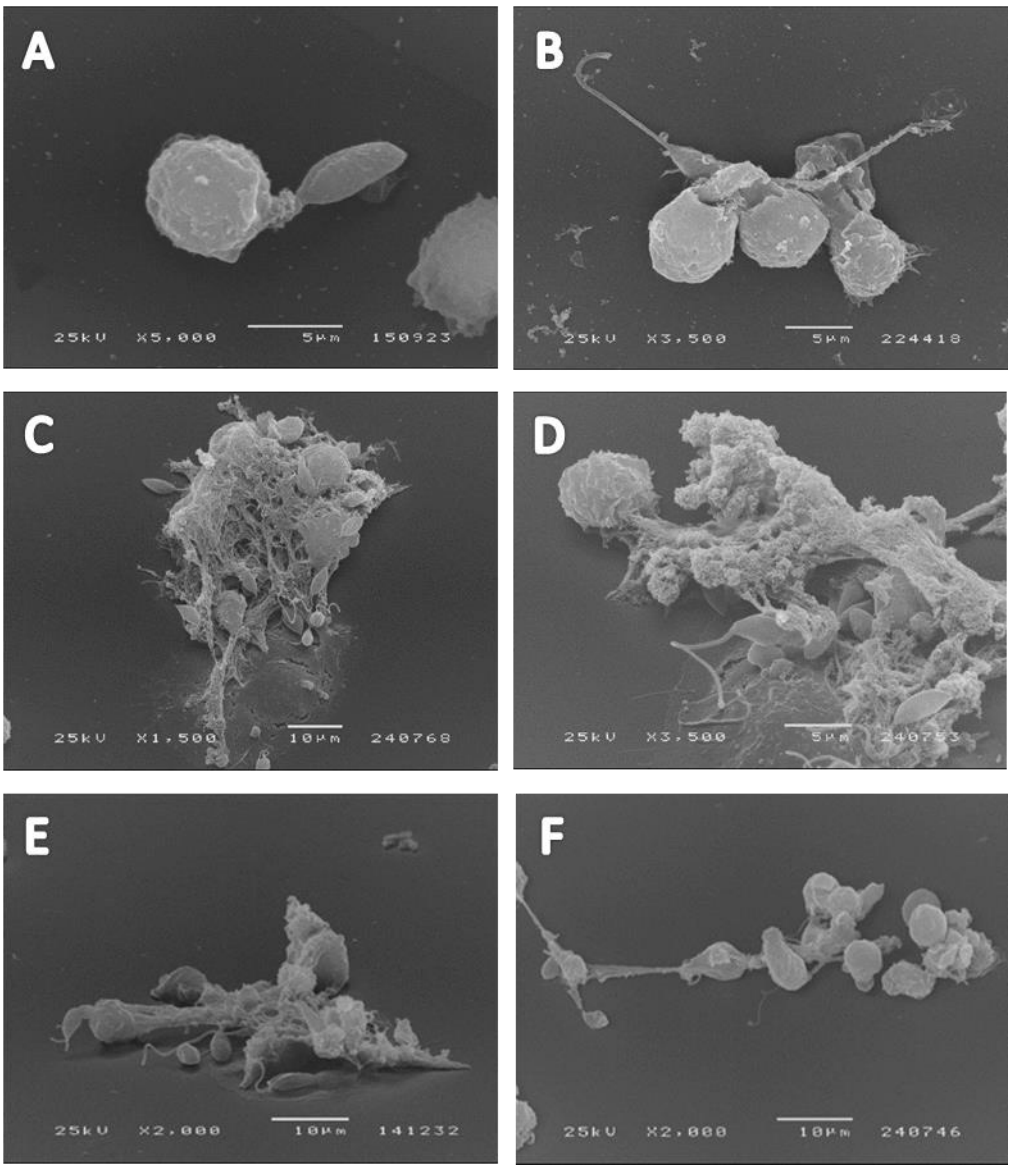

Figure 35. Attachment and phagocytosis of *L. infantum* by dog PMN
Scanning electron microscopy images showing attachment and engulfment of promastigote via their posterior pole (A) and orientated attachment via the flagellum (B). Extracellular interaction between murine PMN and *Leishmania* promastigotes. Scanning electron microscopy images showing filamentous structures entrapping *L. infantum* promastigotes (C), *L. amazonensis* (D), *L. shawi* (E) and *L. guyanensis* (F).

In vitro, canine PMN rapidly kills *L. infantum* promastigotes (Pereira et al. 2017) and BALB/c PMN destroys the parasite in the spleen (Rousseau et al. 2001) using phagocytosis-dependent mechanisms. Other *in vitro* studies showed that *L. donovani* uptake by mouse and canine PMN via lytic organelle-dependent pathway leads to large phagosomes formation and to parasite degradation, but the uptake via a lytic organelle-independent pathway promotes tight phagosomes formation and parasite survival (Gueirard et al. 2008). It was demonstrated that *L. infantum* promastigotes activate canine PMN to release greater amounts of superoxide (Pereira et al. 2017). The induction of a strong oxidative burst results in the elimination of *L. donovani* and *L. major* promastigotes by human PMN (Pearson and Steigbigel 1981; Laufs et al. 2002).

Granule exocytosis and Neutrophil Extracellular Traps (NETs) release contribute to extracellular parasite killing. *L. infantum* promastigotes stimulates neutrophil elastase (NE) exocytosis by canine (Pereira et al. 2017) and by C57BL/6 mouse PMN (Marques et al. 2015), and *L. braziliensis* stimulates both peritoneal and bone marrow derived BALB/c PMN to release NE (Falcão et al. 2015). *L. infantum*, *L. amazonensis*, *L. shawi* and *L. guyanensis* promastigotes promoted NETs release by murine PMN (Fig. 35C, D, E and F) (Valério-Bolas et al. 2019). However, *L. infantum* seems to reduce NETs formation by canine PMN, indicating that the parasite modulates negatively this effector mechanism, favoring parasite spreading and survival (Pereira et al. 2017).

PMN possess some direct leishmanicidal activity, demonstrated *in vitro* and *in vivo*, capable to reduce parasite burden. However, parasite persistence indicates that promastigote killing is clearly insufficient in controlling the establishment of infection. Indeed, several reports showed that a subset of parasites survives to PMN effector mechanisms (Müller et al. 2001). *L. major* viability and capacity to produce infection in naïve mice following in vivo phagocytosis by PMN was demonstrated by Peters et al. (2008). *In vitro* studies indicated that a considerable proportion of *L. infantum* promastigotes maintain viability and replication capability after canine PMN exposure, indicating that dog PMN are competent effector cells able to reduce the parasite burden (Fig. 36) (Pereira et al. 2017). Indeed, it seems that *Leishmania* promastigotes are well equipped to evade PMN killing. For instance, *L. major* blocks the oxidative burst of human PMN (Laufs et al. 2002) and *L. donovani* prevents the fusion between parasitophorous vacuole and mouse neutrophilic granules (Gueirard et al. 2008). Furthermore, some authors consider that surviving parasites might be transitional forms, better adapted to intramacrophagic life (Ribeiro-Gomes and Sacks 2012).

Although PMN might serve as temporary host cells for the parasites within the first hours/days after infection (Aga et al. 2002), MØ are widely considered the primary host cells of *Leishmania* parasites, ensuring its replication, dissemination and long-term survival. Thus,

the interaction between these two phagocytes seems to be important for the establishment of *Leishmania* infection.

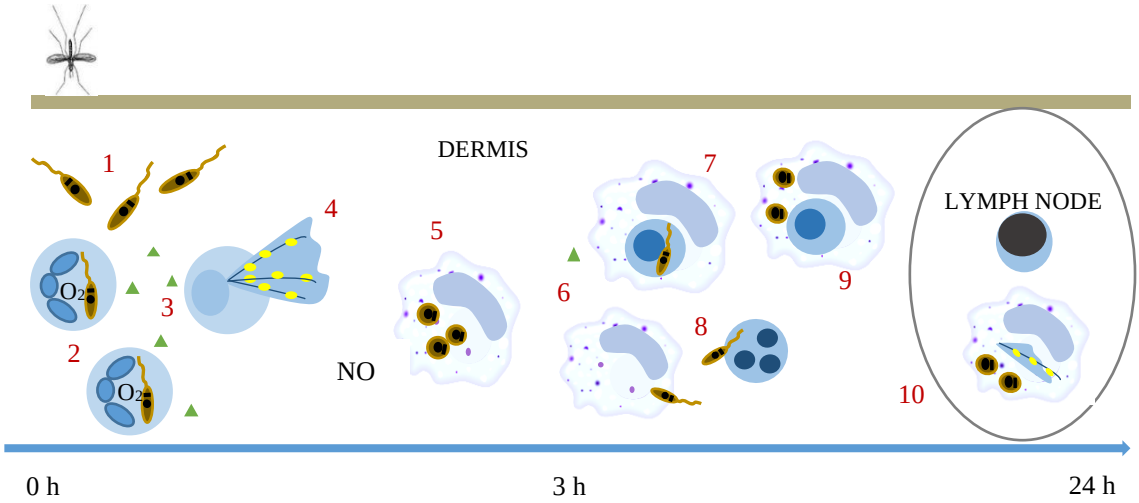

Figure 36. Interaction between dog PMN and MØ at the early phase of *L. infantum* infection
1 - PMN are the first cells to reach the inoculation site and rapidly phagocytize the parasite; 2 - The parasite induces the superoxide ($O2^-$) production; 3 - The parasite induces the exocytosis of neutrophil elastase (triangles); 4 - PMN release neutrophil extracellular traps (NET), containing DNA (lines) and histones (circles); 5 - MØ produce nitric oxide (NO) in response to *L. infantum* infection; 6 - MØ and eventually infected MØ contact with NE that was released by PMN; 7 - Efferocytosis of infected PMN ensure parasite transference; 8 - MØ internalize parasites that escape from dying PMN; 9 - 24 h after inoculation, parasite dissemination takes place; 10 - Eventually in the regional lymph node, parasitized MØ that had removed NET compounds and contacted with NE kill the parasite and present parasitic antigens to lymphocytes.

Although, PMN can undergo spontaneous apoptosis at inflamed sites, *Leishmania* modulates PMN apoptosis, prolonging its life span or accelerating its death (Aga et al. 2002). The parasite uses apoptotic PMN as ‘‘Trojan horses’’ to gain access to MØ. Interestingly, human infected PMN secrete monocyte-attracting chemotactic factors such as MIP1-β, which participate in the recruitment of monocytes. *Leishmania* internalization by MØ via the uptake of infected apoptotic PMN (efferocytosis) prevents the direct interaction with surface receptors, avoiding the activation of MØ effector mechanisms and ensuring parasite survival and replication (van Zandbergen et al. 2004). For instance, *L. major* delays the apoptotic death program of human PMN about 24 h (Aga et al. 2002). When MØ arrive to the inoculation site, they encounter the parasite inside PMN. *In vitro* studies showed that *L. major* infected apoptotic human PMN are readily phagocytized by MØ (van Zandbergen et al. 2004). However, other mechanisms of parasite transference from PMN to MØ have been described. Intra-vital microscopy studies showed viable *L. major* parasites being released from mouse apoptotic PMN in the vicinity of MØ, a mechanism called “Trojan rabbit” (Peters et al. 2008).

In vitro studies showed that efferocytosis of *L. major*-infected apoptotic human PMN promotes transforming growth factor (TGF)-β and suppresses TNF-α release, deactivating MØ effector functions and ensuring intramacrophagic parasite viability and replication (van Zandbergen et al. 2004). However, the interaction between necrotic PMN and *L. amazonensis*-

infected human MØ induces parasite killing via TNF-α NE dependent (Afonso et al. 2008). Another *in vitro* study demonstrated that infected and non-infected canine co-cultures produce NO, a potent microbicide compound, and release extracellular traps (ETs) (Pereira 2016). In the context of infection, ETs clearance can influence MØ phenotype (Boe et al. 2015). Indeed, some studies have shown that the ability of MØ to kill intracellular microorganisms is mediated by the uptake of PMN-derived exogenous proteins. For instance, NE, a NETs component, stimulates *Leishmania*-infected MØ via TLR4 and assists parasite elimination (Ribeiro-Gomes et al. 2007). Thus, the interaction and cooperation between PMN and MØ seems to be complex and influence the outcome of infection, driving either parasite survival or destruction (Fig. 36).

4.5. Do we really know the role of hepatic cells in CanL?

The liver is the largest organ in the mammalian body, and it performs a remarkable number of tasks that support the function of other organs and impacts in all physiologic systems. This organ is, likewise, responsible for several immunological functions as the removal of pathogens and exogenous antigens from the systemic circulation. Its anatomic position and distinctive vasculature contribute to its unique ability to continuously exchange immunological information. In recent years, the liver has been re-discovered and described as a major immunological organ.

In the context of Leishmaniosis, the role of the liver is not yet fully clarified. Few studies on CanL have addressed this question. Most of our current knowledge, on liver's role in disease progression, immune and treatment response is derived from the use of visceral leishmaniosis (VL) murine model and of human VL. The murine model for VL has showed that there is a distinct organ specific pattern of parasite growth during the disease establishment. In humans, dogs and genetically susceptible mice, the liver, the spleen and the bone marrow are major sites of parasite growth and pathology. Evidences regarding the immune response of target organs against *Leishmania* parasites have been accumulated in recent years, pointing out a tissue specific immunity (Alexandre-Pires et al. 2010; Barbosa et al. 2011).

Granuloma formation and a Th1 polarized immune environment, appear to be key in the liver immune response. Indeed, granulomas are poorly formed in the immunodeficient murine model and in humans with progressive VL, which do not develop mature granulomas. The livers of asymptomatic dogs showed an effective immunity with well-organized granulomas able to isolate and restrain parasite spreading in an immune environment of activated effector T cells, dendritic cells (DCs) and central memory cells. In contrast, liver of symptomatic dogs showed a non-organized and ineffective infiltrate of T cells and heavily parasitized Kupffer cells (Sanchez et al. 2004). Furthermore, the highest proportion of activated effector T cells was also observed in the liver of asymptomatic dogs, correlating with

an effective immune response against the parasite. Interestingly, many naive T cells were observed in the liver of symptomatic dogs (Fig. 37). Apparently, central memory T cells sensitized against *L. infantum* may migrate to peripheral tissues, providing protection against these vulnerable sites. In contrast, naive T cells migrate almost exclusively to lymphoid organs, which are designed to receive migrating cells and antigen sampling (Mackay et al. 1990; Mackay et al. 1992).

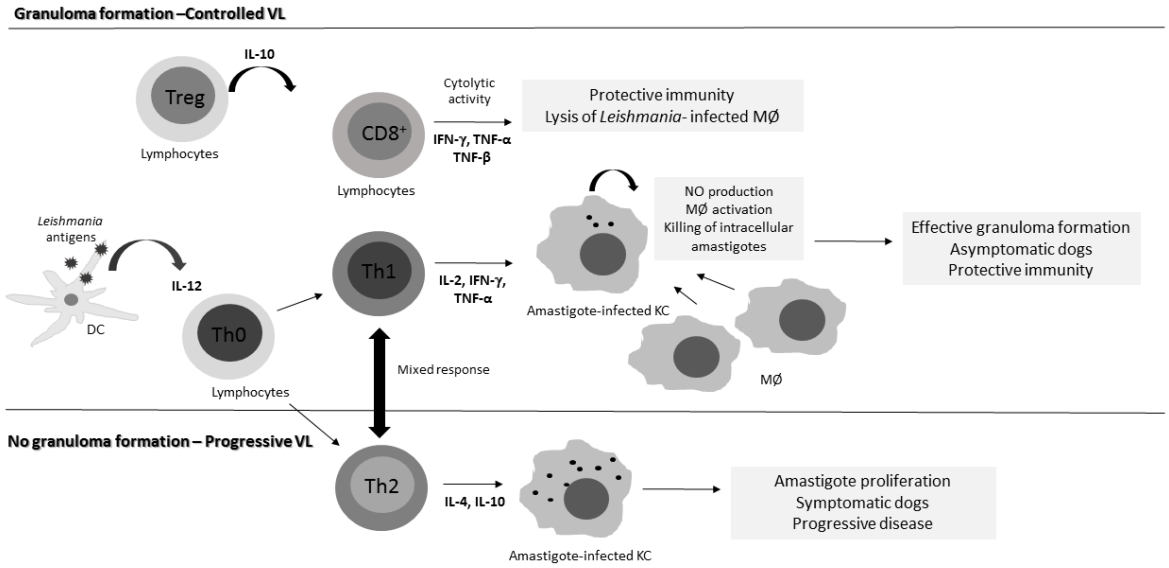

Figure 37. Granuloma formation in a Th1 polarized immune environment is crucial for a protective liver immune response against *Leishmania* infection
The liver response to *Leishmania* infection may lead to the formation of a granuloma, that results in control of parasite growth and dissemination. This response is initiated by IL-12 secreted by activated dendritic cells and results in the activation of lymphocytes and secretion of IL-2, IFN-γ and TNF-α, which will recruit more lymphocytes and lead to the activation of Kupffer cells and recruitment of macrophages. These well-organized granulomas contribute to an effective immunity. In contrast, liver of symptomatic dogs showed a non-organized and ineffective infiltrate of T cells and heavily parasitized Kupffer cells. DC - Dendritic cells; MØ - Macrophages; KC - Kupffer cell; Treg - regulatory T cell; Th1 - T helper cell 1; Th2 - T helper cell 2; Th0 - T helper cell 0 (naïve T cell); NO - nitric oxide; VL - visceral leishmaniosis; IL - interleukin; IFN - interferon; TNF - tumor necrosis factor.

Rodrigues et al. (2017) endorsed the role of the liver as an important immune memory organ in the context of *L. infantum* infection, using the murine model of VL. The phenotype characterization of liver resident T lymphocytes revealed that *L. infantum* infection generates effector and central memory T cells, but these cells did not expand when recalled, demonstrating a parasite silencing effect. The treatment with a leishmanicidal drug (meglumine antimoniate, MG) increases the levels of memory and effector T cells, eliciting a more robust hepatic immune response. This study evidenced the liver's ability to differentiate resident T cells with memory phenotype, emphasizing the role of the liver as an immunological organ. Hepatic leukocyte populations differ from those of other tissues in several interesting ways. Phenotypically, nearly 50% of lymphocytes express the T cell receptor (TCR) and there is an enrichment of $CD8^+$ T cells in the liver. Typically, in the blood, $CD4^+$ T cells outnumber $CD8^+$ T cells, but in the liver this ratio is reversed. The liver also possesses a unique natural killer T

(NKT) cell population. These are important and potent immunomodulatory cell population residing in the liver (Sun et al. 2009). After activation, NKT cells release cytotoxic granules containing perforin and granzyme in a cell directed way. In response to stimulation, these cells also release large amounts of cytokines, such as IFN-γ, and by doing so, shape and direct the immune response and also modulate MHC expression of hepatocytes and hepatic stellate cells (Crispe 2009). As a result, NKT cells have great potential to shape the host immune response, together with additional characteristics of these cells, demonstrate the critical importance of this population for the immune surveillance.

Hepatocytes constitute the majority of the hepatic cells and although the primary roles of these cells are of metabolic nature, hepatocytes express innate immune receptors and, in many cases, have been demonstrated that these cells recognize pathogen associated ligands and display an innate immune response.

Several studies recently conducted also helped to clarify the role of hepatocytes in the orchestration of liver´s innate immune response in the context of *L. infantum* infected canine liver. Rodrigues et al. (2018) has recently contributed to the elucidation of the immune response generated by dog hepatocytes when exposed to *L. infantum*. These parasites presented a high tropism to hepatocytes, establishing strong membrane interactions with these cells. The possibility of *L. infantum* internalization by hepatocytes was raised, although not confirmed. Hepatocytes were able to recognize parasite presence, inducing pattern-recognition receptor (NOD1, NOD2, and TLR2) gene expression and generating a mixed pro- and anti-inflammatory cytokine response. Reduction of cytochrome P (CYPs) 450s enzyme activity was also observed concomitant with the inflammatory response. The addition of leishmanicidal drug, mimicking treatment, increased NOD2, TLR4 and IL-10 gene expression, indicating immune modulation of liver microenvironment. There is evidence for the presence of *L. donovani* amastigotes within hepatocytes in liver biopsies from VL patients having undergone through successful therapy (Duarte et al. 1989). Gangneux et al. (2005) demonstrated *in vitro* that murine, rat and human primary hepatocytes were permissive to L. donovani promastigote infection, but parasites did not massively proliferate. Nevertheless, these findings bring into question a possible role for hepatocytes as a parasite reservoir, during host latent infection, redefining the role of hepatocytes in CanL and, consequently questioning their importance in the epidemiology of zoonotic visceral leishmaniosis (ZVL). Hepatocytes seem to have a major role in coordinating liver's innate immune response against *L. infantum* infection, activating inflammatory mechanisms, but always balancing the inflammatory response in order to avoid cell damage.

Although hepatocytes seem to have a non-negligible role, the main target of *Leishmania* infection in the liver are Kupffer cells (KCs). These cells are the resident MØ population in the liver, located in the vasculature adherent to liver sinusoidal endothelial cells

and directly exposed to the contents of blood circulating through the liver tissue. KCs express an array of scavenger receptors, TLR, complement receptors and antibody receptors, molecules that allow these cells to detect, bind and internalize pathogens. Expressing MHC-I, MHC-II and co-stimulatory molecules needed for T cell activation, KC are important APC. Furthermore, these receptors in part drive the activation of KC, which leads to production of cytokines and chemokines and allows KC to function as immune sentinels, alerting other components of the immune system to the presence of harmful microbes (Bilzer et al. 2006). KCs are also extremely effective in activating the invariant NKT (iNKT) cells that live and patrol the sinusoids of the liver, quickly controlling a potential infection (Jenne and Kubes 2013). Rodrigues, Santos-Mateus, et al. (2017), investigated how canine KCs sense and react to the presence of *L. infantum* promastigotes and amastigotes by evaluating the gene expression of specific innate immune cell receptors and cytokines, as well as the induction of NO and urea production. In addition, the authors also assessed the impact of MG in infected KCs. These cells revealed to be susceptible to both parasite forms and no major differences were found in the immune response generated. *L. infantum* parasites seem to interact with KCs innate immune receptors and induce an anergic state, promoting immune tolerance and parasite survival. MG addition to infected KCs breaks the parasite-imposed silence and increased gene expression of TLR2 and TLR4, possibly activating downstream pathways. Understanding how KCs, sense and react to parasite presence, could bring new insights into the control or even elimination of CanL.

The delicate balance between immunity and tolerance in the liver, results directly from the complex interactions between the various resident immune cells and peripheral leukocyte populations. Under basal conditions, many liver resident cells (LSEC, KCs and DC) have a critical role, maintaining a state of immune unresponsiveness, accomplished, in part, by the low expression of MHC and the absence of co-stimulatory molecules. However, given an appropriate stimulation a robust immune response can be generated in the liver. The anatomical features, blood supply, diverse network of cells and the broad array of receptors enable the liver to act as a frontline immune sentinel. The role of the liver as an important innate immune organ in the context of ZVL and CanL has been growing, accumulating evidences that this organ is key in controlling parasite growth and dissemination to other organs. The liver may function as a safe harbor for *Leishmania* parasites to growth, due to its tolerant immune environment which may have a significant epidemic impact, not only in diagnosis, but also in treatment response and in possible relapses.

4.6. What do we know about the immune response of the dog submitted to CanL treatment?

CanL classic treatments improve the dog's clinical condition, reducing parasite load on the skin and consequently the risk of transmission, but do not eliminate the pathogen (João et al. 2006). The common relapses that occur when therapy is discontinued (Manna et al. 2009) justify the need to improve the efficiency of treatment protocols used for CanL. Some of those protocols include leishmanicidal drugs like MG (N-methylglucamine antimoniate) and miltefosine (1-O-hexadecylphosphocholine, MT), and leishmaniostatic drugs like allopurinol (Ap) (Frézard et al. 2009). MG is a pentavalent antimonial-based drug whose precise mechanism of action is not well understood, being considered a multifactorial drug with probable activity on the molecular processes of the parasite and influence in MØ parasiticidal activity (Frézard et al. 2009). MT is an alkylphosphocholine compound able to induce apoptosis by mechanisms still not entirely clear (Dorlo et al. 2012). Ap is a purine analog of adenosine nucleotide which blocks RNA synthesis, inhibiting *L. infantum* growth (Denerolle and Bourdoiseau 1999). MG in combination with Ap is considered the first line of treatment in Europe and MT plus Ap constitutes the second line of treatment (Solano-Gallego et al. 2009). Nevertheless, with the rising of more reports of drug resistance that lead to either therapeutic failure, unresponsiveness or relapse, a reassessment of the usual therapies is imperative (Pérez-Victoria et al. 2006). Dog clinical signs tend to present type-2 T-helper (Th2) responses associated with the expression of IL-4, IL-5 and IL-6 along with higher levels of specific antibodies (Pinelli, van der Kaaij, et al. 1999; Santos-Gomes et al. 2002). On contrast, protective immunity is thought to depend upon a strong type-1 T-helper (Th1) response characterized by IL-2, IL-12, TNF-α and IFN-γ production. Furthermore, parasites may suppress host immunity by engaging regulatory T cells (Treg) thus enabling the persistence of infection (Rodrigues et al. 2009), with higher expression of regulatory lymphokines (IL-10, TGF-β) (Alves et al. 2009). In our lab, we aim to understand how these most common treatments affect dogs' ability to develop a protective immune response or, if they elicit immune suppression of effector helper T cells, responsible for the orchestration of the immune response, and of cytotoxic T cells that cause the lysis of infected host cells.

For this, several studies are ongoing, namely the effect on cytokine mRNA expression and T cell populations in the blood, lymph node and bone marrow of naturally infected dogs. Published results on cytokine expression (Santos et al. 2019) show that dogs under the MT+Ap protocol presented a protective Th1 response in all tissues, with the maintenance of a high expression of IFN-γ in all tissues, IL-2 in lymph node and TNF-α in bone marrow. This protocol was also able to restore the gene expression of most cytokines, recovering Th2 (IL-4 and IL-5) and Treg (IL-10 and TGF-β) cytokines to normal values. The MG+Ap protocol presented

also a protective Th1 response, but not as pronounced as the MT+Ap. This protocol was also able to, not only, restore the Th2 and Treg to normal values, but also led to a suppression of Th2 and Treg cytokines in blood and of IL-4 and TGF-β in bone marrow beyond normal values. The results also show that changes in cytokine gene expression caused by *L. infantum* in sick dogs seem to be tissue specific, with different tissues presenting different cytokine profiles. Nevertheless, both treatments were able to normalize the cellular immune response and improve the clinical conditions in all dogs. With regard to T cell populations, preliminary results show that sick dogs present specific immunophenotypes in the different organs analyzed, agreeing with what was observed in cytokine expression. Sick dogs presented a predominant pro-inflammatory profile with increase in $CD8^+$ T cytotoxic cell populations. The administration of the treatments seems to cause a shift between CD4 and CD8 cells, with a decrease of $CD8^+$ cells and increase in $CD4^+$ T helper cells, which associated with the increase in IFN-γ previously noted, promoted a Th1 protective response.

4.7. How the neglected feline leishmaniosis should be treated?

Comparing to canine species, information about medical management of feline leishmaniosis is scarce and inconsistent. This is mainly explained by the small number of reported cases in the literature.

The European Advisory Board on Cat Diseases (ABCD) has reported in their guidelines that the medical management of feline leishmaniosis consists of long-term administration of Ap (10-20 mg/kg once or twice daily) (Pennisi et al. 2013). This treatment is usually effective. Information regarding the use of other drugs such as MG, domperidone and MT is scarce (Pennisi et al. 2015). Despite the fact that Ap is actually considered the first-line therapy in feline leishmaniosis, this compound can lead to an unpredictable and overlong response, and eventually several side effects.

Our group has published two clinical cases of feline leishmaniosis, in which Ap therapy did not allow a good clinical management of the disease, and thus required the use of alternative compounds. The first case described a 2-year-old cat with a cutaneous presentation of feline leishmaniosis, diagnosed on skin biopsies (Basso et al. 2016). In this case, Ap was firstly started (Zyloric, Allopurinol, 10mg/kg, *per os*, twice daily, FaesFarma). Two weeks apart, as no improvement has been remarked on the physical exam, MG was added to the therapeutic protocol (Glucantime, 50 mg/kg once daily, subcutaneously, for 30 days, Boehringer Ingelheim Animal Health). This combined therapy, allowed a rapid improvement of the dermatological signs without any side effect reported. No relapse occurred in the following 24 months (date of the last control). The second case reported an unusual presentation of inspiratory dyspnea and stertor in a 12-year-old cat, at which a granulomatous rhinitis

secondary to feline leishmaniosis was diagnosed (Leal et al. 2018). In opposition to previously reported cases, no cutaneous lesions were detected in this cat prior to diagnosis, which was stablished by nasal biopsies. Ap was started (10 mg/kg, *per os*, twice daily) but five days later, a cutaneous adverse drug reaction was strongly suspected, leading to a discontinuation of this compound. MG was then prescribed (50 mg/kg once daily subcutaneously) but three weeks apart, the cat developed acute kidney injury, presumably induced by this drug. Considering this side effect, this drug was also discontinued, and the cat was subsequently treated with nucleotides and active hexose correlated compounds (Impromune, 1/2 tablet once daily, Bioiberica). A relapse of granulomatous rhinitis was suspected 4 months after the onset of this alternative therapy and MT was started (Milteforan, 2 mg/kg, *per os*, once daily, Virbac). Although there was a transitory worsening of azotemia, the cat progressively improved showing stable clinical signs with no relapse of feline leishmaniosis, 16 months apart (date of the last control).

Overall, these two cases contributed to increase the number of reported cases of feline leishmaniosis, highlighting the relevance of continuous clinical and laboratory evaluation. These cases also support that individual response is unpredictable and medical standard therapy should be adapted in a case-by-case scenario.

Acknowledgements

Funding for this work was provided by the Portuguese Foundation for Science and Technology (FCT) through the research projects PTDC/CVT/113121/2009, PTDC/CVT/118566/2010 and PTDC/CVT-CVT/28908/2017, the Centre for Interdisciplinary Research in Animal Health (CIISA - UIDP/CVT/00276/2020), the Global Health and Tropical Medicine (GHTM - UID/Multi/04413/2013) and by awarding the following PhD scholarships: SFRH/BD/73386/2010 (Armanda Rodrigues), SFRH/BD/77055/2011 (Maria Pereira), SFRH/BD/101467/2014 (Marcos Santos) and SFRH/BD/118067/2016 (Ana Valério-Bolas).

CHAPTER V:
Discussion

CanL is a common disease in endemic countries with clinical and epidemiological relevance in itself, but also especially when the human factor is present as well as cases of feline leishmaniosis are becoming less uncommon. Considering this, the role of treatments becomes even more relevant, not only for the health and well-being of dogs, which are an integral part of the modern society, often adopting an important role within the family, but also due to this close contact and, in terms of public health and One Health, their significance as reservoir hosts of zoonotic species of *Leishmania*. Following official guidelines on CanL (LeishVet Guidelines 2018), several possible treatment protocols are recommended, in particular meglumine antimoniate or miltefosine in association with allopurinol, with studies showing different efficacies and effects of these formulations (Denerolle and Bourdoiseau 1999; Manna et al. 2015), from reports of good general efficacy (Nogueira et al. 2019) to failure to remove *Leishmania* parasites (Manna et al. 2009), clinical relapses (Proverbio et al. 2014) and even drug resistance (Yasur-Landau et al. 2016; Yasur-Landau et al. 2018). Beyond that, these treatments seem to constitute a support to the dog's own immune system, as this is what will allow the complete resolution of the disease. Some features of immune stimulation have been attributed to these compounds (Zeisig et al. 1995; Vouldoukis et al. 1996; Wadhone et al. 2009; Barbosa et al. 2011), having not only their anti-leishmanial activity, but also the aforementioned support in the dog's immune system to fight the infection.

Nonetheless, due to different and apparently specific immune responses to *L. infantum* infection in each organ, with studies showing conflicting results, using different methods and infected dogs at different stages of the disease, it becomes difficult to compare and reach a consensual pattern. And whether these compounds influence the dog's immune response during active CanL is what we hope to conclude with this work, by analyzing the profile of cytokines and cell populations in various dog tissues, before and during treatment.

In the present work, the initial objectives were achieved, with some new findings being obtained, but several other new questions also emerged.

5.1. The immunological status of dogs with CanL

Due to the systemic profile of CanL, it was important to evaluate various tissues in order to obtain a general picture of this disease in the dog, in addition to perceiving and confirming the differences reported in previous studies (Alexandre-Pires et al. 2010; Barbosa et al. 2011; Hosein et al. 2017; Giunchetti et al. 2019). Following our findings, it is possible to confirm that, although there are certain similar patterns between the tissues, there are also some marked differences. For instance, IFN-γ is highly expressed by mononuclear cells of peripheral blood, lymph node and bone marrow in sick dogs, being even considered a marker of disease according to our Principal Component Analysis (Santos et al. 2019). This is supported by

others studies that observe this same pattern in these tissues (Pinelli et al. 1995; Quinnell et al. 2001; Manna et al. 2006; Travi et al. 2009; Rodríguez-Cortés et al. 2016). In any case, according to the results of the present work, mononuclear cells of peripheral blood from sick dogs showed a pro-inflammatory cytokine profile with downregulation of IL-2, IL-4, IL-5 and TGF-β, suggesting that *Leishmania* led to a general deactivation of lymphocytes, causing an imbalance of pro and anti-inflammatory immune mediators. In the lymph node, a mixed Th1/Treg profile was observed, with low IL-2 but high IFN-γ, together with down expression of TGF-β, but increased expression of IL-5 and IL-10, pointing to a balance between the differentiation of the inflammatory response mediated by IFN-γ and a regulatory immune response that could favor the persistence of the parasite. The bone marrow, in turn, presented a more pronounced pro-inflammatory response than peripheral blood, with an increase in IFN-γ, IL-12 and TNF-α and low expression of lL-4, IL-5 and TGF-β.

The macrophage's role on intracellular death of *Leishmania* parasites is crucial for the control of infection, but *Leishmania* seem to modulate the repertoire of cytokines secreted by infected macrophages and their ability to act like APC, by suppressing MHC-II expression and preventing the adequate generation of the adaptive immune response (Cecílio et al. 2014; Martínez-López et al. 2018). Thus, signals produced by these APCs, such as IL-12, which are essential for the polarization of naïve $CD4^+$ T cells towards a Th1 subset and subsequent IFN-γ production (Strauss-Ayali et al. 2005; Liu and Uzonna 2012; Rodrigues et al. 2016) are suppressed. Without IFN-γ activation, infected macrophages do not turn into M1 cells and don't engage in the production of NO, essential for active killing of intracellular parasites (Nathan and Hibbs 1991; Liu and Uzonna 2012).

According to previous studies (Bourdoiseau, Bonnefont, Magnol, et al. 1997; Cortese et al. 2015), dogs with CanL are reported to have lower CD4/CD8 ratios, which is also the case in the present work in peripheral blood (<1) and lymph node (≈1) of sick dogs, when compared to healthy dogs (≈2). In the peripheral blood, the reduction in the CD4/CD8 ratio was due to a reduction of $CD4^+$ T cells together with an increase of $CD8^+$ T cells. This contraction of $CD4^+$ T cells has been associated with the inability of the host to control infection, allowing the survival and replication of *Leishmania* parasites in macrophages (Guarga et al. 2000), with studies in mice showing that these parasites negatively interfere with the ability of IFN-γ to induce the expression of MHC-II mRNA, leading to parasitized macrophages with low expression of MHC-II molecules (Reiner et al. 1988). These macrophages, due to their reduced capacity as APC, are therefore unable to provide co-stimulatory signals to naïve $CD4^+$ T cells (Saha et al. 1995; Pinelli, Rutten, et al. 1999), which, in turn, are not stimulated, do not proliferate and do not produce IFN-γ. This can also be supported by the previously mentioned lymphocyte deactivation in this tissue, with downregulation of IL-2, a stimulator of T cell proliferation and Th cell activator, maintaining a pro-inflammatory environment, but with a lack

of signaling for the intracellular production of NO by macrophages. In the lymph node, in turn, the reduced CD4/CD8 ratio is due to an increase in $CD8^+$ T cells, with the cytokine setting, represented by increased IL-5 and IL-10, besides the increased IFN-γ, possibly leading to an activation of infected macrophages to turn into M2 cells, creating a regulatory and anti-inflammatory profile, which favors the survival and growth of parasites (Bhattacharya and Ali 2013; Dayakar et al. 2019)

Following the results of previous reports (Bourdoiseau, Bonnefont, Magnol, et al. 1997; Moreno et al. 1999; Giunchetti et al. 2008), the sick dogs included in the current study also showed significant expansion of $CD8^+$ T cells in blood, lymph node and bone marrow, that together with the previously stated high IFN-γ gene expression found in all these tissues point to the possibility of $CD8^+$ T cells playing a non-negligible role in the production of this cytokine. These findings also suggest that $CD8^+$ T lymphocytes are at the forefront of the fight against *Leishmania* infection. Additionally, sick dogs showed expansion of $CD4^+$ ($CD25^+FoxP3^+$) Treg cells in peripheral blood, along with a decline in $CD4^+$ ($CD25^-FoxP3^-$) effector T cells, signaling a lack of adequate cellular immune response. This could allow the parasite's persistence and, on the other hand, avoid additional inflammation that exacerbates parasite-mediate immunopathology and, consequently, increase the severity of CanL.

Although it is widely believed that Treg cells belong exclusively to the $CD4^+$ fraction, the question of whether $CD8^+$ T cells expressing CD25 and FoxP3 should be considered Treg cells is still a matter of study and debate. While in the murine immunological model, FoxP3 expression is restricted to $CD4^+CD25^+$ T cells, studies in human thymocytes have revealed $CD8^+CD25^+$ cells expressing FoxP3, with functional characteristics similar to CD4 regulatory T cells, such as the suppression of autologous $CD25^+$ T cells through a contact-dependent mechanism (Cosmi et al. 2003; Yu et al. 2018), with other authors confirming that, by stimulation, FoxP3 is expressed in $CD8^+$ T cells, being exclusively limited to those expressing CD25 (Morgan et al. 2005; Stockis et al. 2019). However, with different authors observing both the presence and absence of regulatory functions in these cells, only speculation remains that the production of human FoxP3 in an activated cell may act in part as a natural negative feedback loop to prevent unrestricted production of cytokines and inflammatory reactions in humans (Morgan et al. 2005). In turn, in human visceral leishmaniosis, Peruhype-Magalhães et al. (2006) have proposed that IL-10-producing $CD8^+$ T cells could lead to inhibition of pro-inflammatory cytokines such as TNF-α and IFN-γ, thus blocking the anti-leishmanial macrophage activity. Other studies have shown that a subset of $CD8^+$ Treg cells can inhibit the immune response mediated by $CD4^+$ T cells by inducing the apoptosis of activated $CD4^+$ T cells (Chen et al. 2013). Lastly, without significant information about this subset of Treg cell in dogs, the question still remained whether they exist as in humans or whether FoxP3 is restricted to the fraction of $CD4^+$ T cells as in mice.

This way, the present work finally revealed some insights into this $CD8^+$ Treg population, with the observed expansion of $CD8^+$ ($CD25^+FoxP3^+$) Treg cells in peripheral blood and bone marrow possibly representing another mechanism of immune regulation that may favor the parasite's survival. While in the lymph node of dogs with CanL there were no significant changes in $CD4^+$ Treg and effector T cell subsets, in a study with mice infected with *L. infantum* Rodrigues et al. (2009) found a high frequency of $CD4^+CD25^+$ T cells expressing FoxP3 in lymph nodes in the first weeks of infection, which soon decreased during the chronic phase of the disease, supporting our results. Thus, confirmation of the presence of $CD8^+$ Treg cells in peripheral blood and bone marrow during active CanL raises the question of their role. Previous studies have found that, although the effect of cytotoxic mechanisms lead to the apoptosis of target cells, $CD8^+$ T cell cytotoxicity does not control *L. braziliensis* parasites (C.D.S. Santos et al. 2013; T.M. Campos et al. 2017). The same authors also found that $CD8^+$ T cells co-cultured with macrophages infected with *Leishmania* released granzyme B, but had no effect on parasite death, while $CD4^+$ T cells co-cultured with infected macrophages produced IFN-γ and mediated *Leishmania* killing (C.D.S. Santos et al. 2013). Associated with this, and while the immunoregulatory function of $CD4^+$ Treg cells *in vivo* is to protect the host against the development of autoimmunity, they may also help in mounting an immune response against foreign parasites, such as *Leishmania*. As the responsiveness to IL-2 by $CD8^+$ T cells is a critical factor for cytokine production (IFN-γ) and subsequent cytolytic activity, with $CD4^+$ Treg cells being able to downregulate IL-2 production and CD25 expression in $CD8^+$ T cells (Piccirillo and Shevach 2001), they could control the exacerbated and fruitless immunopathogenesis of these cytotoxic cells in CanL. Thus, and following previous studies as well as our results, $CD8^+$ Treg cells could, in turn, lead to the inhibition of pro-inflammatory cytokines such as TNF-α and IFN-γ through IL-10, blocking the anti-leishmanial macrophage activity (Peruhype-Magalhães et al. 2006), while inhibiting the immune response mediated by $CD4^+$ T cells by inducing the apoptosis of activated $CD4^+$ T cells (Chen et al. 2013; Yu et al. 2018). Following this, we propose that $CD8^+$ Treg cells may have a possible role in the maintenance of *Leishmania* infection (Fig. 38). Future efforts should be made to understand these relationships.

Additionally, this study found that $CD4^+CD8^+$ DP T cells increased significantly in all tissues tested on dogs with CanL, similar to studies on other chronic diseases such as cancer, autoimmune diseases and viral infections (Matsui et al. 1989; Bagot et al. 1998; Kitchen et al. 2004; Desfrançois et al. 2010; Talker et al. 2015). Furthermore, the presence of $CD4^+CD8^+$ DP T cells expressing CD25 and FoxP3 in the peripheral blood of sick dogs revealed a possible regulatory activity (Rothe et al. 2017), while the lymph node and bone marrow showed decreased percentages of CD25, reflecting a possible cytotoxic role of these cells (Rabiger et

al. 2019). However, since the role of these $CD4^+CD8^+$ DP T cells is not yet fully understood *in vivo*, further studies in these subpopulations are still needed to elucidate their modes of action.

Finally, considering all of these reported differences between the tissues studied, the proposed compartmentalization of the immune response against *L. infantum* is supported.

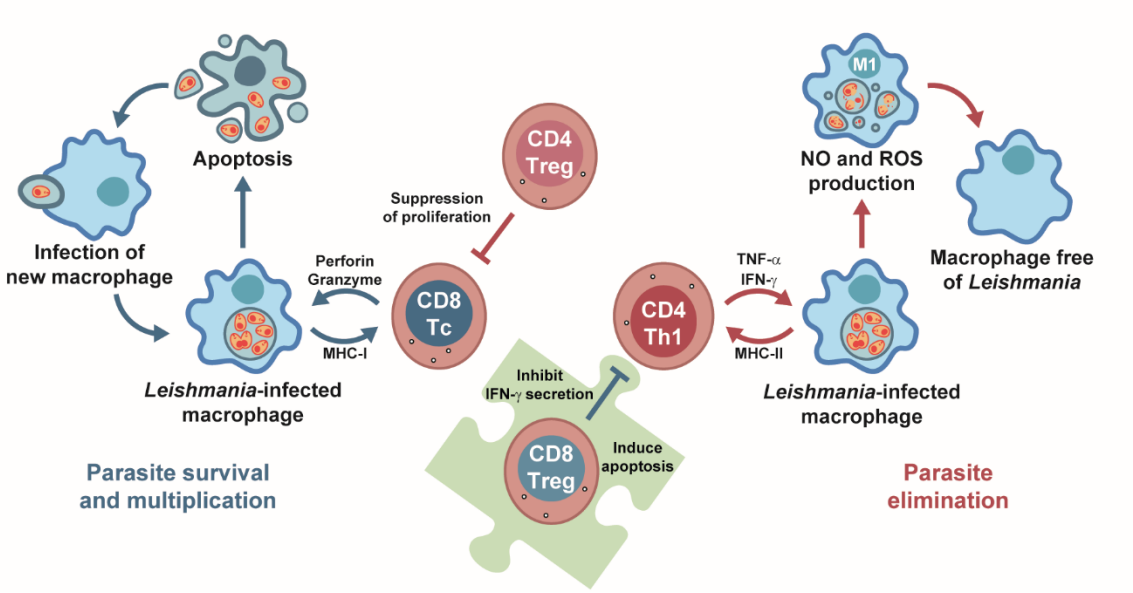

Figure 38. Proposed role of $CD8^+$ Treg cells in CanL
Cross-talk between $CD8^+$ T cells and *Leishmania*-infected macrophages leads to the release of Granzyme B and Perforin to fight infection, activating the programed cell death of the macrophage, but not causing parasite death. These parasites are then available to be phagocytized by new macrophages that perpetuate the infection. To counteract this, $CD4^+$ Tregs can act by suppressing the proliferation of $CD8^+$ T cells, in order to reduce the exacerbated inflammatory response and prevent the perpetuation of parasite survival. In turn, activated TNF-α and IFN-γ-producing $CD4^+$ T cells act on infected macrophages, activating them to become M1 cells, which produce NO and ROS, resulting in the intracellular death of the parasite. IL-10-producing $CD8^+$ Tregs can then lead to inhibition of pro-inflammatory cytokines such as TNF-α and IFN-γ and induce the apoptosis of activated $CD4^+$ T cells, blocking the anti-leishmanial macrophage action previously induced by these cells. Figure based on Piccirillo and Shevach (2001), Peruhype-Magalhães et al. (2006), Chen et al. (2013) and C.D.S. Santos et al. (2013).

5.2. Evidence of increased cellular communication in dogs with CanL

In a recent study, Burel et al. (2019) found that doublet discrimination, an important step when defining the gating strategy in flow cytometry analysis, could be masking information on cell-to-cell contact, in particular T cell/monocyte association, that is not disrupted during sample processing in this technique. These gated events are always excluded from the final analysis in flow cytometry, in the sense of being considered experimental artifacts, originated from cellular aggregates or cellular debris resulting from the damage and disintegration of cells after apoptosis or mechanical disruption (Wersto et al. 2001). Burel et al. (2019) concluded that the cell complexes found in peripheral blood are the result of *in vivo* interaction between T cells and monocytes and, although the origin and location of the formation of these complexes are unknown, they consider that it may be either occurring directly in blood or in tissues and/or draining lymph nodes and migrate to the peripheral circulation. The authors also

recommend this approach as a tool to monitor treatment success and predict possible relapses, using examples from individuals infected with dengue, in which a higher frequency of T cell/monocyte complexes at time of admission was associated with dengue hemorrhagic fever, the more severe form of disease and, in the case of active tuberculosis, all tested individuals showed a significant decrease in the frequency of T cell/monocyte complexes after treatment.

Following these new findings, in the present work, a simple approach was made to compare the frequency of doublets in healthy, sick and treated dogs, in order to find any statistically relevant differences between these groups. As our original experiments did not include additional fluorochrome-conjugated antibody markers for monocytes, such as CD14, we were only able to obtain the complete doublet frequencies according to our gating strategy and compare between groups. In this sense, we observed a significant increase of cell doublets in the blood and lymph node of dogs with CanL when compared to healthy dogs. These results may reflect an increase in the crosstalk between T lymphocytes and APC in these tissues of sick dogs. Subsequently, the administration of any of the treatments led to an increase in this crosstalk in all tissues, including bone marrow, with subsequent decrease to normal values.

We also propose that doublets may increase as a result of Treg/lymphocyte interaction, as Treg cells, which appear to be augmented in CanL, seem to exert immune suppression by mechanisms dependent on cell contact (Lee et al. 2018), with Burel et al. (2019) pointing out the possibility that other types of complex pairings of T cells and other APCs, such as B cells or DCs, may be found.

Although further detailed studies are needed to corroborate this hypothesis, the correlation between CanL and the level of doublets may be used as a possible marker of the disease, to monitor the success of treatment and to predict potential relapses.

5.3. Meglumine antimoniate combined with allopurinol and miltefosine combined with allopurinol led to dog's clinical recovery

The dogs with CanL studied in this work showed a variety of clinical signs, from loss of body weight to local/generalized lymphadenopathy, lethargy, onychogryphosis, cutaneous alopecia, exfoliative dermatitis, erosive-ulcerative dermatitis, decreased/increased appetite, hyperkeratosis, mucous membrane pallor and polyuria/polydipsia, with fewer cases of epistaxis, lameness and masticatory muscle myositis. Sick dogs also showed changes in hematological and biochemical parameters, including mild decrease of hemoglobin values, mild erythropenia, lower hematocrit values, thrombocytopenia, mild renal azotemia, hyperglobulinemia with increased alpha 2 and gamma globulin fractions, and reduced values of alpha 1 and albumin/globulin ratio. Regarding anti-*Leishmania* antibody titers, these dogs

showed values between 1:80 and 1:320. Due to this diversity in clinical signs and laboratorial findings, even within the same animal, all dogs were allocated in a mixed clinical stage I/II, according to the LeishVet Consensus Guidelines (LeishVet Guidelines 2018), having mild to moderate disease, and stage C following the Canine Leishmaniosis Working Group Guidelines (Paltrinieri et al. 2010), being considered sick dogs.

Both miltefosine and meglumine antimoniate protocols in combination with allopurinol were found to be effective in treating CanL, with the two treatment groups exhibiting a successful recovery, with remission of all clinical signs within the three-month observation period. Likewise, three months after starting treatment, all but one dog were negative for anti-*Leishmania* antibodies. This single dog belonged to the group treated with miltefosine and allopurinol and presented a titer of 1:320, which when re-evaluated 6 months after the initial diagnosis became negative. In addition, no amastigote forms were observed in lymph node and bone marrow smears of dogs from both groups. Lastly, it should also be noted that one month after the start of treatment, dogs in the group treated with meglumine antimoniate plus allopurinol exhibited greater liveliness and energy than dogs treated with miltefosine and allopurinol. These results are supported by previous reports on the effectiveness of these treatments in CanL (Denerolle and Bourdoiseau 1999; Nogueira et al. 2019), while also suggesting a possible superior efficacy of meglumine antimoniate compared to miltefosine, as stated in other works (Manna et al. 2015).

In addition, several questions arose regarding which protocol to choose when treating a dog with CanL, of which several considerations must be taken. The potential nephrotoxicity of antimonial compounds, such as meglumine antimoniate, has been considered over the years as a disadvantage and a characteristic to be avoided in dogs with renal pathology (Mancianti et al. 1988; Roatt et al. 2014), recommending the treatment of these animals with alternatives such as miltefosine, which in turn is reported to have teratogenic effects (Sundar and Olliaro 2007; Roatt et al. 2014), but more and more authors consider this to be unfounded. According to some studies, such as those by Manna et al. (2015), the authors suggest that the meglumine antimoniate is more effective compared to miltefosine, with our study demonstrating some evidence of this, with dogs treated with meglumine showing earlier recovery of clinical signs, laboratory findings and some cytokines and cell populations when compared to the miltefosine protocol. On the other hand, other studies propose that miltefosine does not seem to require T cell-dependent immune mechanisms in order to act (Murray 2000), indicating that this drug can be used in cases of T cell deficiency (N. Marques et al. 2008; Haldar et al. 2011), unlike meglumine antimoniate, which appears to depend on a Th1 response by T cells (Murray et al. 1989; Murray et al. 1991). Lastly, more practical issues are usually left to the tutor to decide, such as the price of the medication, which varies according to the dog's weight, and the mode of administration, with miltefosine being easier for tutors to

administer, as it is a liquid formulation usually given with food, *versus* meglumine antimoniate, which requires a daily injection, forcing the tutor to either take the dog to the vet every day for proper administration or for the tutor to learn and voluntarily administer the medication. Thus, the decision falls on a case-by-case basis, with clinicians following the latest scientific developments but ultimately it is the tutors who decide based on their economic power and resourcefulness in administration. In any case, the correct dosage and the treatment period should always be advocated, in order to avoid relapses, associating it with preventive measures, such as collars, spot-on and other formulations, to avoid re-infection and transmission.

5.4. The effect of miltefosine and meglumine antimoniate combined with allopurinol on the dog's immune response to CanL

The analysis of peripheral blood, popliteal lymph node and bone marrow along the two treatment protocols against CanL chosen in the present study revealed that miltefosine combined with allopurinol led to an increase in IFN-γ generation in all tissues, as shown in previous works (Manna, Reale, Picillo, et al. 2008). Furthermore, peripheral blood and lymph node cells also exhibited increased generation of IL-2, indicating possible lymphocyte proliferation, and TNF-α overexpression, suggesting good conditions for macrophage activation into M1 cells, increased production of NO and subsequent parasite elimination (Zeisig et al. 1995). The bone marrow also presented a maintenance of high expression of TNF-α along with IFN-γ and a slight increase in IL-2, promoting the macrophage activation into M1 cells. Parallel to these results, we could observe the normalization of the immune-suppressor TGF-β in all tissues, synonymous with tissue healing, of anti-inflammatory cytokines (IL-4 and IL-5) in peripheral blood and lymph node and the immune-suppressor IL-10 in lymph node, while the bone marrow showed decreased expression of IL-4, IL-5 and pro-inflammatory IL-12.

The administration of miltefosine plus allopurinol in the present study allowed the recovery of the CD4/CD8 ratio in all tested tissues to values equal to the healthy group and, subsequently, the values of $CD4^+$ and $CD8^+$ T cells. With regard to Treg cells, whose functions are to prevent autoimmunity and suppress excessive or misguided immune responses (Furtado et al. 2002; Jawed et al. 2019), there are few studies in CanL (Hosein et al. 2017) and, therefore, such works, as the present study, become crucial. In peripheral blood and bone marrow, treatment with miltefosine led to a rise in $CD4^+$ Treg cells that quickly normalized, while the $CD4^+$ effector T cell subset progressively increased to normal values. The lymph node, in turn, showed a transient growth in $CD4^+$ Treg cells and a progressive decrease in the $CD4^+$ effector fraction, normalizing in the third month. This seems to promote the development

of a suppressive immune response located in the dog's lymph node, possibly to counteract an exacerbation of the pro-inflammatory profile. The expansion of $CD8^+CD25^+FoxP3^+$ T cells observed in the blood and bone marrow of sick dogs in the present study, proposed to be an immune regulation mechanism that could favor the parasite's survival (Novais and Scott 2015), through the administration of miltefosine and allopurinol, directs these $CD8^+$ Treg cells in the blood to progressively return to normal values, possibly releasing the negative regulation of cytokines, such as TNF-α and IFN-γ, important for the activation of macrophages and NO production. In the lymph node, in turn, the miltefosine protocol led to the expansion of the $CD8^+$ Treg cell subset over the three-month period, while causing a rapid contraction of $CD8^+$ effector T cells. This imbalance of the $CD8^+$ regulatory and effector T cell subsets may be a response to increased levels of IL-2 and IFN-γ mRNA in this tissue, in order to restrict the local inflammatory immune response and cytotoxic activity, lessening possible tissue damage. Bone marrow, showed also a decline in $CD8^+$ effector T cells as a consequence of treatment with miltefosine, possibly avoiding augmented cytotoxicity, which associated with increased gene expression of TNF-α and IFN-γ by mononuclear cells present in this tissue, can induce the classic activation of macrophages, leading to parasite death.

The protocol of meglumine antimoniate in combination with allopurinol, in turn, led to the normalization of the pro-inflammatory immune response in peripheral blood cells, restoring IFN-γ and increasing IL-2 expression, while the lymph node presented a maintenance of IFN-γ and increased levels of IL-2 and TNF-α, similar to what occurred with the miltefosine protocol, possibly indicating lymphocyte proliferation and activation of macrophages into M1 cells, as observed by Vouldoukis et al. (1996). The bone marrow, for instance, showed a maintenance of increased expression of IFN-γ one month after the start of treatment. In the lymph node, an increase of anti-inflammatory cytokines, IL-4 and IL-5, could be being expressed to balance the increased Th1 immune response profile of that tissue. On the other hand, IL-4 and IL-5 remained downregulated in peripheral blood and bone marrow mononuclear cells, as well as IL-10 and TGF-β, indicating the inhibition of the Th2 and Treg immune response during clinical improvement of dogs.

In previous studies, in addition to observing a reduced $CD4^+$ T cell count in the peripheral blood of sick dogs, treatment with meglumine antimoniate led to normalization of these T cells (Bourdoiseau, Bonnefont, Hoareau, et al. 1997), with authors like Moreno et al. (1999) and Moreira et al. (2017) reporting an increase in the percentage of $CD4^+$ T cells after treatment with meglumine antimoniate above the values of healthy dogs. The administration of meglumine antimoniate and allopurinol in this study led to the recovery of the CD4/CD8 ratio in all tissues, with the protocol promoting the recovery of $CD4^+$ and $CD8^+$ T cells, especially in peripheral blood, where the recovery occurred a month earlier of the miltefosine protocol. In terms of Treg cells, the peripheral blood and bone marrow of dogs treated with the meglumine

antimoniate protocol showed a similar pattern to those treated with the miltefosine protocol, with an increase in $CD4^+$ Treg cells that quickly normalized, while the $CD4^+$ effector T cell subset increased to normal values, one month earlier, when compared to the other protocol. The lymph node also showed an increase in $CD4^+$ Treg cells and a progressive decrease in the $CD4^+$ effector fraction, normalizing by the third month, which could allow a localized suppressive immune response to neutralize an exacerbation of the pro-inflammatory profile. For the $CD8^+$ fraction of Treg and effector T cells, the meglumine antimoniate showed a pattern similar to the miltefosine protocol, by directing $CD8^+$ Treg cells in the blood to progressively return to normal values, in order to release the negative regulation on cytokines such as TNF-α and IFN-γ, important for macrophage activation and production of NO. While in the lymph node, this protocol led to a lesser expansion of the $CD8^+$ Treg cell subset that rapidly normalized, while maintaining a decrease of $CD8^+$ effector T cells for another month. In the bone marrow, this protocol led to normalization of $CD8^+$ Treg cells, together with a decline in $CD8^+$ effector T cells, possibly balancing the exacerbated cytotoxicity with some gene expression of TNF-α and IFN-γ, in order to induce the classic activation of macrophages, leading to intracellular killing of parasites.

Lastly, these treatment protocols, in addition to their direct effects on *Leishmania* parasites, are probably influencing, in different ways, the development of a protective response. Whether this occurs by simply reducing the *Leishmania* load and allowing the recovery of lymphocyte proliferation signaling, activation of macrophages and intracellular NO production, or an additional direct action on the immune system, such as those reported for miltefosine that act as a co-stimulator of the IL-2-mediated T cell activation process (Vehmeyer et al. 1991) or like those in the pentavalent antimonials family that seem to promote the generation of ROS, by boosting the production of IL-12 and, subsequently, IFN-γ, which activates macrophages (Basu et al. 2006), is still uncertain, with the current work reinforcing the possibility of both concepts being present. Still, further studies on this topic should be considered, in order to elucidate on this issue.

5.5. The roles of PMN and hepatic cells in the control of *L. infantum* infection and the treatment of Feline leishmaniosis (FeL)

Additional studies carried out by the working group on the interaction of *L. infantum* with PMN, the first line of defense of the innate immune system, showed that viable promastigotes and culture supernatants induce a strong chemotaxis of canine PMN, which preferably engulf the parasite through the anterior pole where the flagellum tip is located (Pereira et al. 2017), revealing that *Leishmania* is able to modulate leukocyte recruitment in the initial stage of infection. This *in vitro* work also showed that about a third of PMN of canine

and C57BL/6 mice had internalized the parasite in 3 hours (Marques et al. 2015; Pereira et al. 2017), emulating previous reports with experimental cutaneous injection of *L. infantum* in dogs (Santos-Gomes et al. 2000), proving the early involvement of these cells in CanL. Canine PMN infected with *L. infantum*, in turn, seem to have the formation of NETs reduced, indicating that the parasite could negatively modulate this effector mechanism, favoring parasite spreading and survival (Pereira et al. 2017). This, associated with the considerable proportion of *L. infantum* promastigotes that remain viable and with good replication capacity after exposure to canine PMN (Pereira et al. 2017), reaffirms the role of PMN as temporary host cells of the parasite to be later transferred to the primary host cell, the macrophage.

In the liver, *L. infantum* presents a high tropism to hepatocytes, showing strong membrane interactions, inducing gene expression of pattern-recognition receptors (NOD1, NOD2, and TLR2) and generating a mixed pro- and anti-inflammatory cytokine response (Rodrigues et al. 2018). In this study, although the internalization of parasites by hepatocytes has not been confirmed, other studies on *L. donovani* infection both *in vivo* (Duarte et al. 1989) and *in vitro* (Gangneux et al. 2005) report amastigote and promastigote internalization, respectively, bringing the possibility of hepatocytes being a reservoir of parasites during latent infection in the host. Kupffer cells, the resident macrophages in the liver, in turn, are important APC susceptible to both amastigote and promastigote parasite forms, with *L. infantum* being able to interact with the innate immune receptors and induce an anergic state, promoting immune tolerance and parasite survival (Rodrigues, Santos-Mateus, et al. 2017). The administration of meglumine antimoniate to these infected Kupfer cells, in turn, seems to release the silence imposed by the parasite and increase the gene expression of TLR2 and TLR4, possibly activating downstream pathways.

Feline leishmaniosis, despite being recognized for several decades by the scientific and veterinary communities, due to its scarce presence and the few reported cases has led to the best approach to treatment still being uncertain. Since early guidelines, the use of long-term administration of allopurinol in monotherapy has been recommended as an effective treatment for FeL (Pennisi et al. 2013). But even so, there are reports of side effects and several cases of therapeutic failure, including two clinical cases reported by the working group and where alternative therapeutic protocols were chosen with greater success (Basso et al. 2016; Leal et al. 2018), positively supporting the LeishVet group's decision to include FeL alongside CanL in their guidelines, while proposing some alternative treatments (LeishVet Guidelines 2018). However, as there are no published controlled studies to provide scientific evidence on the best treatment for FeL, the decision rests with the veterinarian, depending on each case (Pennisi et al. 2015; LeishVet Guidelines 2018).

CHAPTER VI:
Conclusions and future perspectives

The classic Th1/Th2 dichotomy of immune response to CanL is still true today, in the sense that a predominant Th1 response appears to confer protection against *Leishmania*, while a strict Th2 response fails to provide that protection. However, following previous studies and our own findings, while a predominant cytokine Th1 response is important, dogs with CanL can still remain sick even when they have that characteristic Th1 response, often presenting a mixed profile between Th1/Th2 and Th1/Treg, as occurred in this work. Allied to this, the idea of compartmentalized immunity in leishmaniosis adds another layer of complexity to this model. This concept, previously advocated by other authors, is strongly supported by the present study, with sick dogs showing some general similarities amongst the tissues studied, such as increased IFN-γ and $CD8^+$ T cells, synonymous with a pro-inflammatory and cytotoxic approach by the immune system to fight *Leishmania* infection, but still with underlying differences in the cytokine profile and in the cell populations between tissues. This way, the different tissues appear to develop their own specific immune profile against *L. infantum*, not being strictly restricted to this "choice" between a Th1 or Th2 response.

Nonetheless, the current work enabled a very detailed profile of cytokines and cell populations in three different tissues along a considerable timeframe, revealing that both meglumine antimoniate and miltefosine in combination with allopurinol are effective in the remission of clinical signs of CanL, with either treatment groups exhibiting a successful recovery with remission of all clinical signs, while seemingly triggering slightly different immune responses. And although we cannot be sure of the direct influence and mechanism of action that these treatment protocols cause through our experimental approach, since the two treated groups presented these different results, we can hypothesize that each therapeutic protocol acts differently on the immune system of the dog. In any case, the immune response of dogs to CanL and the effect of these drugs seems to be a very complex process that involves several requirements, from a specific profile of cytokines to the balance between cell populations, requiring future in-depth studies. At the same time, other studies carried out in collaboration by the working group on the role of PMN, hepatocytes and Kupffer cells, as well as on the treatment of FeL, have allowed the joining of several pieces of this giant puzzle that is leishmaniosis.

Lastly, although the current work has enabled a lot of information to be obtained, after the accomplishment of all objectives there are still some open questions and some new ones to be answered. While we could extrapolate some of the effects of these treatments on the dog's immune system, an *in vitro* approach can be useful to determine the direct effect of these molecules in groups of cultured cells obtained from sick dogs. Namely, through the use of fluorescence-activated cell sorting (FACS) technique in flow cytometry, selected groups of cells could be used not only to test these drugs, but also to be specifically profiled for their cytokine mRNA expression. Studies on flow cytometry doublet populations could open up new

possible avenues to evaluate cell-to-cell communication and, through the use of specific cell markers, it could be possible to detect some of these cellular cross-talks in CanL. Finally, since one of the main steps in the development of a protective Th1 response depends on a correct signal from APCs, such as DCs, the *in vitro* priming of cultures of DCs and re-inoculation in model specimens could reveal interesting new prospects in this area.

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ANNEX 1 - Statement of Responsibility

PTDC/CVT/118566/2010

Statement of Responsibility

Tutor

Name:_____

Adress:_____

ID/Citizen Card/Passport/Other:_____ Nº:_____

Telephone contact:_____ e-mail:_____

Animal

Name:_____ Breed:_____

Age:_____ Gender:_____ Chip Nº:_____

Intervention

Blood, lymph node and marrow collection for hematological, biochemical, parasitic and immunological assessment of the present study (PTDC/CVT/118566/2010).

Declaration

I, the undersigned, declare that authorize the Veterinary Doctor(s):

_____

_____,

to carry out the interventions described, in the animal identified above, for research purposes in the area of Canine Leishmaniosis. I have been informed and assume any risk that may arise from the referred intervention.

As it is true, I sign this disclaimer in accordance with my identification document.

Lisbon, _____ of _____ of 20__

Signature:_____

ANNEX 2 - Animal Welfare and Ethics Committee statement

FACULDADE DE MEDICINA VETERINÁRIA
UNIVERSIDADE TÉCNICA DE LISBOA

COMISSÃO DE ÉTICA E BEM-ESTAR ANIMAL (CEBEA)

Exma. Senhora
Professora Doutora Isabel Maria Soares
Pereira da Fonseca

Lisboa, 23 de Maio de 2012

Assunto: Avaliação de projecto de Investigação

Vimos pela presente informar V.Exa. que a CEBEA, após ter avaliado as actividades que envolvem manipulação de animais, no âmbito do projeto de investigação "Regulação da resposta imunológica em cães em várias fases clínicas de Leishmaniose e submetidos a diferentes protocolos terapêuticos" (refª PTDC/CVT/118566/2010), considerou que estão salvaguardados os princípios éticos e de bem-estar animal exigidos pela legislação vigente e pelo código de boas práticas, pelo que aprovou a execução do protocolo experimental nas instalações e serviços da FMV, conforme requerido por V.Exa.

Com os melhores cumprimentos,

Luis Lopes da Costa
Presidente da Comissão de Ética e Bem-Estar Animal

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