Universidade Nova de Lisboa
Instituto de Higiene e Medicina Tropical

Bridging the gap between innate and adaptive immunity in dog leishmaniasis

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DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS ESPECIALIDADE DE PARASITOLOGIA

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"Knowledge gives wings, curiosity flaps them "

– Nilabhra

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Resumo

A leishmaniose é um grupo de doenças causadas por diferentes espécies de *Leishmania* que afecta maioritariamente populações pobres de países subtropicais e tropicais e também animais selvagens e domésticos, como o cão. A leishmaniose canina (CanL) é uma doença endémica de preocupação mundial causada principalmente pelo protozoário *L. infantum* . O cão é um bom reservatório de *L. infantum*, uma vez que mantém a infeção durante muito tempo antes de desenvolver a doença facilitando a transmissão do parasita. Além disso, pensa-se que o cão também pode ser o reservatório de espécies americanas de *Leishmania,* como é o caso de *L. amazonensis*. A imunidade inata que é a primeira linha de defesa contra agentes patogénicos inclui células fagócitárias e células Natural Killer (NK). As células dendríticas (CD) têm a principal função de capturar agentes patogénicos e processar antigénios, desempenhando um papel crucial na proliferação e activação de células T, estabelecendo assim uma ponte entre a imunidade inata e a adquirida. As células NK influenciam o processo infeccioso através do reconhecimento e eventual destruição de células infectadas e da libertação de mediadores imunitários celulares que promovem microambientes inflamatórios. Considerando a reduzida informação existente sobre o papel das CD e das células NK durante a infeção por *Leishmania*, este estudo visou analisar *in vitro* a atividade das CD derivadas do sangue periférico (moCDs) canino e também abordar o papel das NK na resposta imunitária celular e a sua inter-relação com as moCDs. Após confirmação da diferenciação *in vitro*, as moCDs foram infectadas com *L. infantum* e *L. amazonensis*, e também expostas a vesículas extracelulares (EVs) libertadas por *L. infantum* (*LiEVs*) e *L. amazonensis* (*LaEVs*). A infecção por *L. infantum* aumentou a expressão génica do receptor toll-like (TLR) 4 das moCDs em sinergia com a activação e translocação do factor nuclear (NF)-κB para o núcleo e subsequente geração de citocinas pró-inflamatórias [interleucina (IL)-1β e IL-18]. Este parasita também induziu o predomínio da subpopulação de moCDs expressando moléculas de classe I do complexo maior de histocompatibilidade (MHCI) e aumentou a molécula de co-estimulação CD86 que, juntamente com a libertação da quimiocina CXCL16, poderá atrair e activar linfócitos T citotóxicos ($CD8^+$) que podem causar a apoptose das células infetadas. Em contraste, *L. amazonensis* parece induzir moCDs anergicos, indicando que estes parasitas estabelecem diferentes relações imunitárias com as CDs, o que pode ser uma consequência da co-evolução parasita-hospedeiro. Os moCDs infectados com parasitas em co-cultura com células NK parecem promover a predominio da subpopulação de moDCs expressando moléculas de classe II do MHC, sugerindo a possibilidade de apresentação de antigénios às células T $CD4^+$. Além disso, apesar da desgranulação das células NK e da libertação de perforina, não houve aumento da apoptose dos moCDs, e os parasitas internalizados pelos moCDs mostraram-se viáveis. Quando expostos a EVs, os moDCs também mostram diferenças na actividade imunitária. As *LaEVs* parecem sinalizar moCDs através de TLR2, causando a upregulação de CD80/CD86 e *Li*EVs promovem a expansão de moCDs $MHCI^+$ aumentam a expressão génica de CD86. Ambas as EVs induziram a libertação de CXCL16, atraindo leucocitos. Os moCDs estimuladas por EVs induziram as células NK a gerar quimiocinas, atraindo outros leucócitos e promovendo a desgranulação que, no entanto, não induziam a apoptose dos moCDs. Quando em contacto com as células NK, os moDCs com *LiEVs* provocaram a expansão das subpopulações de $MHCI^+$ e $MHCII^+$ moCDs, enquanto as moCDs expostos a *LaEVs* mostraram aumento de moDCs $MHCI^+$.

Estes resultados indicam que as EVs podem modular a atividade imunitária das DC caninas. *L.infantum* e *L.amazonensis,* assim como as respetivas EVs, modulam a ativação das CD caninas, embora de formas diferentes. A compreensão detalhada das vias de ativação das CDs assim como a interação com as NK pode conduzir ao desenvolvimento de novas estratégias de controlo da leishmaniose.

Palavras-chave: Leishmaniose canina, Células dendríticas, células NK, Imunidade inata, vesiculas extracelulares

Abstract

Leishmaniasis is a group of diseases caused by different species of *Leishmania* that afflicts poor populations of subtropical and tropical low-income countries and causes disease in wild and domestic animals, such as the domestic dog. Canine leishmaniasis (CanL) is an endemic disease of worldwide concern caused mainly by the protozoan *L. infantum*. The dog is a good reservoir of *L. infantum* since keeps the infection for a long time before developing the disease, facilitating parasite transmission. Furthermore, there is a growing belief that dogs also can be the reservoir of the American species of *Leishmania*, as is the case of *L. amazonensis*. Innate immunity, the first line of defence against pathogens, includes phagocytes and natural killer (NK) cells. Dendritic cells (DC) have the principal function of capturing pathogens and processing antigens, playing a crucial role in the proliferation and activation of undifferentiated T cells, thereby establishing a bridge between innate and acquired immunity. NK cells influence the infectious process through the recognition and eventual destruction of infected cells and the release of cellular immune mediators that promote inflammatory microenvironments. Since there is limited information on the role of DCs and NK cells during *Leishmania* infection, this study aimed to analyze *in vitro* the activity of peripheral blood-derived DCs (moDCs) and tackle the NK role in the cellular immune response and its interrelation with moDCs. After confirmation of in vitro differentiation of moDCs cells were infected with *L. infantum* and *L. amazonensis*, and also exposed to extracellular vesicles (EVs) released by *L. infantum* (*LiEVs*) and *L. amazonensis* (EVs). The activity of moDCs and the interaction between moDCs and NK cells were analyzed. *L. infantum* infection increased the expression of toll-like receptor (TLR) 4 genes of moDCs in synergy with the activation and translocation of nuclear factor (NF)-κB to the nucleus and subsequent generation of pro-inflammatory cytokines [interleukin (IL)-1β and IL-18]. This parasite also induced the predominance of moDCs expressing class I molecules major histocompatibility complex (MHCI) and upregulated the co-stimulatory molecule CD86 that, together with the release of the chemokine CXCL16, could attract and activate cytotoxic T lymphocytes ($CD8^+$) that can cause the apoptosis of infected cells. In contrast**,** *L. amazonensis* seems to induce anergic moDCs, indicating that these parasites establish different immune relationships with DCs, which may be a consequence of parasite-dog co-evolution. Parasite infected moDCs in co-culture with NK cells seem to promote the predominance of $MHCII^+$ moDCs, suggesting the possibility of antigen presentation to $CD4^+$ T cells. Furthermore, despite the degranulation of NK cells and perforin release, there was no increase in moDCs apoptosis, and the parasites internalized by the moDCs remained viable. When exposed to parasite EVs, moDCs also show different immune activity. *LaEVs* appear to signalize moDCs through TLR2, causing the upregulation of CD80/CD86 and *LiEVs* promoted the expansion of $MHCI^+$ moDCs and upregulation of CD86. Both EVs induced the release of CXCL16. EVs-primed moDCs induced NK cells to generate chemokines, attracting other leukocytes and promoting degranulation that did not increase moDCs apoptosis. When in contact with NK cells, *LiEVs*-primed moDCs caused the expansion of $MHCI^+$ and $MHCII^+$ moDC subsets, while moDCs exposed to *LaEVs* only showed in the expansion of $MHCI^+$ moDCs. These findings indicate that EVs can modulate the immune activity of canine DCs. Therefore, *L.infantum* and *L.amazonensis*, as well as the respective EVs, modulate the activation of canine DCs, albeit in different ways. A detailed understanding of DC activation pathways and the interaction with NK cells can open new windows on leishmaniasis control strategies.

Abstract

Keywords: Canine Leishmaniasis, Dendritic cells, NK cells, innate immunity, Extracellular vesicles

Table of Contents

List of Figures

List of Tables

List of abbreviations

µg.ml - microgram to milliliter

AaMΦ – Macrophages Activated by the alternative pathway

ABs - Apoptotic Bodies

ACPs - Acid Phosphatases

AD - Activation Domain

ACL – Anergic Cutaneous Leishmaniasis

Ag - Antigen

ALX – LipoXanthine A4

APC – Professional Antigen-presenting Cells

AT - Acetyl Transferase

AVL - Visceral Leishmaniasis Anthroponotic

BAFF - TNF Family Receptor

BAFT3 - Leucine Zipper Transcription Factor

B cells - B lymphocytes

BDCL- Borderline Disseminated Cutaneous Leishmaniasis

BHI - Brain-heart Infusion agar medium

BIRs - Baculovirus Inhibitor Repeats

Bp - Base-Pair number

BM - Bone Marrow

BMDC - Bone Marrow-derived Dendritic Cells

C - Concentration

C3bi - C3b inactive form

CARD - Caspase Recruitment Domain

CaMΦ – Classically activated Macrophages

CanL - Canine Leishmaniasis

CBC - Complete Blood Count

CBD1 - Canine β-Defensin 1

cDCs - conventional DCs

cDNA - Complementary DNA

CL - Cutaneous Leishmaniasis

CLRs - C-type Lectin Receptors

ConA - Concanavalin A

CIITA - Class II molecules of major histocompatibility complex Transactivator

cPCR - conventional PCR

CPDA - Citrate Phosphate Dextrose Adenine 1 solution

CR1 and CR3 - Complement Receptors 1 and 3

CSF – Colony Stimulating Factor

CVBD - Companion Vector-Borne Diseases

DAMPs - Molecular Patterns Associated with tissue damage ()

DAT - Direct Agglutination Tests

DCL - Diffuse Cutaneous Leishmaniasis

DC - Dendritic Cells

DC-SIGN - DC-specific ICAM-3-grabbing -non-integrin

DLA - Leukocyte Antigen

DAPI – fluorescent nuclear dye 4′,6-diamidino-2-phenylindole

Dil Stain - 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine Perchlorate

DNA – Deoxyribonucleic Acid

Dnase – Desoxirribonucleases

dsDNA - double-stranded DNA

dsRNA - double-stranded RNA

EDTA – Ethylenediamine Tetraacetic Acid

EF-1 - Elongation Factor 1

ELISA - Enzyme-Linked Immunosorbent Assay

EMTM - Modified Evans Tobie

EV - Extracellular Vesicles

FBS – Fetal Bovine Serum

FCgR1 - Fc-gamma 1 Receptor

FML- Fucose-Mannose Ligand

fMLP – Formylated Peptides

FW – *foward*

G418 - Geneticin

GFP – Green Fluorescent Protein

GIPL – Phospholipids Glycoinositol

gp63 – glycoprotein of 63 kDa

GM-CSF – Granulocyte-Macrophage Colony-Stimulating Factor

HE - Hematoxylin and Eosin

HD1 - Proximal helical 1 domain

HLA-G - Human Leukocyte Antigen G

HIV – Human Immunodeficiency Virus

HNO_2 – Nitrous Acid

H_2O_2 – Hydrogen Peroxide

HSPs - Heat Shock Proteins

IBMC - Instituto de Biologia Molecular e Celular

iDCs - immature Dendritic Cells

ICOS-L - enhanced Inducible Costimulator Ligand

IDO - Indoleamine 2,3 Dioxygenase

iE-DAP - Detect γ-d-glutamyl-meso-diaminopimelic acid

IFAT - Immunofluorescence Antibody Test

IFN – Interferon

Ig - Immunoglobulin

IH - Invertebrate Host

IKK- IκB Kinase

IκBα - I kappa B alpha

IL – Interleucin

ILVs - Intraluminal Vesicles

IM - Intramuscular

iNOS – Nitric Oxide Synthase

IPGT –*Isopropyl-beta-D –Thiogalactopyranoside*

IRAK4 - Receptor-Associated IL-1 Kinase 4

IRF8 - Interferon Regulatory Factor 8

IV - Intravenous

IS - Immune System

LaEVs - *L. amazonensis* Extracellular Vesicles

LaAg - *L. amazonenesis* Soluble Antigens

KMP -11 – 11 kDa hydrophobic Kinetoplastid Membrane Protein

LB - *Luria-Broth* medium

LC - Langerhans Cells

LCL – Localized Cutaneous Leishmaniasis

LiAg - *L. infantum* Soluble Antigens

***Li*ESP** - Secretion-Excretion Proteins of *L. infantum*

LiEVs - *L. infantum* Extracellular Vesicules

LCF – *Leishmania* Chemotactic Factor

LPG – Lipophosphoglycan

LPS – Lipopolysaccharide

LTB4 – Leukotriene B4

LRR – Leucine-Rich Repeats

MΦ - Macrophages

MA - Meglumine Antimoniate

MAC - Membrane Attack Complex

MCL - Mucocutaneous Leishmaniasis

moDCs - Monocyte-Derived DCs

MDP - Muramyl Dipeptide

MDM - Monocyte-Derived Macrophages

MHC - Major Histocompatibility Complex

MR - Mannose Receptors

mRNA - messenger Ribonucleic Acid

MS - Magnetic Separation

MyD88 - Myeloid Differentiation protein 88

MIP – Macrophage Inflammatory Protein

NTDs - Neglected Tropical Diseases

NRAMP1 - Natural Resistance-Associated Macrophage Protein 1

nPCR - nested PCR

NNN - Novy–MacNeal–Nicolle medium

NO - Nitric Oxide

NET - Neutrophil Extracellular Traps

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NF-κB – Nuclear Factor κB

NK – Natural killer cells

NKT - Natural killer T cells

NLR – *nucleotide-binding domain, Leucine-Rich repeat containing proteins*

NRLP3 - Pyrin domain-containing 3

NF-κB1 - subunit p50 of Nuclear Factor κB

NF-κB2 - subunit p52 of Nuclear Factor κB

NACHT – Nucleotide-binding Core Domain

NaCl – Sodium Chloride

NO_2 – Nitrogen Dioxide

NOD – *Nucleotide – binding Oligomerization Domain*

NOS – Nitric Oxide Synthetase

O_2^- – superoxide ion

OIE - Organization for Animal Health

$ONOO^-$ – Peroxynitrite

p105 - proteins of 105 kDa

p100 - proteins of 100 kDa

PAF - Platelet-Activating Factor

PALS - Periarterial Lymphatic Sheaths

PAMPs - Molecular Patterns Associated with Pathogens

PBS – Phosphate-Buffered Saline

PCR – Polymerase Chain Ceaction

pDNA - Plasmid DNA

PI - Propidium Iodide

PKDL- Post-kala-azar Dermal Leishmaniasis

PMA – Phorbol Myristate Acetate

P-MAPA - Anhydrous Magnesium-Ammoniophospholinoleate-Palmitoleate Protein Aggregate

PMN – Polymorphonuclear cells

PPG - Protophosphoglycan

primers - oligonucleotide sequences

PRR - Pattern Recognition Receptors

PKC - Protein C serine/threonine Kinase

PYD - Pyrin Domain

QA-21 - Quillaja Soponaria

qRT-PCR - real-time PCR

RHD - Rel homology domain

rIL-4 - Recombinant IL-4

RIGI - Retinoic acid-Inducible Gene I

rK39 - Recombinant protein K39

ROS – Reactive Oxygen Species

RNA – Ribonucleic Acid

RV – *reverse*

RT - Room Temperature

SbIII - Sodium stibogluconate trivalent form

SbV - Sodium stibogluconate pentavalent form

SC - Subcutaneous

SCHN – Schneider Drosophila Medium

SEM - Scanning Electron Microscopy

SPLA - Soluble Leishmania Promastigote Antigens

SOC – Super Optimal Broth with catabolite repression

ssRNA – single-stranded RNA

SYBR – Syber Green

TAE buffer - Tris-HCl, sodium Acetate and EDTA

TAN - Annealing Temperature

TAB T- AK1/TGF-β-activated kinase complex

TGF – Transforming Growth Factor

TGP – Transaminase Glutâmica Piruvática

Th – T helper lymphocytes

TIR - Toll-Interleukin 1 Receptor domain-containing adapter protein TIRAP

TLR – Toll-*like* Receptor

TNF – Tumor Necrosis Factor

tolDCs - tolerogenic DCs

TRAM - TRIF Adapter-related Molecules

TRIF - IFN-β-Induced TIRAP

Treg - Regulatory T lymphocytes

U/ml - Units per millilitre

vs **–** *versus*

VL - Visceral Leishmaniasis

VH - Vertebrate Host

v/v – Volume per volume

WHO - World Health Organization

WOAH - World Organisation for Animal Health

WHD - Winged Helical Domain

w/v - Weight by volume

g – G-force

X-Gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

ZVL - Zoonotic Visceral Leishmaniasis

1|General Introduction

1|General Introduction

1.1. Leishmaniasis

In tropical and subtropical countries, where health care is limited and the population is in contact with disease vectors and reservoirs, there is a variety of neglected tropical diseases (NTDs). Leishmaniases are mong the 17 NTDs defined by the WHO (Centers for Disease Control and Prevention, 2020; World Health Organization 2021a).

Leishmaniases are diseases caused by an intracellular protozoan of the family *Trypanosomatidae* Doflin, 1901 and of the genus *Leishmania* Ross, 1903, which may be present as zoonoses or anthroponoses (João *et al.,* 2000, Croft *et al.,* 2006). This parasite is transmitted to vertebrates through the bite of female sandflies. Of the 30 known vector genera, two of them are important in human and veterinary medicine, the genera *Lutzomyia* França, 1924 and *Phlebotomus* Rondani and Berté, 1840. Though approximately 1000 species of sandflies have been identified, more than 90 have been recognized as *Leishmania* vectors (Maroli *et al.,* 2013).

In the Old World (Europe, Africa, the Middle East and Asia) the genus *Phlebotomus* is involved in the transmission of different *Leishmania* species, while in the New World (America) the genus *Lutzomyia* is responsible for the transmission of this parasite (Maroli *et al.,* 2013). Based on the development of the parasite in the invertebrate host (IH), *Leishmania* is classified into two subgenera (Fig. 1): the subgenus *Leishmania* with a suprapylaric development, i.e. in the stomach of the vector, and the subgenus *Viannia* where the parasites are peripylaric, i.e. they are located initially in the hindgut and only later migrate to the sandfly proboscis (Saridomichelakis, 2009).

The disease is geographically distributed across all continents, except for Antarctica, being endemic in 98 countries across Africa, South and Central America, Asia and the Mediterranean region. It is one of the most neglected diseases in the world, with 350 million people at risk, 12 million people infected, 20,000 to 30,000 deaths per year and it has been estimated that 700,000 to 1 million new cases occur annually. Affects mainly the most disadvantaged populations and is associated with malnutrition, population displacement, poor housing conditions, a weakened immune system and lack of resources. The increase and spread of the

disease are related to environmental changes, such as deforestation, construction of dams, irrigation systems and urbanization (WHO, 2022).

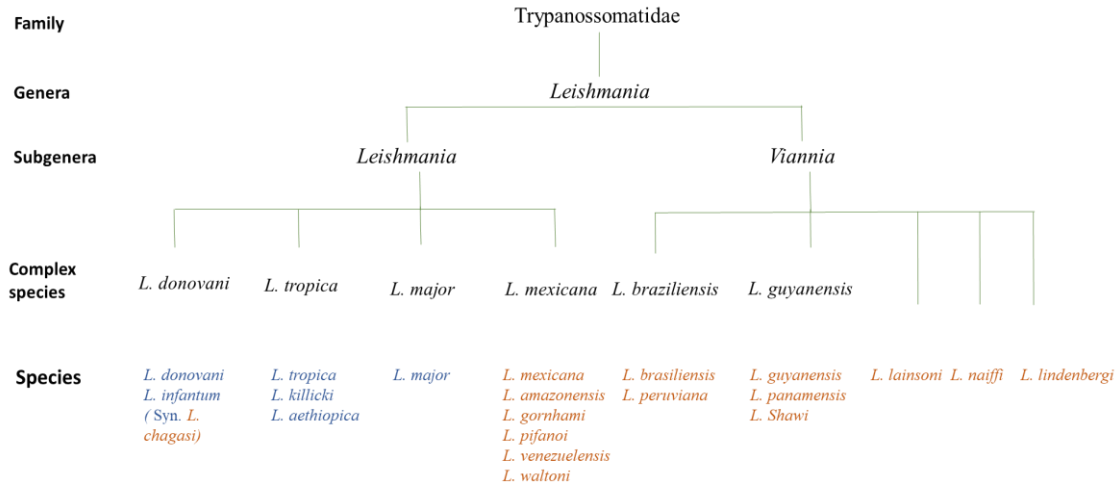


Figure 1. Taxonomic position of pathogenic *Leishmania* species. The parasite belongs to the family Trypanosomatidea, genus *Leishmania* and within this genus, there are two subgenera: the subgenus *Leishmania* which contains thirteen species and occurs in both the New World and the Old World and the subgenus *Viannia* which is restricted to Neotropical region of American continent. The species are organized into six complexes, of which four belong to the *Leishmania* subgenus (*L. donovani*, *L. tropica*, *L. major* and *L. mexicana*) and two to the *Viannia* subgenus (*L. braziliensis* and *L. guyanensis*). Old and New World species are highlighted in blue and orange, respectively. Image of my own authorship.

Of the 30 known *Leishmania* species, 21 are pathogenic to humans (Pace, 2014). Depending on the signs, symptoms and severity, Leishmaniasis can be classified into three main clinical types: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL), which can be zoonotic (ZVL) or anthroponotic (AVL) (Neuber, 2008).

CL presents skin lesions, mainly in exposed areas such as the face, arms and legs, and is caused by several species in the Old World, such as *L. major*, *L. tropica* and *L. aethiopica*, and in the New World by *L. mexicana*, *L. amazonensis* , *L. venezuelensis*, *L. braziliensis*, *L. guyanensis*, *L. panamensis*, *L. lainsoni*, *L. naiffi*, *L. shawi* and *L. peruviana.*

A less common form of CL known as anergic cutaneous leishmaniasis (ACL) has also been described. ACL is manifested by chronic, non-ulcerated and limited lesions. In Ethiopia and Kenya, it is caused by *L. aethiopica*, but there are also reports in Tanzania and Namibia. In South America (Ecuador, Venezuela, Brazil, Dominican Republic, Mexico, Honduras, Nicaragua, Peru, Bolivia, and Colombia) it is caused by *L.amazonensis* and *L.mexicana*

Diffuse cutaneous leishmaniasis (DCL) initially presents localized and nodular lesions or infiltrative plaques that gradually spread throughout the body. This disease can also affect the internal organs. It is endemic in several South American countries, such as Brazil, Venezuela, Mexico, Dominican Republic, Peru and Colombia. It can be caused by *L. amazonensis* , *L. mexicana*, *L. venezuelensis*, *L. pifanoi* and *L. braziliensis*.

The MCL is characterized by the presence of lesions in the nasal and oral mucosa, throat and surrounding tissues and is essentially caused by *L. braziliensis* and *L. panamensis.*

About 95% of CL cases occur in the Americas, the Mediterranean Basin, the Middle East and Central Asia (Fig. 2), while 90% of MCL cases occur in Bolivia, Brazil, Ethiopia and Peru (WHO, 2022).

The VL or Kala-azar is the most severe form of the disease affecting the lymph nodes, spleen, liver and bone marrow. The disease is manifested by irregular bouts of fever, weight loss, splenomegaly, hepatomegaly, lymphadenopathy and anemia. It is a progressive disease, that can be fatal if not treated. The common etiologic agents are *L. donovani* and *L. infantum* (syn. *L chagasi*). From an epidemiological point of view, VL can be classified into zoonotic VL (ZVL) and anthroponotic VL (AVL). ZLV is transmitted from animal to vector and from this to another animal or man, while AVL is transmitted from man to man through the vector. ZVL is caused by *L. infantum* and its reservoirs are canids, mainly the domestic dog. It mainly affects populations in rural areas and has a wide geographic distribution: Mediterranean basin, Middle East, Northwest China and Africa. AVL is caused by *L. donovani* and is distributed across the Middle East, India and East Asia (Maroli *et al.*, 2013). In 2018, more than 95% of new VL cases reported to WHO occurred in 10 countries: Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan (WHO, 2022) (Fig. 3).

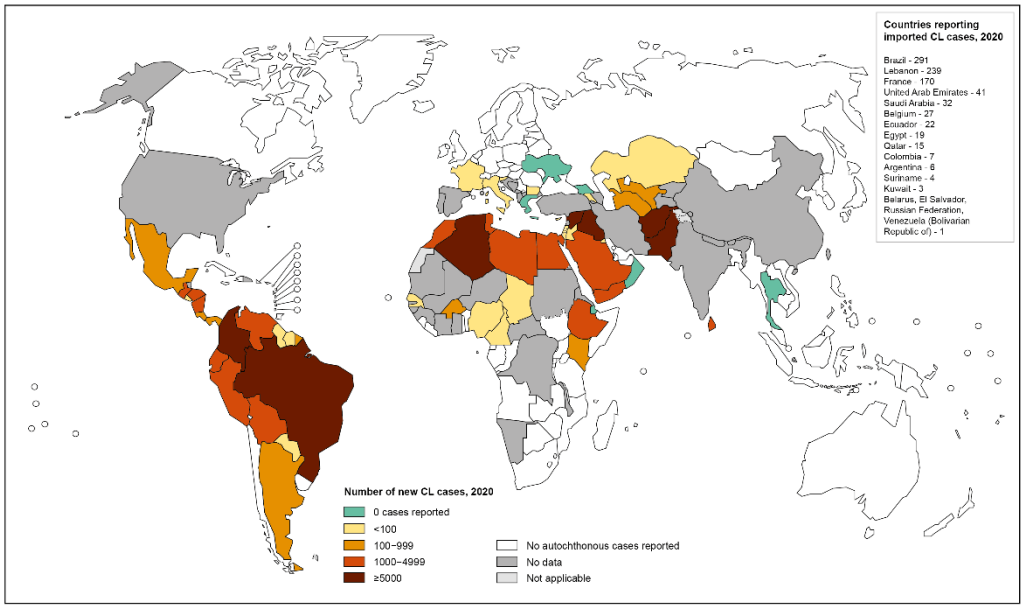


Figure 2. Status of endemicity of cutaneous leishmaniasis worldwide (WHO, 2020; Control of Neglected Tropical Diseases). Reprinted by permission from WHO under the license number 390239 (https://cdn.who.int/media/docs/default-source/2021-dha-docs/leishmani sis_cl_2020.pdf?sfvrsn=716850a8_9)

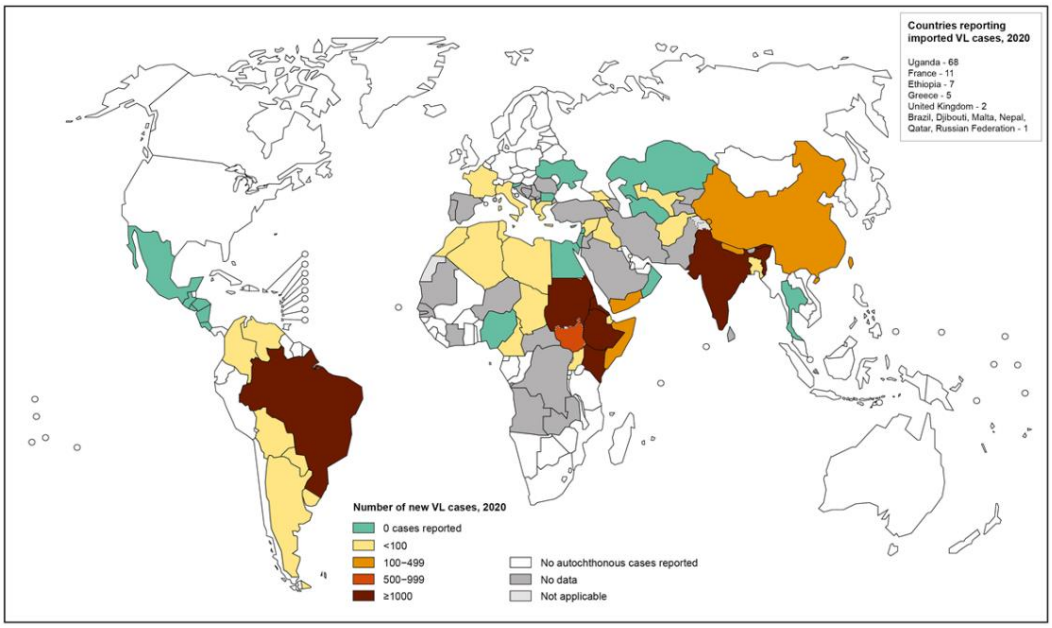


Figure 3. Status of endemicity of Visceral Leishmaniasis Worldwide (WHO, 2020 - Control of Neglected Tropical Diseases). Reprinted by permission from WHO under the license number 390239 (https://cdn.who.int/media/docs/default-source/2021-dha-docs/leishmaniasis_vl_2020.pdf?sfvrsn=17eea7b2_7)

Post-kala-azar dermal leishmaniasis (PKDL) may occur in some patients after leishmaniasis recovery (McGwire and Satoskar, 2014). Cases of PKDL caused by *L. donovani* are mainly found in two specific regions, East Africa and South Asia. This syndrome is characterized by a maculopapular condition, macular or nodular rash around the mouth that spreads throughout the body. In Africa, where PKLD is common (50% to 60%), this clinical form of leishmaniasis usually occurs within 6 months after VL treatment (Zijlstra and el-Hassan, 2001, Zijlstra and Alvar, 2012, Zijlstra *et al.,* 2020). Since PKDL is characterised by lesions rich in parasites, and seems to favour anthroponotic transmission of *L. donovani* in Africa and India (Molina *et al.,* 2017, Mondal *et al.,* 2019), it is believed that these patients can be reservoirs, facilitating parasite transmission. PKDL can be difficult to treat, especially in some East African patients suffering from a severe form of PKDL. In South Asia, this syndrome is relatively rare and can occur several years after the VL has been clinical cured. In these cases, prolonged treatments are required. In India, PKLD is found in 1-3% of VL cases successfully treated (Burza *et al.,* 2018, Rijal *et al.,* 2019). The clinical presentation of PKDL patients from Africa and Asia are different (90% papular eruption in Africa, 90% macular in Asia), as well as the interval between LV and PKDL (0-13 months in Africa, usually 2-3 years in Asia). Approximately 85% of cases in Africa have self-healing lesions but PKDL lesions rarely resolve (Burza *et al.,* 2018), In Asia, although there are some reports of self-healing, all patients are treated. After successful treatment with prolonged courses of antileishmanial drugs, the rash will disappear and patients will develop immunity similar to that of cured VL patients. This immunity is permanent unless there is associated immunosuppression (Zijlstra, 2016).

In the Mediterranean basin and Portugal, ZVL affects mainly small children and immunecompromised people due to infection by the human immunodeficiency virus (HIV) or due to the use of immunosuppressed drugs, but above all, it infects the dog, which is the main reservoir of *L. infantum* and also the main host. In Portugal, the regions of Trás-os-Montes, Beira interior, Lisbon and the Tejo valley, Alentejo and Algarve are recognized focus of Canine leishmaniasis (CanL) (Afonso and Alves Pires, 2008). CanL is important from a veterinary medicine point of view, but also in terms of public health since domestic dogs are the principal reservoir of *L. infantum* parasites (Reithinger and Davies, 1999, Gramiccia and Gradoni, 2005, Solano-Gallego *et al.,* 2009).

1.1.1. Life cycle

During the life cycle (dimorphic), *Leishmania* presents two main morphological forms: the promastigote form that is found in the insect vector and the amastigote form that is found in the vertebrate host (VH). The promastigote form has a fusiform shape, measures about 15×3 μm in length and has a free flagellum in its anterior region that can reach 20 μm in length (Fig. 4).

The obligate intracellular amastigote form is slightly ovoid, measuring 2×4 μm, and the flagellum is intracellular, ending near the cell membrane (Fig. 4). Both morphological forms have a nucleus, a large mitochondrion and a kinetoplast that corresponds to mitochondrial deoxyribonucleic acid (DNA) condensed in a single region near the basal body (Tomás and Romão, 2008).

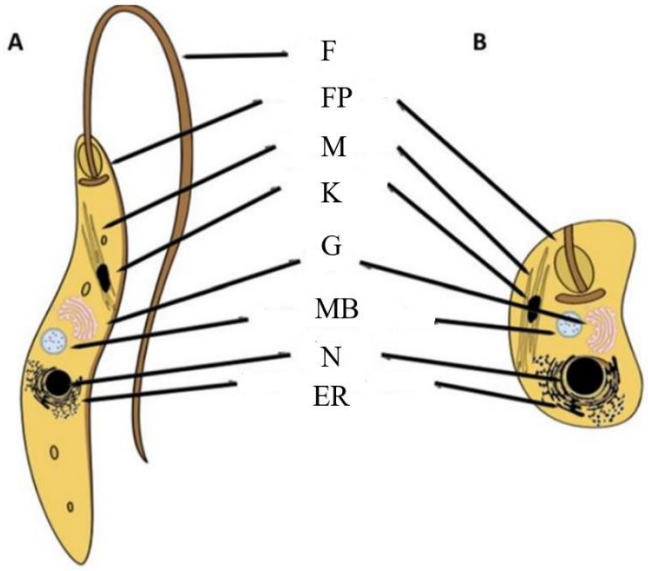


Figure 4. Schematic representation of *Leishmania* evolutive forms. Promastigote, the extracellular form (A) and amastigote, the intracellular form (B). F, Flagellum; FP, flagellar pocket; M, Mitochondrion; K, Kinetoplast; G, Golgi complex; MB, Multivesicular bodies; N, Nucleus; ER, Endoplasmic reticulum (Figure adapted from https://www.researchgate.net/publication/347628924_Carbonic_Anhydrase_in_Acid_Acclimatization_of_Leishmania/figures?lo=1)

When the female of the vector takes a blood meal, it inoculates metacyclic promastigotes, the infective form of the parasite, into the VH dermis, which bind to surface receptors of phagocytic cells. Neutrophils or polymorphonuclear cells (PMN) are the first phagocytic cells to be recruited to the bite site, which can internalise part of the promastigotes inoculated by the vector.

Macrophages (MΦ) are the definitive host cells of the parasite, reaching the inoculation site in the dog approximately 24 h after infection (Santos-Gomes *et al.*, 2000). After phagocytosis, promastigotes transform into amastigotes, which replicate by longitudinal binary fission. By saturation of amastigotes, MΦ end up lysing and releasing the parasites to the extracellular space, which are phagocytosed by other MΦ promoting the spread of *Leishmania* parasites (Solano-Gallego *et al.*, 2011).

The life cycle is completed when infected MΦ are ingested by the female vector during blood meal in the VH (host). Amastigotes differentiate into procyclic promastigotes and attach to the insect's gut where they replicate by mitosis. Non-infectious procyclic promastigotes transform into infectious metacyclic promastigotes and migrate to the esophagus and pharynx, being ready to be inoculated through the proboscis (Fig. 5) in a new VH (Afonso and Alves Pires, 2008).

The route of dissemination of the parasite in VH is depending on the species of *Leishmania* involved. In visceralizing species, infected MΦ transport the parasites to organs rich in phagocytic cells, namely the spleen, liver, bone marrow and lymph node. In the case of cutaneous species, the parasites are limited to the inoculation site or are disseminated through the lymphatic system to other sites in the dermis, mucosa or cartilage (Tomás and Romão, 2008).

Shortly after parasite inoculation, skin dendritic cells (DCs) can take up *Leishmania* and/or *Leishmania* antigens before migrating to the draining lymph nodes where they can stimulate *Leishmania*-specific T lymphocytes (Feijó *et al.*, 2016).

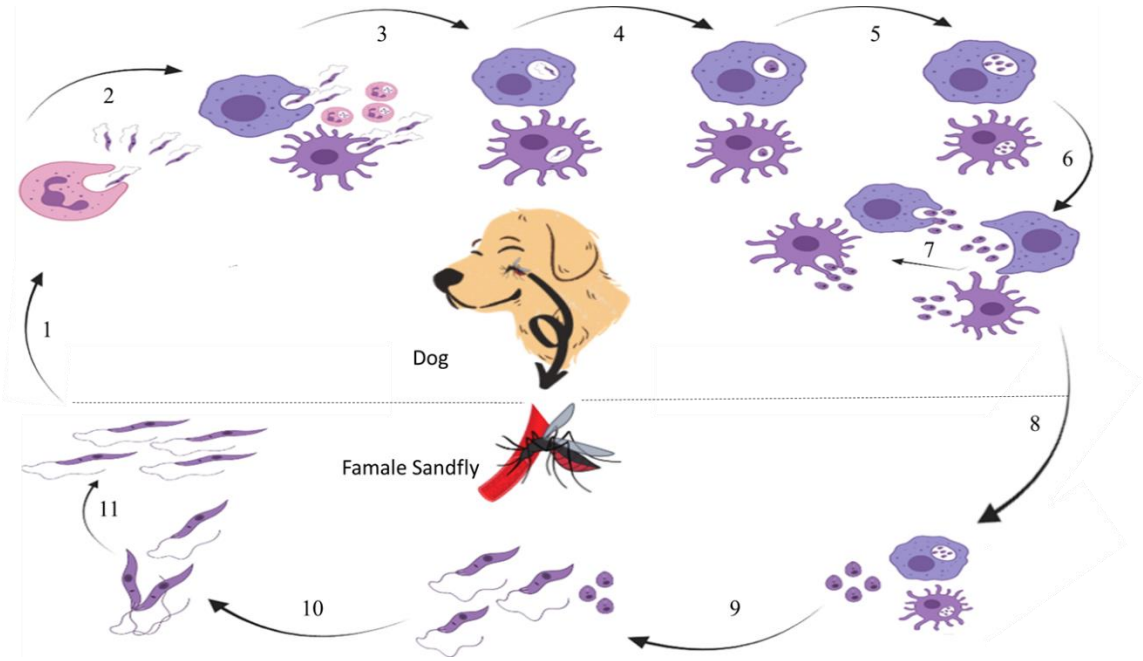


Figure 5. *Leishmania* spp. life cycle. *Leishmania* transmission occurs during the blood meal of female sandflies (1), which introduces metacyclic (infectious) promastigotes in the vertebrate host's dermis, as is the case of the dog. Parasites are phagocytosed by neutrophils and professional antigen-presenting cells (APCs), such as macrophages and dendritic cells (2 e 3). Inside macrophages, promastigotes differentiate into amastigotes (4). Amastigotes replicate inside the cells (5). After several replication cycles, amastigotes induce cell lysis being released (6). Parasites are phagocytosed by othermacrophages . (7). During another blood meal, the phlebotomine female ingests the infected macrophages (8) and in the midgut, the amastigote differentiates into promastigotes (9) and replicates (10). Promastigotes migrate to the proboscis ready to be injected into the mammalian host during the next blood meal (11). Image of my own authorship, created with BioRender.com (accessed may 2022)

1.2. Canine Leishmaniasis

The CanL is one of the main zoonoses in the world, which is a serious and fatal disease of dogs (Solano-Gallego *et al.*, 2009). This disease is endemic in more than 70 countries within the Mediterranean region, South and Central America, Africa and Asia (Baneth et al, 2008, Dantas-Torres et al, 2009, 2012, Solano-Gallego et al, 2009, 2011). It has been estimated that

at least 2.5 million dogs are infected in southwestern Europe (Moreno *et al.*, 2002). The number of infected dogs in South America also is estimated at millions, and there are high infection rates in some areas of Venezuela and Brazil, where a high prevalence of canine infection is associated with a great risk of human disease (Werneck *et al.*, 2006)

In Europe, CanL is restricted to the Mediterranean region, but cases have been reported in Northern countries (Shaw *et al.*, 2009, Ballart *et al.*, 2013, Kaszak *et al.*, 2015).

In dogs, the most widely distributed and well-characterized canine infection is caused by *L. infantum*. *L.infantum* infection was first described in dogs in 1908 by Nicolle and Comte in Tunisia (Nicolle and Comte, 1908, Solano-Gallego et al, 2009). However, other species of *Leishmania* that are responsible for causing CL, MCL and VL in humans have been also found in dogs (Lemrani *et al.*, 2002, Dantas-Torres, 2007, Solano-Gallego *et al.*, 2009). Until now, of the 20 species of *Leishmania* pathogenic to humans, 12 were already found in dogs (Table 1) (Ashford and Snowden, 2000, Baneth, 2005, Saridomichelakis, 2009).

Table 1. Species of *Leishmania* found to infect dogs, geographical distribution and principal vectors involved in transmission. Adapted from Solano-Gallego et al (2009) and Saridomichelakis (2009).

Subgénero	Species	Geographic distribution	Proven or suspected vector
Leishmania	*L. infantum* (Syn. *L. chagasi)*	Europe, America, Asia, Africa	*L. longipalpus, L. evansi, P. neglectus, P. perniciosus,* others
	L. donovani	East Africa	*P. orientalis , P. martini , P. rodhaini*
	L. tropica	India, Iran, Israel, Morocco, Syria	*P. sargenti*
	L. major	Egypt, Saudi Arabia	*P. papatasi*
	L. arábica	Saudi Arabia	*P. papatasi*
Viannia	*L. amazonensis*	Brazil	*L. flaviscutellata , L. nociva , L. whitmani*
	L. mexicana	Ecuador, USA	*L. ayacuchensis , L. olmeca*
	L. pifanoi	Ecuador	*L. flaviscutellata , L. youngi*
	L. braziliensis	Argentine, Bolivia, Brazil, Colombia, Peru, Venezuela	*L. intermedia, L. migonei , L. wellcomei , L. whitman , others*
	L. peruviana	Peru	*L. peruensis , L. verrucarum*
	L. panamensis	Colombia, Ecuador, Panama	*L. hartmanni , L. gomezi , L. panamensis, L. trapidoi*
	L. colombiensis	Venezuela	*L. hartmanni*

1.2.1. Vertebrate hosts and reservoir

The domestic dog (*Canis familiaris*) is considered the main peridomestic reservoir of *L. infantum* in Europe (Millán *et al.,* 2014). This is due to the high number of dogs present in the ecological niche and the close relationship with the vector (Alvar *et al.,* 2004). Dogs cohabit with humans or are present near human dwellings, which favours the maintenance of the domestic cycle of *L. infantum* transmission (Dantas-Torres, 2007). In the Old World, other wild canids infected with *L. infantum* have been identified, such as the fox (*Vulpes vulpes, Vulpes corsac, Vulpes zerda*), wolf (*Canis lupus*), jackal (*Canis aureus*), mongoose (*Herpestes ichneumon*), Iberian lynx (*Lynx pardinus*), marten (*Martes* sp.), genet (*Genetta genetta*), , European badger (*Meles meles*), Mediterranean monk seal (*Monachus monachus*), rat (*Rattus norvegicus*), syrian hamster (*Mesocricetus auratus*), grey hamster (*Cricetulus migratorius*), porcupine (*Hystrix* spp.) among others (Dantas-Torres and Brandão-Filho, 2006, Dantas-Torres, 2007, Pereira, 2008). In the New World, several wild mammals were found as *L. infantum* hosts, as is the case of the opossum (*Didelphis* spp.), common cloth (*Bradypus variegatus*), bush dog (*Speothos venaticus*) and the bat (*Carollia perspicillata*). Although they are responsible for maintaining the sylvatic cycle of *Leishmania*, they are not considered the main source of transmission of *Leishmania* parasites to dogs and humans, due to their small number and distance from human activity (Alvar *et al.,* 2004). Other wild mammals have been more recently found infected with *L. infantum*, such as lagomorphs (Molina *et al.,* 2012), especially those found near humans (Millán *et al.,* 2014). In endemic areas, cases of infection have been reported in other domestic animals such as cats, horses, pigs, sheep, goats and domestic rats (Fisa *et al.,* 1999; Quinneland, 2009).

1.2.2. Invertebrate host

Sandflies are considered the only vectors of *Leishmania* spp. It is estimated that there are more than 800 sandfly species, of which only 90 are potential or proven vectors of *Leishmania* spp.

Sandflies belong to the Class *Insecta*, Subclass *Pterygota*, Order *Diptera*, Suborder *Nematocera*, *Psychodidae* Family and *Phlebotominae* Subfamily. Their elongated body is densely covered with fine silks, has long antennae and its colour varies from light brown to black

(Killick-Kendrick, 2002). When the sandfly is at rest position, its wings assume a characteristic V-shaped position, at an angle of 45º to the body (Killick-Kendrick *et al.*, 1986). Sandflies are holometabolic insects, with four distinct stages of development: egg, larva (with four stages), pupa and imago or adult. During the life cycle, adults are in an aerial phase and immature in a terrestrial phase (Killick-Kendrick, 2002). Adults show marked sexual dimorphism and both sexes feed on the sugary juices of plants. However, females need a blood diet for ovarian maturation and thus to proceed to oviposition and maintenance of the sandfly life cycle.

When oviposition is complete, and there is no larval diapause, new adults will emerge again after approximately 35 to 60 days, with a longevity of 15 to 60 days (Leger and Depaquit, 2001, Killick-Kendrick, 2002).

Most adults have twilight and nocturnal activity. Its period of activity, both annual and daily, is varied and strongly conditioned by climatic factors (Dillon and Lane, 1993). Sandflies are active in the absence of rain and strong winds, and when the temperature varies between 15 and 28°C (Killick-Kendrick, 2002). In the tropics, sandfly activity is observed throughout the year, while in Europe its activity presents a seasonal pattern, usually being more active between spring and autumn (Dantas-Torres *et al.*, 2012).

Resting places for sandflies consist of cool, damp and dark microhabitats such as houses, latrines, cellars, stables, caves, cracks in walls, rocks and soil, areas of dense vegetation, burrows of rodents and other mammals, nests of birds and termites mounds (Killick-Kendrick, 2002). They can go to a maximum distance of about one or two kilometres from their breeding sites, and these distances may vary depending on the species (Afonso and Alves-Pires, 2008). The flight speed of a sandfly is less than one meter per second (Killick-Kendrick *et al.*, 1986), being inhibited to fly with higher wind speeds, which is one of the main factors that limit its dispersion. Females are predominantly exophagic (biting outside) and exophilic (staying outside for ovarian maturation).

Females have a life span between two to six weeks. The preferred places to have their blood meals in the mammal are glabrous areas such as the snout, the auricle and the inguinal and perianal area (Saridomichelakis *et al.*, 2009).

The sandfly species existing in South Europe are zoo-anthropophilic, with a greater preference for dogs than for humans, but in the absence of a preferential host, they may present an opportunistic behaviour, feeding on the available vertebrate (Maroli *et al.*, 2008).

In Portugal, there are two known genera, the genus *Phlebotomus* and genus *Sergentomyia* and five species: *Phlebotomus* (*Phlebotomus*) *papatasi (*Scopoli, 1786); *Phlebotomus* (*Paraphlebotomus*) *sergenti (*Parrot, 1917); *Phlebotomus* (*Larroussius*) *perniciosus* (Newstead, 1911); *Phlebotomus* (*Larroussius*) *ariasi (*Tonnoir, 1921) and *Sergentomyia* (*Sergentomyia*) *minuta* (Rondani, 1843).

The species *P. perniciosus* is the most abundant in Portugal, being only supplanted by *P. ariasi* in the regions of high humidity and lower temperatures of Trás-os-Montes and Alto Douro (Semião Santos *et al.*, 1995; Alves-Pires *et al.*, 2004).

In South America, the best-established vector for *L. infantum* transmission is *Lutzomyia longipalpis* (Lainson and Rangel, 2005; Dantas-Torres, 2009).

1.2.3. Epidemiology

The CanL is a complex disease that in endemic areas can have seroprevalence ranging from 60% to 80%. It is estimated that about half of dogs infected with *Leishmania* do not show clinical signs (Baneth *et al.*, 2008, Campino and Maia, 2010, Solano-Gallego *et al.*, 2011) and only 10% to 30% of dogs show clinical signs (Travi *et al.*, 2018). Thus, it is assumed that apparently healthy animals actively participate in transmission. According to Campino (2002), symptomatic dogs are considered more efficient as reservoir hosts, in parasite transmission.

The prevalence of CanL varies considerably between regions and depends on the serological test used for diagnosis (Leite *et al.*, 2015). The distribution of CanL in endemic areas is not uniform, but focal, with variations in its prevalence between contiguous areas. Its distribution usually follows the distribution of the vectors, which is also not uniform and depends on the existence of specific ecological niches, which supply the biological needs of each sandfly species (Campino, 2002).

In Europe, the average seroprevalence of CanL is described in the table below (Table 2).

Table 2. CanL seroprevalence in some European countries

Country	Percentage	Reference
Spain	8.5%	Amusategui *et al.*, 2004
Portugal and Chipre	20%	Pires *et al.*, 2019; Beyhan *et al.*, 2016
South France	4-20%	Chamaille *et al.*, 2010
Italy	2-15%	Maroli *et al.*, 2008
Greece	25%	Ntais *et al.*, 2013
Turkey	15.7%	Dujardin *et al.*, 2008
Croatia	1.4%	Mrljak *et al.*, 1990

The distribution of CanL in Europe has been changing, probably due to socioeconomic and climate factors (Solano-Gallego *et al.*, 2011). The increase in global average temperature, caused by climate change, has contributed to the geographic expansion of sandfly vectors to new areas. The infection is progressing to northern Europe, reaching the Alps in northern Italy (Maroli *et al.*, 2008), the Pyrenees in France (Chamaille *et al.*, 2010) and northern Spain (Amusategui *et al.*, 2004), as well as Germany, Netherlands, United Kingdom and Poland (Shaw *et al.*, 2009; Solano Gallego *et al.*, 2009, 2011; Ballart *et al.*, 2013; Roura *et al.*, 2015) and some Balkan countries, such as Bulgaria, Kosovo, Romania, Serbia and Slovenia (Vaselek, 2021) as an emerging disease. This expansion was observed before the upsurge of the COVID-19 pandemic and seems to be associated with the increase in pet travel to CanL endemic countries, along with the importation of CanL cases (Zygner 2006; Menn *et al.*, 2010;) and the adoption of animals from endemic areas (Maia and Cardoso, 2015). Importing *Leishmania*-infected dogs to *Leishmania*-free areas might increase the risk of parasite transmission in the case of existing competent vectors, even at low densities. In endemic areas, it is expected that in the future, the climate changes leading to the increase in average temperatures and

higher periods of hot climate, sandflies will increase not only their period of activity but also their number of annual generations and their density (Ready, 2008).

In Portugal, seroprevalence studies carried out through direct agglutination tests (DAT) indicate that Trás-os-Montes and Alto Douro, the sub-region of Cova da Beira, the district of Coimbra, the region of Lisbon and Setúbal, the district of Évora, the Algarve region (Campino *et al.,* 2006; Campino and Maia, 2010) and the districts of Portalegre, Beja and Castelo Branco (Cortes *et al.,* 2012) are endemic regions for CanL. Between 2008 and 2009, the dog seroprevalence in Portugal assessed by DAT and enzyme-linked immunosorbent assay (ELISA), was 21.3% (Sousa *et al.,* 2011). In 2012, a study carried out by Cortes and collaborators estimated a higher prevalence of canine infection in rural areas (8.8%) compared to urban/suburban areas (3.8%), and the opposite situation in the incidence of human infection (Cortes *et al.,* 2012). This phenomenon previously observed by Abranches *et al* (1987) is called trophic deviation in rural areas. Although the vector is predominantly zoophilic, urbanization led to the decrease of stray dogs in urban areas, which can make the human population more vulnerable to accidental infections (Abranches *et al.,* 1987).

In other studies, the ELISA seroprevalence rate of CanL in naturally exposed dogs was estimated to be 15.4% in Iran (Mahshid *et al.,* 2014), 10.5% in the west of Iran (Gharekhani *et al.,* 2020), 5.5% in Palestine (Abdeen *et al.,* 2002) and 26.6% in Pakistan (Rab *et al.,* 1995). Through the use of immunofluorescence antibody test (IFAT), the seroprevalence ranged between 42.9% and 74.3% in Sudan (Dereure *et al.,* 2003)**.** In previous studies carried out in Egypt, the prevalence rate of infected dogs was estimated to be 66.6% by polymerase chain reaction (PCR) (Abuowarda *et al.,* 2021) and 10% by immunochromatography (Rosypal *et al.,* 2013). However, a more recent study using ELISA indicated a seroprevalence of 21.3% (Selim *et al.,* 2021).

The CanL cases were distributed across West Africa, with reports occurring in three countries, Gambia (Desjeux *et al,* 1983), Senegal (Faye *et al,* 2011) and Burkina Faso (Andre *et al.,* 1978). Epidemiological surveys of dogs and clinical cases of CanL have been reported in North Africa, as is the case of Tunisia (Ben Said *et al.,* 1992) and Algeria (Harrat *et al.,* 1996), and in East Africa, such as Kenya (Mutinga *et al.,* 1980), Sudan (Dereure *et al.,* 2000), Ethiopia (Kalayou *et al.,* 2011) and Uganda (Millán *et al.,* 2013). Data from Southern Africa are still scarce.

Among all Latin American countries where *L. infantum* has been found in dogs, Brazil, Argentina and Paraguay show the greatest evidence of CanL expansion. CanL seroprevalence in endemic areas of Brazil ranges from 3.1% to 36.0%, Paraguay between 23% and 32% (Canese *et al.,* 2000; Miret *et al.,* 2010; Portillo *et al.,* 2011) and in 2010 it reached values of 69% in stray dogs (Miret *et al.,* 2011). In 2006, after the first case of CanL has being reported in Posadas, Argentina, the prevalence based on serology or PCR was 57.3% (Cruz *et al.,* 2010). Studies on CanL seroprevalence reported levels between 1.7% and 15.7% in Mexico (Arjona-Jiménez *et al.,* 2012; López-Céspedes *et al.,* 2012), 5.6% and 40.0% in Venezuela, (Delgado *et al.,* 1998; Zerpa *et al.,* 2000, 2001, 2003; Feliciangeli *et al.,* 2005), and 1.6% and 36.0% in Colombia (Fernández *et al.,* 2002; Cortés, 2006; Rosypal *et al.,* 2007; Paternina-Gómez *et al.,* 2013). CanL is comparatively rare in Bolivia compared to CL, but dogs can be infected with *L. infantum* (Le Pont *et al.,* 1989; García *et al.,* 2009). Although French Guiana has not reported any autochthonous cases of VL in humans so far the first autochthonous case of CanL was described in 2005 (Rotureau *et al.,* 2006).

Moreover, in certain areas, the enzootic transmission cycles of different species of *Leishmania* may overlap and dogs may be coinfected (Torras-Dantas, 2009) and co-infections may have diagnostic relevance due to the possibility of serological cross-reactions between different *Leishmania* species (Vale *et al.,* 2009).

1.2.4. The role of host factors in canine leishmaniasis

In dogs, *L. infantum* infection causes a variety of clinical signs ranging from no signs, to mild or very severe signs in stablished disease. Several factors such as the immune status and the genetic history of the host can influence the outcome of CanL (Solano-Gallego *et al.,* 2011).

In endemic areas, several host factors such as age, gender, genetics, nutritional status, or concurrent infections, as well as the virulence of *Leishmania* strains, play an important role in the dog's response to infection (Alvar *et al.,* 2004, Solano-Gallego *et al.,* 2009).

The age of the animal (Alvar *et al.,* 2004) appears to be a very important factor. According to Acedo-Sánchez *et al.* (1996), the prevalence of leishmaniasis by age group usually presents a bimodal distribution, with 80% of infected dogs younger than three years old, and another,

less significant peak, when dogs begin to experience immune depression from 8-10 years of age. Other factors, such as exposure to sandflies, as is the cases of dogs that are often outside the house or stray dogs, as well as short-haired dog breeds, increase the predisposition to develop the disease (França-Silva *et al.,* 2003, Moreira Jr. *et al.,* 2003, Rosypal *et al.,* 2004). Crossbreed dogs are also less prone to infection than purebred dogs (Cortes *et al.,* 2012).

Theoretically, all dog breeds are susceptible to *Leishmania* infection, although it is recognized that genetic factors play an important role in the development of the disease. Furthermore,the competence of the immune response, which seems to be determinant to the progression from a subclinical to a patent clinical state, is related to the resistance to infection (Barbiéri, 2006, Pinhão, 2009). A series of studies revealed a high prevalence of infection in some dog breeds, such as Boxer, Cocker spaniel, English and American foxhound, Rottweiler, German shepherd, Doberman, and Pinscher (Sideris *et al.,* 1999, Gaskin *et al.,* 2002, Franca-Silva *et al.,* 2003, Sanchez-Robert *et al.,* 2005, Duprey *et al.,* 2006). On the other hand, other breeds such as the Mediterranean Ibizan-hound, a breed from the Balearic Islands of Ibiza rarely develop clinical signs of CanL, pointing to some degree of resistance associated with a strong immune response (Solano-Gallego *et al.,* 2000).

Polymorphisms in the canine β-defensin 1 (CBD1) gene have been previously associated with respiratory and cutaneous diseases in dogs (Van Damme *et al.,* 2009, Erles and Brownlie, 2010), and this gene may also be associated with the canine immune response against *L. infantum* (da Silva *et al.,* 2017). β-defensins may function as an important chemoattractant for immature dendritic cells (iDCs) (Hazlett and Wu, 2011). In this context, macrophages and dendritic cells are known to play a key role in initiating, developing, and maintaining the protective immune response against *Leishmania* (Srivastava *et al.,* 2016). DCs seem to have a dichotomous role in modulating the immune response in cutaneous leishmaniasis, as it appears that they can cause both resistance and susceptibility (Feijó *et al.,* 2016). Of these facts, the Single Nucleotide Polymorphisms (SNPs) in the CBD1 gene that was reported by Da Silva and collaborators (2017) may be a potential genetic marker for the study of susceptibility/resistance to *L. infantum* infection. The VL resistance candidate gene was linked to a polymorphism in the natural resistance-associated macrophage protein 1 (NRAMP1) gene, later named the Slc11a1 gene (Bucheton *et al.,* 2003). The Slc11a1 gene encodes a bivalent proton cation antiporter with a role in regulating macrophage function, including upregulation of chemokines and cytokine genes, such as tumor necrosis factor (TNF) and interleukin-1β (IL-1β), as

well as the induction of oxide synthase that is linked to anti-leishmanial immune mechanisms (Blackwell *et al.*, 2001). To better understand the possibility of the Slc11a1 gene influencing susceptibility to the genus *Leishmania*, in a study involving 97 dogs (40 dogs diagnosed with leishmaniasis and 57 healthy dogs) the polymorphism of promoter regions and the microsatellite located in intron 1 were analyzed. From this study, 14 haplotypes were generated, with significant differences between the two groups of animals, mainly due to the polymorphisms found in point nucleotides of the genome. Of the haplotypes found, the most frequent were TAG-8-141 present in all breeds, and TAG-9-145, fundamentally found in the Boxer breed. In this breed, the TAG-9-145 haplotype was observed preferentially in homozygous animals and healthy animals. On the other hand, the TAG-8-141 haplotype was significantly associated with infected animals, and 50% of Boxers with CanL carrying this haplotype were heterozygous (TAG-8-141/TAG-9-145). Thus, Boxer dogs that carry the TAG-8-141 allele were more susceptible to contracting the disease. It is concluded that the distribution of the genotype of the Slc11a1 gene and the differences in the prevalence of disease in the Boxer breed emphasize the importance that breed genetics has on CanL susceptibility (Sanchez-Robert *et al.*, 2005).

In 2001, Kennedy and colleagues reported that the major histocompatibility complex (MHC) is a candidate region for controlling CanL susceptibility. Several MHC class II alleles were discovered in dogs, namely 67 dog leukocyte antigen (DLA)-DRB1 alleles, 18 DLA-DQA1 alleles and 47 DLA-DQB1 alleles. In 2003, Quinnell and colleagues conducted a study in dogs and demonstrated a statistically significant association between the presence of the DLA-DRB1*01502 allele and susceptibility to CanL. They found that dogs with this allele had significantly higher IgG levels, and were more likely to be positive for *Leishmania* by the PCR technique. Among the breeds studied, the Labrador breed showed the highest percentage of DLA-DRB1 alleles (Quinnell *et al.*, 2003).

1.2.5. Immune reponse to Leishmania infection

Mechanisms of the innate and acquired immune response are initiated after contact with the parasite, being acquired mechanism the main responsible for the degree of resistance or susceptibility to infection, specifically, the cellular immune response, which is mediated by helper T lymphocytes (Th) (Reis *et al.,* 2006; Pires *et al.,* 2012). Although there are descriptions of dogs presenting pro-inflammatory Th1 and anti-inflammatory Th2 response, the competence of the immune response seems to depend on the evolutionary stage of the infection, since dogs in the early stages of the disease have a predominance of pro-inflammatory response (Santos *et al.,* 2019**)**. The imbalance of these two immune responses defines parasite replication, disease progression or cure (Carrillo and Moreno, 2009; Tabanez, 2016).

Th1 immune response leads to secretion of TNF, as well as interleukin (IL) -1, IL-2, IL-12 and interferon-gamma (IFN-γ), which enhance the phagocytic and microbicidal capacity of MΦ , leading to the destruction of the parasite by related mechanisms, the respiratory burst that directs nitric oxide (NO) production (Alvar *et al.,* 2004, Solano-Gallego *et al.,* 2009, Alexandre-Pires *et al.,* 2010, Travi and Miró 2018). On the other hand, susceptible dogs show a predominantly Th2 immune response, promoting the secretion of anti-inflammatory cytokines, such as IL-4, IL-10, IL-13 and transforming growth factor (TGF-β), leading to the polyclonal proliferation of B cells and further differentiation in immunoglobulin-secreting plasma cells, which do not play a protective role, but rather contribute to immunopathology development (Kaye and Scott, 2011, Tabanez, 2016).

Not every infected dog develops clinical manifestations, and the progression from a subclinical to another clinical state is determined by the host's immune response. Clinical signs are related to immunopathogenic mechanisms, such as deposition of immune complexes, B lymphocyte hyperreactivity and immunosuppression. The deposition of immune complexes occurs mainly in the vessel wall in the microcirculation. This deposition activates the complement system response and attracts more inflammatory cells, leading to tissue damage (Ferrer, 1999; Tabanez, 2016).

Considering these strict relationships, an imbalance of the dog's immune response in the direction of one of the poles can lead to a clinic cure or establishment of the disease. However, given that subclinical animals can, at some point, develop disease due to immunosuppression

or other concomitant infections (Baneth *et al.*, 2008, Solano-Gallego *et al.*, 2009), it is difficult to progress toward a true parasitological cure, with healthy and subclinically infected dogs constituting relevant *L. infantum* reservoirs.

1.2.6. CanL clinical manifestations

CanL is a systemic disease that can involve any organ, tissue or body fluid. Dogs can present no clinical signs (that can reach more than 80% of cases in some endemic areas) or they can progress to severe disease, with a wide variety of clinical manifestations (Alvar *et al.*, 2004, Otranto *et al.*, 2013, Solano-Gallego *et al.*, 2011).

After inoculation by the sandfly in the dog's dermis, *L. infantum* parasites are spread throughout the dog's body, initially reaching the lymph nodes and spleen by the bloodstream and lymphatic system, later on to the kidneys and liver, and finally reaching the reproductive organs, bladder, digestive and respiratory systems, to going back to the skin (Strauss-Ayali *et al.*, 2007). The presence of the parasites in different organs and tissues triggers proliferative inflammatory reactions, leading to progressive changes and functional imbalance of affected organs, generating skin lesions and other clinical manifestations characteristic of CanL (Alvar *et al.*, 2004). Among the general clinical signs are muscle atrophy and exercise intolerance (Gharbi *et al.*, 2015), apathy, decreased or increased appetite, weight loss, pale mucous membranes, splenomegaly, polyuria and polydipsia, fever, vomiting, diarrhea (including chronic colitis) and generalized lymphadenopathy (Ciaramella *et al.*, 1997, Koutinas *et al.*, 1999, Baneth *et al.*, 2008, Solano-Gallego *et al.*, 2011), mainly affecting popliteal, prescapular and submaxillary lymph nodes (Lima *et al.*, 2004, Couto, 2015, LeishVet, 2018, Ribeiro *et al.*, 2018) (Table 4). Skin lesions, such as nonpruritic exfoliative dermatitis with or without generalized alopecia or localized alopecia on the face, ears and limbs, erosive-ulcerative dermatitis on bone prominences, mucocutaneous transitions, paws and ears (Ferrer *et al.*, 1988, Koutinas *et al.*, 1992), nodular and papular dermatitis, onychogryphosis (Solano-Gallego *et al.*, 2011) and pustular dermatitis due to superficial or deep bacterial and fungal co-infections (Gharbi *et al.*, 2015) are considered CanL manifestations. Moreover, ocular manifestations include exfoliative, ulcerative or nodular blepharitis, nodular or diffuse conjunctivitis, keratoconjunctivitis, anterior or posterior uveitis, glaucoma and panophthalmitis (Amara 2003,

Baneth *et al.*, 2008, Solano-Gallego *et al.*, 2009, Gharbi *et al.*, 2015). Disorders in the lungs, nervous system, joints and cardiovascular system in dogs with leishmaniasis also have been described, although these are not considered tissues in which lesions associated with *Leishmania* infection are commonly found (Rosa *et al.*, 2014; Pinto, 2016; Leishvet, 2018). Dogs may also present with renal alterations and hemorrhagic diathesis (epistaxis, hematuria, petechiae, ecchymosis and hematomas) (Arruda, 2010). Renal lesions are characterized by tubular and glomerular damage resulting from the deposition of immune complexes, that lead to proliferative membrane glomerulonephritis and interstitial nephritis with impaired renal function (Lopez *et al.*, 1996, Luvizotto, 2006) that can progress to chronic renal failure, which is the most severe disease manifestation and the leading cause of CanL mortality (Alvar *et al.*, 2004, Ribeiro, 2007, Solano-Gallego *et al.*, 2011).

Along with characteristic physical manifestations, some hematological and biochemical changes can be found. Laboratory analysis of parameters related to hematopoiesis, renal function and serum electrophoretic profile are used as tools for clinical diagnosis (Ribeiro *et al.*, 2018). The common blood examination includes complete blood count (CBC), serum biochemical analysis, serum protein electrophoresis and urinalysis (Slappendel 1988, Koutinas *et al.*, 1999, Paltrinieri *et al.*, 2010).

Table 3. Clinical manifestations associated with *L. infantum* -CanL according to LeishVet and CLWG

	LeishVetGuidelines	CLWG Guidelines
General	Generalized lymphadenomegaly Loss of body weight Decreased or increased appetite Lethargy Mucous membranes pallor Splenomegaly Polyuria and polydipsia Fever Vomiting Diarrhea	Poor nutritional state or cachexia Muscular hypotrophy Lethargy Pale mucous membranes Mild to moderate enlargement of palpable lymph nodes Epistaxis Hepatosplenomegaly Lameness and joint swellings Fever
Cutaneous	Non-pruritic exfoliative dermatitis with or without alopecia Erosive-ulcerative dermatitis Nodular dermatitis Papular dermatitis Pustular dermatitis Onychogryphosis	Desquamative dermatitis (localized or generalized) Ulcerative dermatitis with varying appearance and distribution (eg, mucocutaneous junctions, skin, covering the extremities and traumatized sites) Papular dermatitis Nodular dermatitis Lupus- or pemphigus-like nasal lesions Onychopathy Nasodigital hyperkeratosis Pustular dermatitis
Ocular	Blepharitis (exfoliative, ulcerative or nodular) and conjunctivitis (nodular) Keratoconjunctivitis, either common or sicca Anterior uveitis Endophthalmitis	Palpebral lesions: See cutaneous and mucocutaneous findings Diffuse or nodular conjunctival lesions Corneal lesions, mainly associated with the conjunctiva (keratoconjunctivitis) Nodular keratitis and keratoconjunctivitis sicca Scleral lesions (diffuse or nodular scleritis and episcleritis) 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) Granulomatous orbital lesions or myositis of extrinsic muscles
Others	Mucocutaneous and mucosal ulcerative or nodular lesions (oral, genital and nasal) Epistaxis Lameness (erosive or non-erosive polyarthritis, osteomyelitis and polymyositis) Atrophic masticatory myositis Vascular disorders (systemic vasculitis and arterial thromboembolism) Neurological disorders	Gastrointestinal or neurologic involvement

Anemia is one of the main alterations in the blood count. Most patients (50 to 70%) have normocytic/normochromic and non-regenerative anemia, which suggests, at the very least, the participation of chronic inflammatory disease and/or impairment of erythropoiesis by infection-induced changes in the bone marrow and/or kidneys. There is a possible relationship between anemia and the clinical forms of the disease. Dysproteinemia is considered one of the most important alterations of the disease (Ribeiro *et al.*, 2013). CanL is often characterized by an increase in total serum proteins (hyperproteinemia), azotemia, hypergammaglobulinemia (polyclonal B-cell response), hypoalbuminemia (renal and/or liver failure) (Paltrinieri *et al.*, 2016) and albumin/globulin ratio below the lower reference values (Ribeiro *et al.*, 2013, Paltrinieri *et al.*, 2016). Moreover, it is recognized that is almost inevitable that sick dogs develop kidney damage (Ribeiro *et al.*, 2013). Liver enzyme activity is generally within reference values for the canine species, although biochemical findings in infected dogs may include changes in AST (aspartate amino transferase), ALT (alanine amino transferase), and FAS (serum alkaline phosphatase) enzymes (Paltrinieri *et al.*, 2010, Solano-Gallego *et al.*, 2011).

In a study by Pereira and colleagues (2020), the majority of dogs had hyperproteinemia, mean normocytic normochromic anemia, normal urea and creatinine levels as classified as stage 1 with the International Interest Society (IRIS) guidelines at the time of diagnosis. Therefore, survival analysis showed that patients classified as IRIS 1 at the time of diagnosis survived more than four years, in contrast to dogs classified as IRIS 2 which survived about two and half years and dogs classified as IRIS 3-4 that survived about a month.

1.2.7. Diagnosis

The CanL diagnosis is complex and difficult since its clinical signs can be confused with other diseases (Solano-Gallego *et al.*, 2009). Several diagnostic strategies have been implemented based on parasitological, molecular or serological methods in association with clinical and epidemiological parameters (Oliveira *et al.*, 2009). Thus, performing a thorough physical examination and collecting a complete anamnesis is fundamental to direct the most adequate diagnostic techniques. The most common diagnostic methods at the parasitological level are cytology/histology and immunohistochemistry. At the molecular level, the methodologies

based on polymerase reaction (PCR), which includes conventional PCR (cPCR), nested PCR (nPCR) and real-time PCR (qRT-PCR), the latter being the most sensitive of all. Regarding serological diagnosis, there are mainly two classes of tests: quantitative tests, as is the case of ELISA and IFA and qualitative tests, such as rapid tests.

Parasitological Tests

Parasitological diagnosis is always used to evidence the presence of the parasite in biological samples from dogs with suspected LCan. For this purpose, indirect methods or direct methods can be used to evidence its presence.

Direct methods: Cytology, Histology and Immunohistochemistry

Cytological evaluation allows microscopic detection by staining, such as Giemsa staining, of *Leishmania* amastigotes in macrophages of parasite target tissues, as is the case of skin, lymph nodes and bone marrow (Alvar *et al.*, 2004, Saridomichelakis *et al.*, 2005, Solano-Gallego *et al.*, 2009) and, less frequently, in other tissues and body fluids (Agut *et al.*, 2003, Dantas-Torres *et al.*, 2006, Solango-Gallego *et al.*, 2009), with lymph nodes and bone marrow were the samples of choice (Barrouin-Melo *et al.*, 2004; Paltrinieri *et al.*, 2016; Akhoundi *et al.*, 2017, Ribeiro *et al.*, 2018, Taylor, 2018). The sensitivity of this technique is not very high, with sensitivities of 30 to 50% being reported for lymph node samples, and 60 to 75% for bone marrow samples (Ferrer, 1999, Alvar *et al.*, 2004). However, another study identified amastigote forms in 93% of bone marrow and lymph node samples from dogs naturally infected with *L. infantum* (Rosypal *et al.*, 2005). Sensitivity depends not only on the density of parasites present in the biological sample (Saridomichelakis *et al.*, 2005) but also on the quality of the cytology preparation, the examiner's experience and the time devoted to its analysis, as well as, the number of fields observed (Noli and Saridomichelakis, 2014). Furthermore, it does not allow the distinction between *Leishmania* species (Solano-Gallego *et al.*, 2011, Akhoundi *et al.*, 2017). In the absence of visualization of the parasite, other complementary tests are required for diagnosis, such as immunohistochemistry and/or PCR (Solano-Gallego *et al.*, 2011).

The histopathological analysis is performed on biopsies of parasite-target organs, such as the spleen, liver, bone marrow and popliteal lymph node (Paltrinieri *et al.*, 2010). Samples are stained with hematoxylin and eosin (HE) and are used when the cytology result is negative

but the dog exhibit characteristics highly consistent with leishmaniasis (Paltrinieri *et al.*, 2016). However, a detailed observation can be necessary to be able to observe amastigotes since this technique presents lower sensitivity than cytology (Maia and Campino, 2008b), especially due to the reduced size of the parasite (amastigote forms reduce their size with formalin fixation) and the suboptimal properties of HE (Noli and Saridomichelakis, 2014).

Immunohistochemical methods, using antibodies (Ab) against *Leishmania* antigens, have been suggested as a supplementary method to confirm the diagnosis, particularly in organs with a low parasitic load. However, these methods are invasive, time-consuming (Gomes *et al.*, 2008) and may give false-negative results, as their sensitivity depends on the parasite load.

Culture

In vitro isolation of parasites from aspirates, scrapings or tissue biopsies allow confirmation of whether the suspected dogs harbour the parasites, but also if the parasites are viable. The methods of isolation and culture of the parasite can be performed *in vitro*, on selected culture media such as blood agar, Novy–MacNeal–Nicolle (NNN) medium, brain-heart infusion (BHI) agar medium, Modified Evans Tobie (EMTM) or Schneider's medium supplemented with fetal bovine serum (FBS) among others (Taylor, 2018). It has the disadvantage of requiring a long period (up to 30 days) before results are obtained, being subject to contaminations by other microorganisms and requiring an invasive procedure to obtain the biological material. Currently, this test is only used for research purposes (Maia *et al.*, 2008, Paltrinieri *et al.*, 2010).

Conventional methods

The detection of *Leishmania*-specific IgG antibodies through serological tests is the most frequent initial approach in the diagnosis of CanL (Bourdeau *et al.*, 2014) and also in conducting scientific studies (Solano-Gallego *et al.*, 2014). The most common serological methods for the detection of anti-*Leishmania* Ab are indirect IFAT, ELISA and immunochromatography. Serological methods like IFAT and ELISA allow quantification of Ab titers or observed optical density, respectively. Its diagnostic performance is superior to qualitative methods (Solano-Gallego *et al.*, 2014). Seroconversion can occur a few months after infection. According to Paltrinieri and colleagues (2010), the seroconversion interval can range from 1 to

22 months (average 5 months) after infection. Thus, whenever the presence of a high Ab titer is verified, this result is consistent with the presence of the infection, but if the Ab titer is low, it is indicative of an early stage of the infection, or only the exposure of the animal to the parasite. A negative result does not exclude the existence of infection, as there are animals that take some time to develop a humoral response and reach positive Ab titers. Thus, animals clinically suspect, or with dubious results, should repeat the test after 4 to 6 weeks, or resort to another type of test (Solano-Gallego *et al.*, 2014). There is still a disadvantage of serological diagnostic techniques, which is the appearance of false positives. This is due to cross-reactions in dogs with Chagas disease (*Trypanosoma cruzi*), ehrlichiosis and rickettsiosis, but with other *Leishmania* species (Gomes *et al.*, 2008, Solano-Gallego *et al.*, 2009). A current problem with 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), except for those vaccinated with Letifend®.
The IFA is highly specific and sensitive in the detection of clinical leishmaniasis; however, it may be less sensitive in the detection of infected but clinically healthy animals (Mettler et al. 2005, Solango-Gallego *et al.*, 2009). It is considered the "gold standard" of serological tests (Maia and Campino, 2008b, Paltrinieri *et al.*, 2016) and has been recommended by the World Organization for Animal Health (WOHA) (Taylor 2018). This technique is performed through a series of serum dilutions on slides coated with promastigote forms of *Leishmania*. Subsequently, the presence of Ab is revealed through the use of secondary Ab conjugated to a fluorochrome. In CanL, IFA Ab titers between 1:40-1:80 are suggestive of exposure to the parasite but not necessarily of infection, in turn, titers of 1:160 and above are indicative of infection and established disease in dogs with clinical suspicion (Paltrinieri *et al.*, 2010).

ELISA is a classic method used in the detection of Ab against *Leishmania*, with no limitations regarding the use of specific laboratory conditions and techniques. In a study carried out by Solano-Gallego *et al.* (2014), the ELISA test showed a sensitivity of 92.5-95.3% and a specificity of 86.9-100%, but it seems that using recombinant peptides, such as proteins K9, K26, and K39, the technique of ELISA becomes very specific (100%) and sensitive (98%) in detecting clinically healthy infected dogs (Mettler *et al.*, 2005; Inno, 2012). Among the ELISA kits tested, the one that presented the best diagnostic performance was LEISCAN® (Solano-Gallego *et al.*, 2014), which is essential for the detection of infected animals that remain without clinical signs.

Rapid tests

Rapid tests are based on the immunochromatography technique and have the advantage of being quick and easy to read. However, they have lower specificity (30-70%) but high sensitivity (61-100%) when compared to ELISA and IFA techniques. There are several commercial kits, each using a different antigen (Ag) and different reagents (Paltrinieri *et al.,* 2010). In Portugal, there are several rapid tests, namely the WITNESS Leishmania® test and the Speed Duo Leish/Ehrli®, to search for anti-*Leishmania* Ab using purified Ag of the *Leishmania* genus. To perform the test, samples of whole blood, serum or plasma are required. However, the result obtained is only qualitative and if the result is positive, another serology is required to quantify the level of Ab (Solano-Gallego *et al.,* 2014).

Molecular diagnostic

Molecular diagnosis is performed through methodologies based on PCR, which allows the detection of *Leishmania* deoxyribonucleic acid (DNA). DNA collection can be performed through a blood sample, aspirative biopsies of bone marrow aspirate, lymph node, spleen, liver and skin. Can be performed with fresh, frozen or 95% ethyl alcohol pre-conditioned samples (Paltrinieri *et al.,* 2010). The sensitivity and specificity of these molecular methodologies reach parameters close to 100% (Ferrer, 1999; Luviozotto, 2005). This technique is characterized by the amplification of a specific DNA target, using oligonucleotide sequences (primers) selected from the small subunit ribonucleic acid (RNA) gene (Mathis and Deplazes 1995), kinetoplast DNA minicircles (de Bruijn and Barker 1992) or other highly repetitive genomic DNA sequences. The most commonly used techniques are cPCR, nPCR and qRT-PCR, which can provide quantitative data (Paltrinieri *et al.,* 2010). Qualitative PCR can be useful when an immediate and sensitive diagnosis is needed, especially in cases with dubious serological results (Gomes *et al.,* 2008). Despite this high sensitivity of the technique, it should be considered that different samples may have different concentrations of parasites and even variable chances of containing leishmaniasis DNA, For example, several samples routinely used in decreasing order of sensitivity are: bone marrow, lymph nodes, skin, conjunctiva, buffy coat, and the least 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). However, these molecular methodologies can present several, false positives that can occur due to DNA contamination and are expensive techniques that requires

specific reagents, specialized equipment and highly trained technicians. It should not be performed as the sole diagnostic test, as a positive result only confirms the presence of *Leishmania* DNA, which indicates a possible infection but is not necessarily an indicator of disease (Solano-Gallego *et al.*, 2011).

Xenodiagnosis

Xenodiagnosis is a technique that allows the detection and isolation of the parasite using a sandfly colony. These sandflies will feed on the blood of infected dogs and are then examined for the presence of promastigotes in the intestine (Paltrinieri *et al.,* 2016). Despite its high specificity and reasonable sensitivity, it is a time-consuming, non-quantitative and non-viable technique that requires animals and insectaries, being mainly restricted to investigation and not recommended for routine use (Akhoundi *et al.*, 2017).

1.2.8. Treatment

Due to its complex pathogenesis, CanL can manifest with different clinical signs. The immune response plays an important role in the development of infection and response to treatment (Olivia *et al.*, 2010). All known anti-*Leishmania* drugs used in dogs can lead to temporary or permanent remission of clinical signs, increasing life expectancy and improving quality of life, in addition to a reduced parasite load that leads to lower infectivity to vectors (Ribeiro *et al.*, 2018), but the clinical and parasitological cure is rarely achieved, not to mention the possibility of drug resistance (Travi, 2014, Marcondes and Dia, 2019). Before deciding to administer the therapy, it is necessary to evaluate the dog's ability to respond to therapy by assessing the serological, hematological, biochemical, and urological profile, mainly, the bone marrow, renal and hepatic status, as well as the ability and/or will of the dog tutor to comply with treatment protocols.

Current therapies rely on three main drugs: pentavalent antimonials (first drug of choice), amphotericin B, and miltefosine (Hefnawy *et al.,* 2017). Thus, the first-line treatment for

CanL is the combined use of leishmanicidal agents, such as pentavalent antimonials, miltefosine and allopurinol (Miró *et al.,* 2008; Solano-Gallego *et al.,* 2009; Oliva *et al.,* 2010). These drugs are used in the treatment of human leishmaniasis and have already shown clinical resistance in dogs (Miró *et al.,* 2017) (Table 5). Some published studies indicate that anti-*Leishmania* drugs reduce or eliminate the infection for at least 4 months (Miró *et al.,* 2017). The combination of a leishmaniostatic, allopurinol, with a leishmanicidal agent, meglumine antimoniate or miltefosine promotes an increase in the number of Th1 lymphocytes. However, all drugs currently available for the treatment of this disease are unsatisfactory in terms of efficacy, cost, easy administration and safety (Murray, 2010; Mcgwire and Satoskar, 2014; Hendrickx *et al.,* 2019). Even so, the response to treatment is usually fast, with weight gain, reduction of skin lesions, and blood values tending to normal, leading to a general improvement in the animal's condition (Alvar *et al.,* 2004; Santos *et al.,* 2019).

Antimony compounds

Antimony compounds were initially used in their trivalent form (SbIII). However, due to its side effects, the pentavalent form (SbV) prevailed (Meireles, 2008). There are two drugs that stand out within the group of antimonials: meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®), but the most used compound is Glucantime. It can be used alone (monotherapy) or in combination with allopurinol to potentiate the therapy effect against *Leishmania* spp. Glucantime selectively inhibits glycolysis and fatty acid oxidation, thus inhibiting two enzymes within the parasite: phosphofructokinase and pyruvate dehydrogenase (Meireles, 2008; Oliva *et al.,* 2010). They are considered a prodrug, which is converted into the trivalent active form under low pH conditions in host phagocytes (Singh *et al.,* 2012). This mechanism of action within the organism generates toxicity against both the host and the parasite (Kato *et al.,* 2014). SbV also exhibits antiparasitic activity through the inhibition of topoisomerase activity (No, 2016). The topoisomerases are a group of enzymes responsible for controlling the topological homeostasis of DNA during the processes of replication, transcription, and chromatin condensation (Sun *et al.,* 2020). In *Leishmania*, the topoisomerase is present in the nucleus and into the only mitochondria. There are clear differences in struc.

Table 4. Therapies used in CanL and drug resistance (Miró *et al.*, 2017)

Drug and dose	Mechanism of action	Dose	Duration	Side effects	Relapse	Drug resistance
Meglumine antimoniate*	Blocking parasite metabolism through inhibition of phosphofructokinase enzyme	100 mg/kg SC, SID	two doses for 4-6 weeks	Pain and inflammation at the injection site, pancreatitis, panniculitis nephrotoxicity	6–12 M	Reported by: Gramicciaet al. (1992), Aït-Oudhia, *et al*. (2012)
Miltefosine*	Impairment of signaling pathways and cell membrane synthesis	2 mg/kg PO, once a day for 28 days	4 wks	Anorexia, vomiting, diarrhea	4–6 mo	Not reported
Allopurinol	Interference with purine pathway	10 mg/kg PO, twice a day for at least 6-12 months	6–24 mo	Xanthinuria, urolithiasis, renal mineralization	Not well documented	Yasur-Landau *et al.* (2016)

* Both drugs are commonly recommended in combination with allopurinol

ture and expression between parasite and host enzymes, thus demonstrating the great therapeutic potential of topoisomerase inhibitors as a drug with leishmanicidal activity (Reguera *et al.*, 2019). Sb^{III} derived from metabolism is responsible for most undesirable side effects such as gastrointestinal disorders, nephrotoxicity, skin irritation, sporadic hyperproteinemia, fatigue and relapses. In addition to these effects, the administration itself causes severe local pain as well as thrombophlebitis, muscle fibrosis, and abscesses. If the pharmaceutical formula contains high molecular weight polysaccharides (such as dextran), hypersensitivity reactions may also occur. The half-life of this substance in dogs is relatively short: 21, 42 and 122 minutes according to the route of administration, intravenous (IV), intramuscular (IM), and subcutaneous (SC), respectively. Within 6 to 9 hours after administration, 80 to 95% of the active substance is eliminated through the kidney (Oliva *et al.*, 2010).

The combination of meglumine antimoniate and allopurinol is considered the gold standard for the treatment of CanL, although it does not result in a complete parasitological cure, dogs demonstrate an improvement in clinical signs and a good prognosis. It also leads to a normalization of the pro-inflammatory immune response, restoring the gene expression levels of IFN-γ and IL-2 in blood cells, IL-2, IL-12 and TNF-α in the lymph node and IFN-γ in the bone marrow, as well as normalizes immunosuppressive cytokines (Santos *et al.,* 2019). This combination of drugs makes it possible to reduce the time of taking meglumine antimoniate, improves its tolerance and makes the treatment protocol less expensive. (Saridomichelakis *et al.,* 2005; Pierantozzi *et al.,* 2013). The most common protocol administers meglumine antimoniate, SC, once daily (SID) for 4 weeks in combination with allopurinol given orally for several months (Solano-Gallego *et al.,* 2009; Olivia *et al.,* 2010). Associated adverse effects reported are pain at the injection site, skin abscess/cellulitis, vomiting, diarrhea, uveitis, and potential nephrotoxicity (Miró *et al.,* 2008; Solano-Gallego *et al.,* 2009).

Miltefosine

Miltefosine is an alkylphosphocholine that was developed as an antineoplastic agent and later used to treat leishmaniasis in humans. It has a direct toxic effect on promastigote and amastigote forms and also stimulates the activation of MΦ and T cells as well as the production of reactive oxygen species (ROS) and oxide nitric (NO) metabolites (Virbac, 2009, Oliva et al., 2010, Reguera et al., 2016).

This drug is administered orally to dogs, being almost completely absorbed and presenting an absolute bioavailability value of 94%. After treatment, the parasite load is efficiently reduced in most infected organs. However, complete elimination of parasites from the bone marrow is not achieved, which implies that clinically cured dogs remain parasitologically positive (Manna et al., 2015, Reguera *et al.,* 2016). One of the disadvantages of this compound is the possibility of relapse or slower normalization of clinicopathological variables (Manna *et al.,* 2015). One of the current concerns is the development of parasites resistant to miltefosine, as has already been reported in human medicine, but so far no resistance has been reported in naturally infected dogs (Mondelaers *et al.,* 2016, Reguera *et al.,* 2016). The recommended dose of miltefosine as monotherapy is 2.0 mg/kg per day for 30 days (Noli *et al.,* 2014). Iarussi and collaborators (2020) carried out a pilot study, where they used the oral administration of Miltefosine at a dose of 1.2 mg/kg, once a day, for 5 days, followed by a dose of 2.5 mg/kg, once a day, for 25 days in combination with oral allopurinol at a dose of 10 mg/kg twice daily

for 6 months. Concerning the reduction of parasite load, recurrences and improvement of clinical and pathological parameters, the results show that the proposed protocol has a good level of safety and tolerability similar to or tends to have better efficacy than the standard protocol (Iarussi *et al.,* 2020).

Allopurinol

This substance was prescribed for the treatment of gout in humans (Sivera *et al.,* 2014), but in 1974 it was first described as having anti-leishmanial activity (Pfaller and Marr, 1974). Allopurinol or 4-Hydroxypyrazolo(3,4-d) pyrimidine (Zyloric®) is a purine analogue used as a xanthine oxidase inhibitor to reduce serum urate concentration. This enzyme is important in the parasite's purine recovery pathway, converting dephosphorylated purines into nucleoside monophosphates (Chawla and Madhubala 2010). Allopurinol is metabolized by *Leishmania* to inosine, which is incorporated into the RNA of the parasites, leading to the synthesis of abnormal messenger ribonucleic acid (mRNA) and consequently to the synthesis of aberrant proteins, thus inhibiting parasite replication (Plumb *et al.,* 2011, Lamoureux *et al.,* 2016). However, as this is not the only purine rescue route from the parasite, Allopurinol has a limited leishmanicidal effect (Chawla and Madhubala 2010) and is considered a leishmaniostatic. This is why, although allopurinol is sometimes used as a monotherapy, its effectiveness is questioned, since never truly eliminates the parasite from the host (Miró *et al.,* 2011, Miró and López-Vélez, 2018). For this reason, therapeutic guidelines recommend the use of allopurinol in combination with meglumine antimoniate or miltefosine. Allopurinol has low toxicity, being reported that it helps to reduce proteinuria and does not cause liver or kidney damage. However, it induces the formation of xanthine crystals in almost all dogs and sometimes urolithiasis, which in turn can lead to postrenal azotemia and, occasionally, renal failure (Meireles, 2008, Torres *et al.,* 2011, Noli and Saridomichelakis, 2014, Reguera *et al.,* 2016). When used in monotherapy, allopurinol results in the remission of clinical signs and improvement of clinicopathological changes, associated with a decrease in parasite load (Saridomichelakis *et al.,* 2005). There also has been reported resistance to allopurinol in dogs, especially after disease relapse (Yasur-Landau *et al.,* 2016). The METK gene, which encodes S-adenosylmethionine synthetase in *L. infantum*, appears to be linked to this resistance (Yasur-Landau *et al.,* 2018).

Amphotericin B

Amphotericin B has fungicidal and leishmanicidal activity, due to its interaction with a complex of 24 sterols, mainly ergosterol and episterol, abundant in the plasma membrane of fungi and *Leishmania* (Oliva *et al.,* 2010). Its mechanism of action leads to the generation of pores in the cell membranes of the parasites, through the binding on ergosterol Then, there is a change in membrane permeability (allowing the passage of cations and anions) that causes metabolic disturbance and cell lysis (Ramos *et al.,* 1996). As it also has an 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, putting dogs that already have renal pathology at risk (Baneth and Shaw, 2002, Miró *et al.*, 2008). Some studies have shown that dogs treated with this drug showed a rapid improvement in clinical signs, similar to the administration of antimony compounds (Oliva *et al.,* 1995). In contrast, other studies have shown that it causes several relapses and the need to monitor renal function during therapy. According to Noli and collaborators (2005) there is not sufficient evidence to recommend the administration of this drug to dogs.

1.2.9. Control measures

Environmental control

Preventive measures are essential to avoid infection in dogs, but also is a matter of public health. According to Miró and coworkers (2017) there are additional measures that involve the control and reduction of sandflies present in the dog environment. These measures consist of using nets on windows and doors with a mesh size between 0.3 and 0.4 mm^2 (Molina *et al.,* 2006, Miró *et al.,* 2017), removing sandfly breeding sites in sites close to inhabited areas (Sharma *et al.,* 1993¸ Miró *et al.,* 2017), application of residual insecticides or screens impregnated with permethrin in homes and respective surroundings, mainly in hyperendemic areas (Basimike and Mutinga 1995, Quinnell and Courtnay 2009, Miró *et al.,* 2017) and lastly, keeping dogs indoors from dusk until dawn during the high-risk season, which in the Mediterranean basin takes place between March and November (Rossi *et al.,* 2008, Martin-Sanchez *et al.,* 2009, Gálvez *et al.,* 2010, Otranto and Dantas-Torres 2013, Alten *et al.,* 2016, Miró *et al.,* 2017).

Some natural compounds are known to be insect repellents. However, its activity against sandflies is generally unknown. These products have not been tested on dogs, and the duration of repellency is considered very limited (Sharma *et al.,* 1993). Neem oil or lavender lotions tested against human kala-azar vectors showed protection for only 7 h. Citronella (lemon grass) extract and geraniol were also tested with very low repellent efficacy against sandfly bites (Muller *et al.,* 2008).

Vector control

Transmission of metacyclic promastigotes to dogs occurs during the *phlebotomine* bite. Thus, preventive measures taken to interrupt the transmission of the parasite to the vertebrate host include the use of topical insecticides, belonging to the group of synthetic pyrethroids, such as permethrin and deltamethrin (Miró, 2008) impregnated collars, spot-on, and sprays (Miró *et al.,* 2017). They have two important actions: a repellent action, which prevents the vector insect from approaching the vertebrate host, preventing blood ingestion, and an insecticidal action, which leads to the death of the sandfly that has managed to feed on the canid (Miró, 2008).

The mechanism of action of these synthetic pyrethroids involves two main aspects: sandflies that remain on the dog's skin long enough will absorb a lethal dose of insecticide, and those that have only had brief contact with insecticide-treated skin can still be affected by irritation and disorientation, which results in reduced rates of blood-feeding (Killick-Kendrick *et al.,* 1997). The active ingredients in these necklaces act against several species of sandflies present in Europe, Asia, and South America. Widespread use in dogs as a control measure significantly reduced the canine and human seroprevalence of leishmaniasis. Population protection rates ranged from 50% to 86%. Collars impregnated with 4% deltamethrin (Scalibor®) have a great effect against *P. perniciosus* and kill up to 60% of insects within 2 hours of exposure. The use of these collars prevents about 80% of sandfly bites (Maroli *et al.,* 2010). Due to the slow release of the insecticide from the collar, activity is reached one to two weeks after its application (Maroli *et al.,* 2010, Molina *et al.,* 2012). Collars impregnated with 4.5% of flumethrin and 10% imidacloprid (Seresto®) were tested in a kennel in a hyperendemic area in southern Italy and showed effective preventive effects against *L. infantum* infection in puppies under 6 months of age (Otranto *et al.,* 2013, Brianti *et al.,* 2014). Currently, Seresto®

is used to help reduce the risk of infection with *Leishmania infantum*, with its effect lasting up to 8 months.

Spot-on

These products have protective activity on the entire body surface, 24 to 48 hours after being applied. Spot-on products should be applied about a month before reaching the peak of vector activity to achieve the maximum level of protection (Otranto *et al.*, 2010). However, they have a shorter duration than collars, being effective until the twenty-first day after application (Molina *et al.*, 2006; Maroli *et al.*, 2010). It was demonstrated in dogs that the association between permethrin and pyriproxyfen (an insect growth inhibitor) had a repellent effect for *P. perniciosus* and that it lasted up to a maximum of 3 weeks (Corrales and Moreno, 2006). A combination of 10% imidacloprid and 50% permethrin (Advantix®) was developed in a topical dermal formulation as a prophylactic agent against ticks, fleas, mosquitoes, and sandflies. The insecticidal efficacy of this spot-on formulation against *P. papatasi*, *P. perniciosus* and *Lutzomyia longipalpis* bites was experimentally determined to range between 92.7% to 97.7% and confers protection for 3 weeks (Maroli *et al.*, 2010). Other spot-on formulations, with a 65% solution of permethrin (Exspot®), are effective against *P. perniciosus* and *Lutzomyia migonei* bites for 4 t 8 weeks, respectivelly (Maroli *et al.*, 2010).

Sprays

Insecticidal lotions marketed as sprays based on 65% permethrin or permitrine in combination with pyriproxyfen offer good repellent and insecticidal properties (Reithinger *et al.*, 2001, Molina *et al.*, 2001, 2006) and offer immediate insecticidal protection after its application. Its residual effect is less than that of other products and applications with 1 or 2 weeks of interval are required for protection (Molina *et al.*, 2006). The use of insecticide spray is of particular importance in cases of short exposure, or to immediately protect a dog in case of transient loss of protection from other products (during and immediately after grooming) (Dantas-Torres *et al.*, 2019).

Finally, another alternative is systemic compounds in the form of chewable tablets, usually containing isoxazolines, a new class of compounds that target the central nervous system and neuromuscular junctions of arthropod vectors, blocking the ligand-controlled chloride chan-

nels that lead to death after the blood meal (Weber and Selzer, 2016). Although these compounds are marketed as anti-flea and anti-tick systemic ectoparasiticides, several studies have demonstrated their effectiveness against sandflies. This is the case of a study using an oral dose of fluralaner (Bravecto®) that resulted in 100% mortality of *P. perniciosus* 24 hours after two applications with 28 days of interval , with a significant insecticidal efficacy (>50%) still being observed on day 84 after the application (Bongiorno *et al.,* 2019). In another study, oral administration of afoxolaner (NexGard®) resulted in insecticidal efficacy against *P. perniciosus* of 100%, 95.9%, and 75.2%, 48 hours after its application on days 1, 14, and 28, respectively. And after 72 hours showed 100% of efficacy on days 1 and 14 and 86.3% on day 28 (Perier *et al.,* 2019). Thus, a single chewable tablet provides protection between 30 (NexGard®) and 84 (Bravecto®) days. The major drawback is that while topical insecticides can act as a result of physical contact with the arthropod, systemic compounds involving arthropod biting and feeding do not prevent infection but only transmission (Jongejan *et al.,* 2016).

1.2.10. Immunotherapy

Currently, immunomodulatory therapies using immunosuppressive or immunostimulating drugs together with antileishmanial drugs are used aiming to obtain a more sustained clinical improvement with a low dose of anti-*Leishmania* drugs, thus minimizing adverse effects and reducing the risk of developing resistance. Immunodepressive drugs, such as glucocorticoids (namely prednisone and prednisolone) have been used in dogs with lesions resulting from the deposition of immune complexes. These drugs act by decreasing humoral immunity, consequently decreasing the production of antibodies and the formation of immune complexes (Alvar *et al.,* 2004). Immunostimulant drugs are used to activate cellular and macrophage immunity (Alvar *et al.,* 2004), such as anhydrous magnesium-ammoniophospholinoleate-palmitoleate protein aggregate (P-MAPA) and domperidone (Leishguard®) used in prophylaxis and CanL therapy. Domperidone is a dopamine antagonist, which blocks dopaminergic D2 receptors, causing the release of serotonin which, in turn, stimulates the production of prolactin. Prolactin, in addition to stimulating milk production, also plays an important role in the $CD4^+$T helper 1 (Th1) immune response, leading to the secretion of IL-2, IL-12, IFN-γ and

TNF-α, which directs the activation of NK cells and MΦ initiating a cellular immune response (Oliva *et al.*, 2010; Noli and Saridomichelakis, 2014). Domperidone is often used for prevention, as it induces the activation of phagocytes that persist for at least one month after administration. It can also be used as a medication in the early stages of the disease, aiding the remission of clinical signs and decreasing Ab titers although without achieving a complete parasitological cure. It has the advantages of oral administration and can be used in animals with renal failure (Gómez-Ochoa *et al.*, 2009). In turn, P-MAPA induces stimulation of cellular immunity, reducing clinical signs, decreasing the parasite load on the skin and IL-10 serum levels and increasing IL-2 and IFN-γ (Gómez-Ochoa *et al.*, 2009, Santiago *et al.*, 2013, Miró *et al.*, 2017).

The decision of administer immunotherapeutic agents must be made recognizing that sick dogs may have depleted T cell response, therefore immunomodulatory therapy must always be done in conjunction with antileishmanial therapy since by itself immunotherapy may present a limited efficacy when the parasite load is high (Miró *et al.*, 2017).

1.2.11. Euthanasia and Alternatives

There is no justification for mandatory euthanasia of infected or sick dogs (Otranto and Dantas-Torres, 2013). In addition to being ethically questionable, several studies have demonstrated a lack of efficacy since euthanasia of infected dogs does not result in the efficient control of the human VL source. In China and Brazil, the massive slaughter of dogs did not change the incidence of human VL. In China, this measure is currently not applied and it is no longer mandatory to euthanize sick dogs, although cases must be reported and treatment controlled by a veterinarian (Wang *et al.*, 2011). Since in many environments, the dog is not the only species that serves as a potential reservoir for *L. infantum* (Millán *et al.*, 2014, Molina 2012). WHO recommends mandatory euthanasia for stray or feral dogs, and veterinary treatment and follow-up of *L. infantum* -positive dogs (WHO, 2010). The World Companion Vector-Borne Diseases Forum recommended the following measures:

1. Companion animals must be protected from sandfly bites to prevent leishmaniasis infection or parasite spread from infected dogs. Additional control measures, including

environmental vector control, vaccination, and prophylactic drugs (Wylie *et al.,* 2014, Miró *et al.,* 2017) should be used when available.

2. The slaughter of dogs in areas where VL is endemic should be replaced by alternative non-terminal measures that can prevent infection in dogs.
3. Improve the general health and nutritional status of dogs
4. Implement the latest concepts on the clinical procedure in canine leishmaniasis, including diagnostic and treatment approaches.
5. Improve environmental and housing conditions to provide greater sandfly control and reduce human exposure to vectors (Dantas-Torres *et al.,* 2019)

1.2.12. Immunoprophylaxis

Protective and effective vaccines constitute an essential tool for the prevention of infectious diseases and, therefore, are important for the prevention of CanL. Immunity to all *Leishmania* species, as intracellular pathogens, depends on the production of a Th1-type cellular immune response, including IL-12 production by DC and MΦ, efficient antigen presentation, and subsequent IFN-γ production by T cells (Kaye and Aebischer, 2011, Engwerda and Matlashewsks, 2015).

The ability of a vaccine to maintain a low parasite load is essential to limit transmission among dogs, other animals and people. The cumulative work of many research groups has led to multiple vaccine/adjuvant combinations with the potential to induce a cell-mediated immune response against *Leishmania* infection (Palatnik-de Sousa 2008). CanL vaccines have been tested and licensed in Brazil and Europe, but there is still a lack of effective vaccines available worldwide (OIE, 2018). In the last decades four vaccines for CanL prevention are or have been commercialized: Leishmune® and Leish-Tec® in Brazil and CaniLeish® and LetiFend® in Europe (Velez *et al.,* 2020).

In 2004, Leishmune® was the first licensed vaccine for CanL in Brazil. It is a 2nd generation vaccine made of fucose-mannose ligand (FML) from *L. donovani* and the ajuvant saponin (Velez *et al.,* 2020). The vaccination protocol consisted of three doses, administered subcutaneously, every 21 days in dogs younger than 4 months, followed by annual boosters (Ceva, 2019). Studies have demonstrated a selective profile for $CD8^+$ T cells (Araujo *et al.,* 2008,

2011) as well as increased levels of IFN-γ, nitric oxide and anti-*L.donovani* IgG2 (Araujo *et al.*, 2009, Araujo de Lima *et al.*, 2010, Araujo *et al.*, 2011, Moreira *et al.*, 2016). Vaccination also induced a protective innate immune response profile through stimulation of neutrophils and monocytes (Araujo *et al.*, 2008, Moreira *et al.*, 2016). In 2014, the Brazilian Ministry of Agriculture removed the production and marketing license for this vaccine, due to the lack of efficacy in phase III trials (Ministry of Agriculture, Livestock and Supply, MAPA, 2014).

Currently, the only vaccine authorized in Brazil is Leish-Tec®, which was licensed in 2007. Leish-Tec® is formulated with *L. donovani* amastigote A2 recombinant protein and adjuvanted with saponin. This vaccine is to be administered to dogs aged 4 months or older and consists of taking 3 doses, subcutaneously, with an interval of 21 days between doses and annual boosters (Ceva, 2019). Leish-Tec® in dogs experimentally infected with *L. infantum* developed a partially protective immune response against CanL, showing positive medullary parasitism 9 months after challenge (Fernandes *et al.*, 2008). A more recent study on dogs living in a highly endemic area did not show great efficacy of the vaccine in inducing clinical protection, as 43% of vaccinates developed the disease over time (Grimaldi *et al.*, 2017).

Only in 2011 was the first vaccine licensed in Europe, CaniLeish®, commercialized by VIRBAC laboratories, composed of secretion-excretion proteins of *L. infantum* (LiESP) and purified extract of *Quillaja soponaria* (QA-21) as adjuvant (Lemesre *et al.*, 2007, European Medicines Agency, 2010). The vaccination protocol is identical to those described above, subcutaneous administration of three doses with intervals of 21 daysfollowed by the annual booster for dogs older than 6 months (Virbac, 2011). The vaccine directs the Th1 immune response (Moreno *et al.*, 2014). The number of specific lymphocytes and the leishmanicidal capacity of MΦ increase after 21 days of the last dose (Moreno *et al.*, 2014). This response remained effective for one year. Infected vaccinated dogs show slower progression and a less severe form of the disease. It has an efficacy of 68.4% in preventing the development of clinical signs (Oliva *et al.*, 2014). Vaccinated dogs also have a lower bone marrow parasite load compared to unvaccinated animals. However, the commercialization of this vaccine was discontinued.

LetiFend® (Leti Laboratories, Spain) vaccine was licensed in Europe in 2016, being the most recent vaccine against CanL (European Medicines Agency, 2016). It is a recombinant vaccine containing a chimeric protein (Q protein) formed by five antigenic fragments of four different

proteins from *L. infantum* (ribosomal proteins LiP2a, LiP2b, LiP0 and the histone H2A). This is also the only vaccine that did not have the addition of an adjuvant (Carcelén *et al.,* 2009; Dantas-Torres *et al.,* 2020). The vaccine is for dogs aged 6 months and over, administered in a single dose followed by annual booster. This vaccine does not protect the infection but protects against the development of the disease from the 28th day after vaccination and has prevention effectiveness of 72% (Leti, 2016). In a large-scale study, this vaccine proved to be safe and effective in the active immunization of uninfected dogs, reducing the risk of developing CanL after natural infection by *L. infantum* (Wylie *et al.,* 2014, Cotrina *et al.,* 2018).

Vaccination should be considered as part of a comprehensive control program for CanL, as well as the concomitant application of sandfly repellents in vaccinated dogs to decrease potential transmission (Miró *et al.,* 2017).

1.3. Immune Response

Immunity refers to the set of immune system (IS) strategies that promote protection against external agents. The IS is constituted of organs, tissues, cells and immune mediators that develop integrated functions against the attack of pathogenic microorganisms, defending immune homeostasis. The coordinated reaction of the IS is known as the immune response (Nylén and Gautam, 2010) against infectious agents and relies on innate and adaptive immune responses.

The innate immune response represents the bodymin.s first line of defense and includes anatomical and physiological barriers, phagocytic cells (neutrophils and monocytes/MΦ), NK cells, APC such as DC, and inflammatory mediators, as is the case of the complement system. Innate immune cells have pattern recognition receptors (PRR) to recognize structures of pathogens, the so-called molecular patterns associated with pathogens (PAMPs) and molecular patterns associated with tissue damage (DAMPs). The main PAMPs are polysaccharides, mannose residues and teichoic acids that can be found on the surface of microorganisms and activate innate immune cells, promoting phagocytosis and inducing the secretion and release of inflammatory mediators (Cruvinel *et al.,* 2010, Male, 2014). Although the innate immune

response can fight against many pathogens, preventing infection establishment, microorganisms have evolved and adopted strategies to evade this nonspecific response. Therefore, the mammals had to adapt and develop other forms of combat, thus giving rise to adaptive immunity (Arosa *et al.,* 2008; Nylén and Gautam, 2010).

Adaptive immunity is based on defensive strategies specific to each pathogen, being able to recognize and selectively eliminate different microorganisms and antigens, keeping the memory of the microorganism antigen that had triggered the immune response. The components of the adaptive IS are B and T lymphocytes, Ab and several soluble immune mediators (Santos-Gomes *et al.,* 2008).

1.3.1. Innate Immunity

The immune response is initiated after inoculation of the promastigote forms of *Leishmania* in the dermis. Promastigotes interact with serum components, activating the complement system, both via the classical and alternative pathways. All these pathways result in the common activation of the C3 convertase that cleaves C3 to generate C3b. Deposition of C3b on the surface of *Leishmania* induces the deposition of C5b-C6-C7-C8-C9, assembling the membrane attack complex, (MAC) that promotes the lysis of *Leishmania* promastigotes. However, virulent *Leishmania* expresses protein kinases that phosphorylate C3, C5 and C9 components, inhibiting complement activation (Hermoso *et al.*, 1991, Nunes *et al.,* 1997, Costa-da-Silva *et al.,* 2022). Parasite opsonization plays a major role in promoting phagocytosis through the binding of C3b, which results from the cleavage of C3, to MΦ complement receptors 1 and 3 (CR1 and CR3). In contrast, *Leishmania* parasites have the cell surface covered by the glycoprotein of 63 kDa (gp63), which can convert C3b into its inactive form (C3bi). C3bi binds to MΦ-CR3 preventing complement-mediated lysis promoting parasite phagocytosis and avoiding MΦ activation. In this way, complement-*Leishmania* interaction favours the establishment of infection and parasite survival inside the MΦ phagolysosome (Liese *et al.,* 2008).

Neutrophils

PMN constitute the majority of leukocytes in peripheral blood, representing 60 to 75% of the total leukocytes in an adult dog. After leaving the bone marrow where are produced, circulate in the bloodstream with a lifespan of a few days (Borregaard 2010; Tizard, 2012). They are the first cells to migrate from vessels to tissues, representing an important step in the early stages of the inflammatory reaction. PMN migration is induced by the expression of chemotactic agents such as complement products (C3a and C5a), chemokines (IL-8 / CXCL-8, cytokines (TNF-α), lipids, as is the case of platelet-activating factor (PAF) and leukotriene B4 (LTB4), and bacterial substances such as formylated peptides (fMLP) and lipopolysaccharide (LPS). In *Leishmania* infection, the *Leishmania* chemotactic factor (LCF) has been identified as a recruitment factor of PMN (Forsberg *et al.*, 2001). The interaction of LCF with lipoxin A4 (LXA) chemotactic receptor favours the silent internalization of *Leishmania* by phagocytes, contributing to the intra-phagocytic survival of the parasite (Ritter *et al.*, 2009). Depending on the species of the parasite and the host's innate immunity, PMN can protect or fight pathogens. When PMN phagocyte *Leishmania* promastigotes and become infected, they can be phagocytosed by MΦ, releasing the parasites inside these cells without promoting their activation. MΦ is the definitive *Leishmania* host cell ensuring the differentiation of the parasite in its intracellular form, the amastigote, and allowing several cycles of amastigote replication (Costa *et al.,* 2013; Freitas and Pinheiro, 2010, Cunha *et al.,* 2020). The mechanism of infection of MΦ via infected neutrophils is commonly called the "Trojan Horse" (Freitas, 2010). Previous studies have shown *L. major* parasites being released from apoptotic mouse neutrophils in the vicinity of surrounding MΦ (Peter *et al.,* 2008.), facilitating the access of the parasite to MΦ and enabling phagocytosis. This mechanism was called the "Trojan rabbit". PMN can have a protective role against leishmaniasis, since this cell has intracellular and extracellular antimicrobial mechanisms, such as the production of ROS, enzyme-rich granules, and can emit traps (neutrophil extracellular traps, NET) in a process known as NETosis (Carneiro, 2021). NETosis is a process in which the neutrophil emits an extracellular network of sticky fibres, which are mainly constituted of chromatin and proteolytic enzymes that restrain and inactivate pathogens. An *in vitro* study showed that neutrophils from mice exposed to *L. guyanensis*, *L. shawi* and *L. amazonensis* produced superoxide, released enzymes into the extracellular space and generated NETs. However, *L. guyanensis* and *L. shawi* inhibited enzymatic activity while *L. amazonensis* reduced NET emission, pointing to the

modulation of extracellular neutrophils effector mechanisms by cutaneous *Leishmania* species (Valério-Bolas *et al.*, 2018).

Santos-Gomes and collaborators (2000) showed that 3 - 4 hours after dermal experimental inoculation, *L.infantum* promastigotes already had been internalized by canine neutrophils, proving the early involvement of these cells with *Leishmania* parasites. Recently, an *in vitro* study demonstrated that *L.infantum* parasites internalized by dog neutrophils maintained their viability and replication capacity (Pereira *et al.*, 2017) and, therefore, the ability to infect MΦ (Pereira *et al.*, 2019). Although neutrophil produces NO, this may be related to the increase in *L. infantum* -induced neutrophil necrosis (Pereira *et al.*, 2017) and the subsequent efferocytosis of necrotic neutrophils. The transfer of *L. infantum* from neutrophils to MΦ is well established in the domestic dog according to the study performed by Pereira and colleagues(2019) as had already been proven by Peters *et al* (2008) and Van Zandbergen *et al.*, (2004) in *L. major* murine model.

Monocytes/Macrophages

Monocytes temporarily circulate in the blood, evolving into MΦ as they migrate to tissues. When monocytes evolve to MΦ, there is an increase in their phagocytic capacity and the number of lysosomes carrying hydrolytic enzymes (Arosa *et al.*, 2008). Moreover, MΦ also presents foreign antigens to lymphocytes, being professional antigen-presenting cells (APC) (Santos-Gomes *et al.*, 2008).

After inoculation of promastigotes into the host's dermis, MΦ is the second line of defense to be recruited to the infection site. The promastigote surface proteins [lipophosphoglycan (LPG), gp63 and proteophosphoglycan (PPG)] are crucial for phagocytosis and subsequent intracellular survival of the parasite (Liu and Uzonna, 2012). *Leishmania* binds to surface molecules of MΦ, such as RC1 and RC3 (MAC-1), fibronectin and mannose (MR) receptors, followed by phagocytosis (Fig. 6). Once in the mammal, the parasites need to adapt to the mammal's temperature (37ºC) and, after being internalized by MΦ, to the acidic and enzyme-rich environment of the phagolysosome, acquiring the amastigote form to survive and replicate (Lamour *et al.*, 2012). In the presence of the parasite, MΦ may be activated, triggering leishmanicidal mechanisms, leading to parasite death, or they may not be activated, allowing the parasite to survive, replicate and spread to other cells and tissues. Classically activated MΦ (CaMΦ) by stimulation of Th1-type cytokines such as TNF-α, IL-12 and IFN-γ generate

reactive nitrogen and ROS species, including NO and its derivatives, such as nitrogen dioxide (NO_2), nitrous acid (HNO_2) and peroxynitrite ($ONOO^-$) through the action of inducible NO synthetase (iNOS). ROS production, such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) require L-arginine, oxygen and the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, during a reaction catalyzed by NO synthetase (NOS) (Liese *et al.*, 2008). In this case, the infected MΦ can control the parasite's fate (Horta *et al.*, 2012). In contrast, MΦ exposed to IL-4, IL-13 and IL-21, considered Th2-type cytokines, stimulate arginase transcription and subsequently the generation of polyamines via the L-ornithine pathway, which supports parasite replication. These MΦ are considered to be activated by the alternative pathway (AaMΦ) (Lamour *et al.*, 2012).

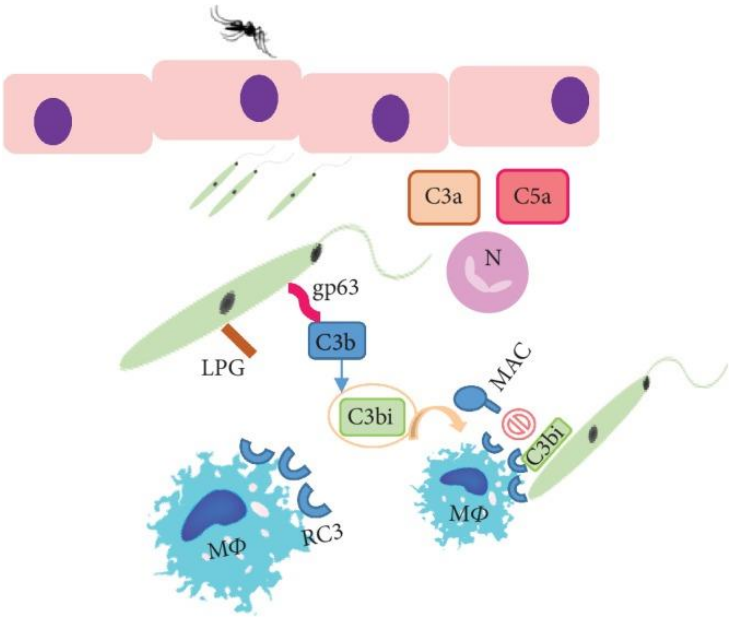


Figure 6. Activation of the complement cascade. The 63 kDa surface glycoprotein (gp63) can convert C3b to the inactivated form (C3bi), which prevents the assembly of the lytic complex (MAC) on the parasite surface. In turn, C3bi binds to the MΦ receptor (RC3), promoting rapid phagocytosis of the parasite (Gabriel *et al.*, 2019, *https://doi.org/10.1155/2019/2603730*, *Copyright/License*)

iNOS uses the substrate L-arginine to produce NO, the same substrate utilized by arginase to produce ornithine and urea (Wanasen and Soong, 2009). Ornithine in turn is the immediate precursor of the synthesis of polyamines essential for *Leishmania* growth. Thus, the balance between iNOS and arginase activity determines whether the intracellular environment is microbicidal or whether it is favourable to parasite survival and replication (Gaur *et al.*, 2007).

The iNOS enzyme is positively regulated by Th1-type cytokines, IFN-γ and TNF-α, toll like receptors (TLR) and by ligands such as LPS.

Studies carried out *in vitro* suggested that iNOS has high importance in the regulation and in the effective process of controlling the intracellular replication of *Leishmania* amastigote forms (Freitas *et al.,* 2010). Canine MΦ infected *in vitro* and incubated with lymphocytes from previously immunized dogs showed an increase in NO production, after the release of IFN-γ, as well as MΦ apoptosis (Holzmuller *et al.*, 2006). In *in vivo* studies, in which human and mouse samples were used, it was suggested that iNOS expression by activated MΦ was the leading effector mechanism in the control of leishmaniasis (Serarslan and Atik, 2005). Another recent study with *L. infantum* -infected canine MΦ showed that the initial activation of MΦ was subverted by *L. infantum* parasites, that lead to parasite silencing and reduction of MΦ microbicide defense, as well as prolonged lifespan of infected cells (Rodrigues *et al.,* 2022). Moreover, according to Poulaki and collaborators (2021) *Leishmania* can manipulate the hypoxia-inducible factor 1 pathway in MΦs, which enhances glucose availability and drops immune response, favouring parasite survival and ultimately prolonging the lifespan of the infected cell.

Pattern Recognition Receptors

Toll-like Receptors

The function of TLRs in innate immunity is to bind to antigens (ligands), induce antimicrobial activity and the production of pro-inflammatory cytokines (Vidya *et al.,* 2017; Li *et al.,* 2010). Furthermore, TLRs activate APCs, linking innate and adaptive immunity and coordinating T cell and B cell responses (Iwasaki and Medzhitov, 2010).

The TLR family are transmembrane molecules that recognize common PAMP, including endosomal and surface compartment components, such as LPS, peptidoglycans and nucleic acids (unmethylated CpG dinucleotides) as well as DAMPs. These sensors are type I transmembrane proteins that contain three domains: an extracellular portion with leucine-rich repeats (LRRs), a transmembrane domain, and an intracellular portion with a Toll-Interleukin 1 receptor (TIR) domain that represents a conserved model of intracellular signalling. LRRs are responsible for recognizing pathogens, while the TIR domain interacts with signal transduction adapters and initiates signalling (Takeda *et al.,* 2003; Nie *et al.,* 2018).

Currently, ten TLRs are described for humans and thirteen for mice, and TLR10 is not functional in mice (Wang *et al.*, 2015). TLR1, TLR2, TLR4, TLR6 and TLR11 are associated with the cell membrane, while TLR3, TLR7 and TLR9 are associated with the membranes of endocytic compartments, such as the endosome, lysosome and endoplasmic reticulum (Tuon *et al.*, 2008). Members of the TLR family are expressed on innate immune cells, including MΦ, NK cells, DCs, and circulating leukocytes such as monocytes and neutrophils (Kawai and Akira, 2011, Prince *et al.*, 2011), as well as on adaptive immune cells, such as T and B lymphocytes, and in non-immune cells, as is the case of fibroblasts and epithelial and endothelial cells (Delneste *et al.*, 2007). TLR1, TLR2 and TLR6 recognize lipo-, glyco- and acyl-peptides. TLR4 recognizes LPS and also endogenous ligands, such as heat shock proteins and the extracellular matrix components, including fibronectin, hyaluronic acid and sulfated heparin in response to injured tissues. The subfamily of nucleic acid sensors TLR3, TLR7 and TLR9 recognize nuclear material, eg. single-stranded RNA (ssRNA), double-stranded RNA (dsRNA) and double-stranded DNA (dsDNA). TLR adapter molecules are myeloid differentiation protein 88 (MyD88), TIR (domain-containing adapter protein TIRAP, also known as MAL), IFN-β-induced TIRAP (TRIF), and TRIF adapter-related molecules (TRAM) (Smith, 2014). These adapters are used in various combinations of TLRs, but the signalling pathways can be MyD88-dependent or independent (Faria *et al.*, 2012). Generally, the TRIF-dependent pathway is considered specific for only a few TLRs, such as TLR3 and TLR4 in mammals, but TLR4 also signals via the MyD88 pathway (Akira, 2001).

After activation by PAMPs or DAMPs, TLR dimerization occurs and the MyD88 binds to the TIR domain of TLR via homotypic/heterotypic interactions. Subsequently, receptor-associated IL-1 kinase 4 (IRAK4) is recruited through the MyD88 death domain, leading to the formation of a myddosome complex (Lin *et al.*, 2010), and autophosphorylation of IRAK1. Then TNF associated with receptor factor 6 (TRAF6) is activated, which in turn activates the TAK1/TGF-β-activated kinase complex (TAB) via K-63-linked polyubiquitination of TAK1 and TRAF6 (Gorjestani *et al.*, 2012). This is followed by IκB kinase (IKK)-mediated phosphorylation and degradation of I kappa B alpha (IκBα). The degradation of this inhibitor finally leads to the nuclear translocation of the nuclear factor κB (NF-κB) (Wang *et al.*, 2001), which induces the transcription of genes encoding pro-inflammatory cytokines. Each transcription factor is responsible for the transcription of specific genes that encode different sets of proteins, such as pro-inflammatory cytokines [TNF-α, IL-1β and IL-6] and type I interferon

(IFN-α, IFN-β), chemokines (CXCL8 and CXCL10), and antimicrobial peptides, and influence the generation of ROS and nitrogen (RNS) species, increase cell survival, and phagocytosis (Kawai and Akira, 2011, Hayashi *et al.*, 2003).

TLR responses to *Leishmania* ligands play a protective role, but may also serve to promote infection (Faria *et al.*, 2012). Although *Leishmania* species express several PAMPs, some parasite-derived molecules have been reported to activate TLR receptors and most studies to date have focused on the activation of TLR2, TLR3, TLR4 and TLR9 (Hayashi *et al.*, 2003, Faria *et al.*, 2012). The reported *Leishmania* ligands are phospholipids glycoinositol (GIPL) and LPG, made up of repeating units of phosphorus disaccharides that are attached to the outer surface of the plasma membrane by the GP anchor.

Flandin and collaborators (2006) demonstrated with *in vitro* studies that TLR2 and TLR3 of *L. donovani* infected MΦ are involved in the immune response, specifically in the production of TNF-α and NO, both are important for protective immunity against *Leishmania*. Furthermore, transfection studies (293T cells transfected with various TLRs) demonstrated that TLR2 is the receptor for *Leishmania* LPG (de Veer *et al.*, 2003). Becker and colleagues, (2003) with studies in human neutrophils suggested that TLR2 can bind directly to LPG to promote signaling events, and more recently Srivastav and collaborators, (2012) demonstrated that anti-TLR2 antibody can inhibit NF-κB induced by LPG signaling events. Taken together these studies indicate LPG as the direct ligand for TLR2.

Glycoproteins and glycosphingo phospholipids from *L. donovani* have been suggested as potential ligands for TLR4 based on their ability to induce effector proteins such as ROS and IL-12 (Karmakar *et al.*, 2012, Paul *et al.*, 2012). According to Kropf and coworkers (2004), $TLR4^{-/-}$ mice are highly susceptible to infection by *Leishmania* spp. showing increased parasite load.

The parasite load of *L. amazonensis* infected C57BL/6 $TLR2^{-/-}$ mice was reduced when compared to the highly susceptible C57BL/6 mice (Guerra *et al.*, 2010). *In vitro* studies carried out by Nogueira *et al.*, (2015) showed that peritoneal MΦ isolated from a clinical case of ADCL exposed to *L. amazonensis* were able to activate TLR4. TLR4 was observed not only in the ACL clinical form but also in the other clinical forms like LCL and borderline dissem-

inated cutaneous leishmaniasis (BDCL) caused by *L. amazonensis* although without differences in its expression. Also recently, it has been shown that *L. amazonensis* promotes its survival by inducing the expression of CD200, an immunoregulatory molecule that inhibits MΦ activation (Liu *et al.*, 2012, Cortez *et al.*, 2011, Costa-da-Silva *et al.*, 2022). This same situation was observed in TLR2$^{-/-}$, TLR3$^{-/-}$ and TLR 4$^{-/-}$ MΦ, but not in MyD88$^{-/-}$, TRIF$^{-/-}$ and TLR9$^{-/-}$ MΦ. When these MΦ have been treated with TLR agonists a strong increase in TLR9 activation was observed, suggesting that TLR9-mediated signaling is required to induce CD200 by *L. amazonensis* (Sauter *et al.*, 2019). This may explain the greater interaction of TLR9 in the *L.amazonensis* BDCL and ACL clinical forms.

Recent *in vitro* studies performed with canine liver spheroids in contact with *L. infantum* promastigotes show TLR2 upregulation, confirming that this innate immune receptor has a non-negligible role in *Leishmania* infection, at least in the early stages of infection (Rodrigues *et al.*, 2020). Another *in vitro* study performed by Rodrigues and collaborators (2022) on canine blood MΦ revealed a strong upregulation of TLR4 during the first 3 h of *L.infantum* infection. In turn, canine Kupffer cells exposed to *L. infantum* axenic amastigotes, despite having lower levels of PRRs than blood MΦs, TLR2 and TLR4, were upregulated. This evidence corroborates previous findings that *L. infantum* parasites induce the expression of more than one TLR in dog MΦ (Figueiredo *et al.*, 2014). In turn, Hosein and colleagues (2017) described an increase in TLR2 transcription and downregulation of TLR9 in all liver tissue of dogs experimentally infected with *L. infantum*. Together, these finding points to a very significant role for TLRs in signaling *Leishmania* infection in dogs.

Nod-Like Receptors

Nucleotide-binding leucine-rich repeat-containing proteins (NLRs) are one of the crucial components of innate immunity, recognizing endogenous and exogenous cytoplasmic ligands. To date, 22 NLRs have been identified in humans and 34 in mice. They have an N-terminal effector domain required for signal transduction, a central nucleotide-binding domain (NOD/NBD, also known as NACHT domain) involved in protein oligomerization, and a C-terminal LRR-rich domain essential for the detection of PAMPs and DAMPs (Moreira and Zamboni, 2012). The NOD domain contains a proximal helical 1 domain (HD1), a distal helical 2 domain (HD1) and a winged helical domain (WHD).

Based on distinct N-terminal effector domains, including transactivation domain (AD), baculovirus inhibitor repeats (BIRs), caspase recruitment domain (CARD) or pyrin domain (PYD), NLRs are divided into four subgroups: NLRA, NLRB, NLRC and NLRP. The Class II molecules of major histocompatibility complex transactivator (CIITA), the only member of the NLRA, are involved in anti-apoptotic functions and transcription activation of MHCII through its intrinsic acetyltransferase (AT) activity. The NLRB subgroup contains only a single member in humans, the NLR family apoptosis inhibitor protein (NAIP), and seven members (NAIP1-7) in the mouse.

The NLRC subgroup includes NOD1 and NOD2, which are well-characterized PRRs that recognize bacterial peptidoglycan. They detect γ-d-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively.

NLRs recruit and activate the inflammatory protease caspase 1 required for the maturation process of the pro-inflammatory cytokines IL-1β and IL-18 and participate in the apoptosis cascade. However, NLRs, such as NOD1 and NOD2, do not directly engage with inflammatory caspases but trigger NF-kB, mitogen-activated nuclear protein kinase (MAPKS), IFN regulatory factors (IRFs), which stimulate innate immunity. NOD1 and NOD2 are encoded in caspase recruitment domain (CARD) 4 and 15, respectively, and like NLRs, both have NODs and share LRR domains in addition to the terminal CARD amino acid (Zhong *et al.*, 2013). Although there is a great similarity between these two NODs, NOD1 has one CARD and is detected in a variety of cells and NOD2 has two CARD and is present in monocytes, granulocytes and DC (Gutierrez *et al.*, 2002). Activation of NOD1 and NOD2 follows cytosolic recognition of peptidoglycan ligands that trigger receptor oligomerization through the NOD domain (Zhong *et al.*, 2013). For adequate activation, post-transductional modifications such as phosphorylation and ubiquitination must occur in the signalling pathway of these two receptors. These receptors are also associated with the induction of apoptosis. NOD1 is associated with caspases 8 and 9 and the RIP2 protein. Activation via NOD1 and NOD2 induces increased expression of MHCII on the surface of APC (Antosz and Osiak, 2013).

Along with its essential role in innate immunity, NOD2 plays a critical role in the adaptive immune system. MDP-induced NOD2 activation has been shown to promote Th17 cell development from memory T helper (Th) cells (Caruso *et al.*, 2014) and increase the level of IL-17 and IL-22.

Dos Santos and collaborators (2017) assessed the role of genetic variation in NOD1 and NOD2 in the immune response to *Leishmania* antigens by applying a functional genomics approach (Li *et al.*, 2016). They showed that a particular genetic variation in NOD2 (Leu1007finsC) down-regulates the production of cytokines, such as TNF-α, IL-1β, IL-6, IL-8, and IFN-γ, by monocytes exposed to *L. amazonensis* or *L. braziliensis* parasites. Notably, they further demonstrated that IL-17 production by human peripheral blood mononuclear cells (PBMC) was NOD2 dependent only after exposure to *L. amazonensis*. These findings are in agreement with the results reported by Nascimento and coworkers (2016) in *L. infantum* murine model, where a Th1 response along with IFN-γ secretion was NOD2 dependent. However, these authors also concluded that the NOD2 pathway was not relevant for Th17 production. These apparent controversial findings can be attributed to differences between *Leishmania* species as well as differences in human and mouse immune responses (Gollob *et al.*, 2014). This evidence strengthened the involvement of NOD2, in the immune responses induced by *Leishmania* spp. and also indicates that NOD2 can also shape the adaptive immune response against this parasite (dos Santos *et al.*, 2017)

A more recent study showed that canine MΦ and KCs exposed to *L. infantum* strong upregulate NOD1 during the first 3 h of infection, emphasizing the role played by NOD1 in *Leishmania* infection (Rodrigues *et al.*, 2022). The role of NODs in CanL is still unclear as previous studies considered the transcription levels in monocyte-derived canine MΦs after 24 h and 72 h of *L. infantum* infection as low or negligible (Turchetti *et al.*, 2015). These observations may reflect the effect of parasite silencing observed after 5 h of infection (Rodrigues *et al.*, 2022). Therefore, dog MΦs can initially recognize *L. infantum* parasites and transiently increase NOD1 expression, which is later silenced. KCs also activate NOD1 gene expression in the presence of the parasite, but later on (after 5 h of exposure to *L. infantum* parasites), confirming that the NOD1 cytoplasmic receptor, together with other PRRs, may be of great importance for the development of dog immune response during *L. infantum* liver infection.

1.3.2. Adaptive Immunity

Innate immunity alone is not sufficient to confer protection against *Leishmania* infection, and the development of the adaptive immune response is crucial (Mesquita *et al.*,

2010, Male, 2014). Lymphocytes (T and B cells) belong to the adaptive immune response and are found in lymphoid organs, blood, and scattered under mucosal surfaces (Tizard, 2012). The adaptive immune response, in turn, is subdivided into functional groups that represent humoral and cellular immunity. Humoral immunity involves B-lymphocytes, which synthesize and secrete Ab, while cellular immunity involves effector T lymphocytes that release immune mediators after interaction with APCs that present antigens 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 the lymphocytes in the dog's bloodstream (Tizard, 2012). B cells can develop in the bone marrow, bursa, or Peyer's patches and account for 10 to 40% of blood lymphocytes (Tizard, 2001).

T lymphocytes help in the recruitment and activation of other cell subtypes, by the production of cytokines and other soluble substances, and in the destruction of target cells in some specific cases, such as infection by viruses and intracellular parasites. These cells recognize antigens that have been processed and presented by APCs. The T lymphocyte population includes two subtypes: $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes.

After activation, $CD4^+$ T lymphocytes or Th cells migrate to the site of infection, where can activate MΦ and other immune cells to destroy pathogens. Th cells may have different phenotypes according to the cytokines they produce. Th1 lymphocytes go to the site of infection producing IFN-γ and IL-2. Together, IFN-γ and TNF-α activate MΦ and IL-12, which play a role in T cell differentiation, promote increased leishmanicidal activity (Scott–*et al.,* 2016, Blackwell *et al.,* 2018, Almeida *et al.,* 2013). $CD4^+$ T lymphocytes may not be present in lesions caused by *Leishmania* and cannot interact with all infected cells. Therefore, IFN-γ has a long-action range, stimulating NO production by the infected MΦ, thus suppressing the missing distribution of $CD4^+$ T cells (Scott *et al.,* 2016).

The Th2 profile is stimulated by IL-4. $CD4^+$ Th2 lymphocytes can produce anti-inflammatory cytokines, such as IL-4, IL-5, IL-10 and IL-13 (Almeida *et al.,* 2013), which are associated with susceptibility to *Leishmania* infection (Gomes *et al*. 2017, Cunha *et al.,* 2020, Araujo and Giorgio, 2015). Leishmaniasis studies have shown that the cytokines IFN-γ, IL-27, IL-10 and IL-6 are associated with hepatosplenomegaly, neutropenia and thrombocytopenia, and high levels of IL-6 are strongly related to death (Freitas and Pinheiro, 2010)

Th17 cells are stimulated by IL-6, TGF-β, IL-21 and IL-23, and secret IL-17 (Tizard 2012, Halwani *et al.,* 2017). This T cell subset plays a role in host defense against extracellular pathogens by mediating the recruitment of neutrophils and MΦ to infected tissues. Furthermore, aberrant regulation of Th17 cells may play a significant role in the pathogenesis of various inflammatory and autoimmune disorders (Korn *et al.,* 2009). IL-22 is also produced by Th17 cells and is particularly involved in immunity on epithelial and mucosal surfaces. IL-17 and IL-22 are pro-inflammatory cytokines that play a protective role against intracellular parasites, such as *Leishmania* (Nascimento *et al.,* 2015).

Cytotoxic T cells or $CD8^+$ T cells directly promote the death of infected cells, including cells hosting intracellular parasites. After activation through antigen complexed with MHCI $CD8^+$ T cells can shape the initial adaptive immune response to leishmaniasis by producing IFN-γ. However, this depends on the intensity of the initial infection (Vasconcelos *et al.*, 2019). According to Scott and collaborators (2016), after a high dose of *L.major* inoculum mice lesions healed in the absence of $CD8^+$ T cells but lesions caused by low doses of *L.major* were prolonged by the presence of $CD8^+$ T cells (Scott et al., 2016).

T regulatory lymphocytes

Another subpopulation of T cells is the regulatory T cells (Treg). These cells play a key role in IS regulation, maintaining immune tolerance and homeostasis, being particularly relevant in the prevention of autoimmunity (Taams *et al.,* 2006, Cortese *et al.,* 2015). Tregs are characterized by the expression of CD4, CD25 and the Forkhead box P3 (Foxp3) transcription factor, playing a key role in stabilizing regulatory properties (Tizard, 2012, Bhattacharya and Ali 2013, Ferreira *et al.,* 2019). Treg cells account for about 5% of circulating T cells and 10% of lymph node T cells (Tizard. 2012). The role of Treg cells in *Leishmania* infection is still being elucidated, although it is involved in the pathology of the disease and the persistence of the parasite depending on the experimental rodent model used. Treg cells have different roles in the regulation of CL. For example, while in *L. major* infection the differentiation of Treg cells increases mice susceptibility or is responsible for reactivation of the infection, in *L. amazonensis* Treg cells seem to participate in the control of immunopathological processes. Furthermore, research on chronic lesions caused by *L. panamensis* or *L. braziliensis* has shown that Treg cells are impaired (Scott *et al.,* 2016).

B lymphocytes

B lymphocytes are the cells protagonist of humoral response since they are responsible for the production and release of immunoglobulins (Ig) or Ab, initially of the IgM class and later of IgG, IgA and IgE (Tizard, 2012, Nera *et al.,* 2015) that have high specificity and affinity for inflammation-promoting antigen. Ab are molecules that act in the defense of the body and are produced by plasma cells formed from the differentiation of B lymphocytes. Ab presents different ways of acting against an antigen, such as neutralization and opsonization. B cells are responsible for immunological memory, since, after a second exposure to the pathogen (called secondary response), there is differentiated a pool of B cells with the ability to specifically recognize the Ag that originated the pool in the primary response, promoting a faster and more effective response (Mesquita Júnior *et al.,* 2010, Male, 2014). During the early *Leishmania* infection, it is believed that the production of antibodies does not play a role in controlling the parasite, as *Leishmania* is an intracellular parasite. However, some studies indicate that B cells can regulate protective and pathogenic immune responses during *Leishmania* infection, depending on the infecting species and the animal model used (Panahi *et al.,* 2021). The production of Ab against *L. major* proved to be important for parasites phagocytose by DCs, whereas the absence of Ab resulted in larger lesions in mice without B cells, higher parasite load, low production of IFN-γ and decreased cell-mediated immune response (Woelbing *et al.,* 2006). Other studies have reported an exacerbating role of B-cell disease during *L. donovani* and *L. amazonensis* infection (Smelt *et al.,* 2000, Wanasen *et al.,* 2008, Elmahallawy *et al.,* 2021). IgM transmembrane domain (μMT) deficient mice, which lack mature B cells, clear *L. donovani* infection faster than wild-type (WT) mice due to enhanced granuloma formation associated with increased Th1 response in the infection site (Hurdayal *et al.,* 2017). However, μMT deficient mice exhibited more severe hepatic necrosis when compared to WT mice, due to increased neutrophil recruitment.

1.4. Scape Mechanisms of *Leishmania spp.* to Immune Response

Leishmania parasites have evolved methods of escaping the host´s immune response. During the blood meal, the insect vector deposits metacyclic promastigotes on the skin of the host. Once in the dermis, the parasites are exposed to a new environment, needing to overcome a variety of obstacles to establish infection within the MΦ phagolysosomes.

The parasite's and survival strategies are mediated by virulence factors, modulating functions of the host, thus ensuring that the parasite can invade the target tissue, survive, disperse, proliferate and be transmitted, completing its life cycle (Yao *et al.,* 2003, Cuervo *et al.,* 2008).

Parasite and host proteinases affect the dynamics of *Leishmania* infection (Olivier-*et al.,* 2012, Silva-Almeida *et al.,* 2012). *Leishmania* parasites present several classes of proteinases, including cysteine proteinases, metalloproteinases, and serine proteinases (Naderer *et al.,* 2004, Silva-Almeida *et al.,* 2012). Although the exact impact of these *Leishmania* components on the mammalian host immune system is not yet fully understood, there is evidence that these components modulate the interplay between the parasite and the host's immune cells, favoring infection (Silva-Almeida *et al.,* 2012)

- **Lipophosphoglycan**

LPG is among the most abundant cell surface glycoconjugate molecules and it is mainly present in the promastigote form of the parasite (Forestier *et al.,* 2014). Promastigote LPG plays several roles, including resistance to the complement system, inhibition of oxidative burst, promote inflammation, and prevent NK cells from recognizing *Leishmania*-infected macrophages (Svarovska-*et al.,* 2010). LPG also impairs the nuclear translocation of the nuclear factor-kappa light chain potentiator in monocytes, resulting in a decrease in the production of IL-12, which may influence the host's initial immune response by modulating the DC activity, inhibiting the antigen presentation and promoting the early release of IL-4 (Liu *et al.,* 2009).

- **Glycoinositolphospholipids**

GIPLs are the predominant class of glycolipids synthesized by all developmental stages of *Leishmania* (Naderer *et al.,* 2004). These molecules are important in the survival of *L. major* within MΦ as they inhibit protein kinase C (McConville *et al.,* 19991, Brandonisio *et al.,* 2000, Silva-Almeida *et al.,* 2012). A previous study documented that inter and intraspecies

polymorphisms in GLPs and GIPLs are not only important for host interaction in Old World *Leishmania* species, but also in New World species, revealing their role as one of the main key elements for the survival of the parasite within the vector in addition to its role in modulating the host's immune response, favoring parasite infection (de Assis *et al.*, 2012, Passero *et al.*, 2015).

- **Proteophosphoglycan (PPG)**

PPG is secreted by the amastigote stage (Piani *et al.*, 1999) and belong to serine and threonine-rich proteins that are extensively modified by phosphodiester-linked phospholigosaccharides and terminal manno-oligosaccharides. The secretion of a mucin-like gel, called promastigote secretory gel (PSG), influences the catabolism of L-arginine to NO, which represents the most effective mechanisms of MΦ to kill parasites (Bogdan, 2001). However, other studies have documented that PSG targeted alternative macrophage activation through increased expression and increased activity of arginase-1, explaining the possible competition between PSG and iNOS for L-arginine. Thus, both PPG and PSG play an important role in protecting parasites from proteolytic damage, favoring *Leishmania* transmission and infection progression (Rogers, 2012, Secundino *et al.*, 2010).

- **KMP-11**

KMP-11 is an 11 kDa hydrophobic kinetoplastid membrane protein associated with LPG and which has shown immunoregulatory properties (Moody, 1993, Jardim *et al.*, 1995) in both parasite stages, increasing its expression during metacyclogenesis (Matos *et al.*, 2010). Some previous reports have suggested the involvement of KMP-11 in the following functions: parasite mobility, attachment to the host cell surface, stimulation of T cell proliferation, and cytoskeletal regulation through interaction with subpellicular microtubules (Tolson *et al.*, 1994, Mukhopadhyay *et al.*, 1998).

- **ACPs**

Acid phosphatases (ACPs) form a group of enzymes released in both forms of *Leishmania*, particularly in promastigote (Pfeiffer *et al.,* 1978, Ilg *et al.,* 1991). This membrane-bound ectoenzyme reduces oxidative burst and the production of superoxide anions by PMN and free radicals by MΦ, thus favoring the survival of parasites within the host cell (Remaley *et al.,* 1985, Chang *et al.,* 1990, Ellis *et al.,* 1998, Kane and Mosser, 2000, Stafford *et al.,* 2002).

- **Proteinases**

Proteinases are among the most important virulence factors, being responsible for the hydrolysis of peptide bonds and degradation of proteins and peptides in host cells (Barrett, 1994, Silva-Almeida *et al.,* 2012). They are classified based on their catalytic domains as serine-, threonine-, aspartyl-, metallo- and cysteine proteinases (Rawlings *et al.,* 2010). Cysteine proteinase (CP) seems to be involved in mechanisms of survival and growth of amastigotes within MΦ (North *et al.,* 1990), in addition to its intracellular degenerative action for proteins, favoring parasite survival (Silva-Almeida *et al.,* 2012). The expression of aspartyl proteinase changes between the morphological forms of the parasite, appearing to be related to the survival of the parasite in different microenvironments (Alves *et al.,* 2005). Recently, it was reported that *L. braziliensis* promastigotes express serine proteinases that have distinct subcellular distribution and expression and may contribute to the maintenance of the parasite's lifestyle at physiological pH (Santos-de-Souza *et al.,* 2019). Another study reported that the small myristoylated protein 3 is a potential virulence factor for *L. amazonensis* (Oliveira *et al.,* 2018).

The gp63 also known as leishmanolysin, was originally identified as a 63-68 kDa glycoprotein anchored to parasite membrane via a GPI anchor (Etges *et al.,* 1986, Bouvier *et al.,* 1987, Chang *et al.,* 1990, Hey *et al.,* 1994, Pandey *et al.,* 2004). This virulence factor is a zinc-dependent proteolytic enzyme (a metalloprotease) and is involved in parasite-MΦ interactions (Russell and Wilhelm, 1986). Gp63 has been identified in both stages of all pathogenic *Leishmania* species, but it is mainly found in the surface membrane of promastigotes (Colomer-Gould *et al.,*1985, Etges *et al.,*1986, Bouvier *et al.,*1987, Chang *et al.,* 1990, Ellis *et al.,* 2002). A previous study revealed that gp63 from *L. mexicana* promastigotes coming into contact with

subcutaneous tissue resulted in the degradation of several extracellular matrix proteins which in turn influence MΦ signalling mechanisms, altering cell functions and favoring parasite survival (McGwire *et al.,* 2003). Moreover, gp63 has been reported to confer protection to the amastigote form when the parasite is in the harsh phagolysosome environment of MΦ (Chen *et al.,* 2000). The proteolytic activity of this metalloenzyme on the amastigote surface protects parasite membranes from cytolytic damage, ensuring parasite replication in MΦ phagolysosomes (Chaudhuri *et al.,* 1989). gp63 is associated with protein C (PKC) serine/threonine kinase, which in turn is involved in cell proliferation, differentiation and apoptosis (Corradin et al., 1999). Through its proteolytic properties, gp63 interferes in other signaling pathways of the host cell, changing their related functions (Isnard *et al.,*2012), such as cleavage and degradation of various kinases and transcription factors, promoting the inhibition of relevant enzymes (Etges *et al.,*1986, Chaudhuri *et al.,* 1988, 1989, Santos *et al.,* 2006, Lieke *et al.,* 2008, Moradin *et al.,* 2012). Interestingly, gp63 is also immunogenic and therefore was used as antigen for immunodiagnosis and immunoprophylaxis (Xu *et al.,* 1995, Reed *et al.,* 1987, Vale *et al.,* 2009).

- **Nucleotidases**

Nucleotidases are a group of membrane-anchored proteins facing the extracellular medium. Several studies have reported that 5-nucleotidase and 3-nucleotidase/nuclease are involved in parasite nutrition through the generation of nucleotides and phosphate from nucleic acids (Joshi *et al.,* 2007, Leite *et al.,* 2012, Freitas-Mesquita *et al.,* 2014). Furthermore, ectonucleotidases are involved in the generation of nucleosides that can cross the plasma membrane via specialized transporters (purine receptors) protecting *Leishmania* parasites from death caused by NETs. Therefore, these enzymes participate in parasite infectivity and clinical outcome (de Souza *et al.,* 2010, Leite *et al.,* 2012, Freitas-Mesquita *et al.,* 2014, Guimaraes-Costa *et al.,* 2014). However, these enzymes require an alkaline pH to properly function, and this may suggest that are associated with promastigote infection rather than amastigotes, which live in the acidic environment of MΦ phagolysosome (Chang *et al.,* 1999, De Souza *et al.,* 2010, Paletta-Silva *et al.,* 2011, Freitas-Mesquita *et al.,* 2014).

- **Heat shock proteins**

Heat shock proteins (HSPs) are molecules with different molecular weights that play a role in the folding and unfolding of other proteins, conferring protection (Feder *et al.*,1999). *Leishmania* species have a complete set of HSPs that may play important roles in parasite differentiation and survival during infection (Adhuna *et al.*,1997). These proteins are crucial in protecting parasite structures during temperature-induced promastigote to amastigote differentiation when parasites move from the vector's low temperature to the higher mammal temperature, in addition to their role in parasite survival within the mammalian host (Morales *et al.*, 2010, Hombach *et al.,* 2013, 2014, Kröber-Boncardo *et al.,* 2020). The development of resistance to therapy may be related to modifications in HSPs that play key role in the survival of the parasite in the mammalian host (Skeiky-*et al.*,1995, Morales *et al.,* 2010).

1.5. Extracellular Vesicles

Extracellular Vesicles (EVs) have attracted significant interest in recent years due to their ability to transfer potentially important intercellular communication mediators, including proteins and nucleic acids (Yáñez-Mó *et al.*, 2015). This function is of particular interest as EVs can be critical mediators of host-pathogen communication and contribute to pathogenesis. Based on their biogenesis, biophysical properties and functions, EVs can be separated into distinct subgroups: apoptotic bodies, microvesicles (MVs) and exosomes. These nanovesicles carry a variety of molecules, including lipids, proteins, and genetic material such as DNA and non-coding RNA (Andre *et al.,* 2002). EVs vary in size and function and are released by different cellular mechanisms.

Apoptotic bodies (ABs) released by apoptotic cells are the largest extracellular vesicles, ranging from 1–5 μm (Chistiakov *et al.,* 2016, Caruso *et al.,* 2018, Rackov *et al.,* 2018). These nanovesicles stimulate the phagocytosis of apoptotic cells before the induction of secondary necrosis (Chistiakov *et al.,* 2006, Caruso *et al.,* 2018). Moreover, are enriched with various damage-associated molecular pattern proteins that can induce inflammation.

MVs are a heterogeneous group of spherical vesicles with a diameter of about 100-1000 nm, formed by budding and fission of the cell membrane, and are enriched in phosphatidylserine,

integrins, selectins and CD40 (Shedden *et al.,* 2003, Pilzer *et al.,* 2005, Obregon *et al.,* 2006; Cocucci *et al.,* 2009, Schara *et al.,* 2009, Antwi-Baffour *et al.,* 2010, Muralidharan-Chari *et al.,* 2010, Shantsila *et al.,* 2010, Sekula *et al.,* 2011). MVs are predominantly derived from platelets and are abundant in the bloodstream wherether blood cells and endothelial cells also shed small amounts of MVs (George *et al.,*1982, Caby *et al.,* 2005).

Exosomes, the endosome-derived membrane vesicles ranging from 30-100 nm in diameter are the smallest EVs (Bhatnagar *et al.,* 2007, Moldovan *et al.,* 2013). Exosomes can transfer proteins and nucleic acids through direct cell-to-cell contact as well as long-range signalling (Moldovan *et al.,* 2013). Although most cells secrete exosomes of endocytic origin (Honegger *et al.,* 2018) their composition is heterogeneous, as it depends on the type of cell from which they originate. The differentiated composition of EVs in different body fluids is a prerequisite for their potential as biomarkers of different diseases.

Exosomes can be secreted by a wide variety of cells, such as mast cells, DCs, T cells, B cells, stem cells, astrocytes, endothelial cells, tumor cells, and epithelial cells (Zhou *et al.,* 2008, Eldh *et al.,* 2010, Corrado *et al.,* 2013). These nanovesicles have several surface molecules which can activate cells allowing them to participate in the exchange of materials between cells (proteins, lipids, carbohydrates and pathogens).

Exosomes are the only secreted cell vesicles that are formed from inner membranes (Meckes *et al.,* 2011). The formation of intraluminal vesicles (ILVs) after the inner budding of endosome membranes initiates exosome biogenesis (Frydrychowicz *et al.,* 2015). Multivesicular bodies (MVBs) transit toward the cell surface, fuse with the plasma membrane and release intraluminal vesicles into the extracellular environment (Gyorgy *et al.,* 2011, Meckes *et al.,* 2011). As a consequence of their endosomal origin, almost all exosomes, regardless of the cell type from which they originate, contain proteins involved in membrane transport and fusion (e.g. Rab GTPases, annexins, flotillin), and in the formation of MVBs (e.g. Alix and Tsg101). They also can carry heat shock proteins (HSP 70 and HSP 90), integrins, tetraspanins (e.g. CD63, CD9, CD81 and CD82), and cytoskeletal components (e.g. actin and tubulin). Exosomes shed by antigen-presenting cells may also contain proteins involved in specific cell functions, as is the case of MHC (Thery *et al.,* 2002, Wubbolts *et al.,* 2003). Tetraspanins facilitate cell fusion, migration, signaling, and cell-to-cell adhesion (Charrin *et al.,* 2014), and were originally identified in B lymphocytes EVs (Raposo *et al.,* 2013). As a consequence of

its biogenesis, exosomes are especially enriched in proteins derived from the plasma membrane, endosomal compartment or cytosol and also incorporate some lipids such as phosphatidylethanolamine (Kruh-Garcia *et al.,* 2015), ceramide (Abrami *et al.,* 2013), cholesterol (Fleming *et al.,* 2014, Kruh-Garcia *et al.,* 2014) and saturated fatty acids (Maurin *et al.,* 2012). Furthermore, exosomes have been found to carry mRNA (Valadi *et al.,* 2007), miRNA (Valadi *et al.,* 2007) and small non-coding RNAs (Nolte Hoen *et al.,* 2012). The inclusion of miRNA in exosomes is not random and appears to be regulated by a 4-nucleotide motif (GGAG) (Villarroya-Beltri *et al.,* 2013). The target cell can take up this cargo from exosomes by phagocytosis, direct fusion with the plasma membrane, or receptor-mediated endocytosis. Receptor-mediated endocytosis involves the direct binding of exosomes to receptors in the cytoplasm or to the membrane of an endocytic organelle of the host cell (Schorey *et al.,* 2015). Direct fusion allows the release of cargo directly into the cell while it is fused to plasma membrane receptors.

1.5.1. Extracellular vesicles of *Leishmania* spp.

Leishmania parasites shed a variety of extracellular vesicles, including exosomes, that allow them to interact and respond to their environment. It is demonstrated, that *Leishmania* species release exosomes in culture and on the midgut of their sandfly vector. The *Leishmania* promastigotes shed vesicles with a mean diameter of 30-70 nm, consistent with exosomes released by mammal cells (Silverman *et al.,* 2010).

The molecular composition of parasite-derived exosomes has been shown to contain homologs of some mammalian exosome markers, as well as molecules that can increase the success of infection. Up to 329 molecules were identified in exosomes released from axenic pro-

mastigotes, of which 52% are representatives of the parasite's secretome. Vesicles of *Leishmania* spp. contain virulence factors (Gp63, HSP10, HSP70, TRYP1, 14-3-3-like protein, sti1 stress-induced protein), LPG and elongation factor 1 (EF-1) (Silverman-*et al.*, 2010, Hassani *et al.*, 2011, Olivier *et al.*, 2012, Hosseini *et al.*, 2013). The chaperone, HSP 100, which was identified in exosomes from *Leishmania*-infected hosts, seems to mediate the packaging of specialized proteins (including virulence factors) in the exosomes (Silverman *et al.*, 210)

Atayde and collaborators (2015) showed that *L. major* parasites secrete exosomes into the midgut of the sandfly and that when inoculated with parasites during the initial bite of a mammalian host, they increase infection and lesion development due to the synthesis of important pro-inflammatory cytokines such as IL-17a. IL-17a is known to recruit PMN during the development of *Leishmania*-induced human and murine lesions (Lopez Kostka *et al.*, 2009, Boaventura *et al.*, 2010). Previous *in vivo* studies by Deatherage and Cookson (2012) evidenced the recruitment of neutrophils to the site of inoculation of *Leishmania* exosomes.

Exosomes produced by axenic *Leishmania* promastigotes modulate chemotactic activity and cytokine secretion by *in vitro* MΦ, which can impair the immune response and increase permissiveness to infection (Silverman *et al.*, 2010). Vesicles also can activate the host's immune system. *L. major* exosomes induce a Th2 polarization in mice, indicating that parasite-derived vesicles have an immunosuppressive nature, favoring disease progression (Gioseffi *et al.*, 2021).

Leishmania EVs (Gavinho, 2015) also carry gp63. EVs with different gp63 loads differ in the immunomodulatory capacity of protein expression by *in vitro* MΦ. The diverse impact on cutaneous leishmaniasis confirmed gp63 as a primary component of EVs ability to enhance inflammatory response (Filho *et al.*, 2021). Exosomes of *Leishmania*-infected MΦ impact the host immune system due to the downregulation of pro-inflammatory macrophage genes (Silverman *et al.*, 2010).

Silverman and colleagues (2010a,b) observed that *L. donovani* exosomes can modify IL-10 and TNF-α secretion by human monocytes subjected to IFN-γ stimulation. Furthermore, they found that mice treated with *L. donovani* exosomes increase IL-10 and IL-4 producing $CD4^+$ T cells, which could in part explain the exacerbated inflammation of the skin (Silverman *et*

al., 2010b). These studies suggested that *Leishmania* exosomes are primarily favoring an immunosuppressive state, allowing the parasite to better propagate within its hosts.

EVs released by different *Leishmania* species appear to induce different responses in human MΦ (Nogueira *et al.,* 2020). *L. infantum* and *L. braziliensis* EVs failed to induce inflammatory response in human MΦ. However, EVs from *L. amazonensis* stimulate human MΦ to produce NO, TNF-α, IL-6 and IL-10 via TLR4 and TLR2 signalling (Nogueira *et al.,* 2020).

Through exosome secretion, *Leishmania* spp. interferes with both innate and adaptive host immunity (Silverman *et al.,* 2010). *L. donovani* EVs exhibited an immunosuppressive effect and exacerbated the disease in infected animals, but EVs derived from *L. donovani* HSP100 KO (*L. donovani* HSP100$^{-/-}$) were able to induce a pro-inflammatory response (Bhatnagar *et al.,* 2007). This evidence leads to the possibility that exosomes containing *Leishmania* antigens can be a vaccine candidate in high-risk areas of *Leishmania* transmission. On the other hand, EVs shed by drug-resistant *L. infantum* parasites showed differences in morphology, size, distribution and protein content. The identification of resistance-related proteins in EVs derived from resistant parasites may bring new avenues for leishmaniasis treatment (Douanne *et al.,* 2020).

The early innate immune response during *Leishmania* infection is influenced by exosomes, as they are proactive in infection, both *in vitro* and *in vivo,* mainly to favor the survival of the parasite, allowing it to fully establish itself within the mammalian host. *Leishmania* exosomes are fully established as virulence factors (Dong *et al.,* 2019).

2|Chapter 2

Immunomodulation of moDCs by *Leishmania* spp.

2|Chapter 2: Immunomodulation of moDCs by *Leishmania* spp.

2.1. Dendritic Cells

DCs were first described in 1973 by Ralph Steinman. After extensive studies, Steinman identified cells expressing high levels of MHCII in contrast to MΦ that showed a lower expression. He also found that these cells were powerful initiators of immunity, being at least 100 times more potent than other APCs. Steinman in partnership with Maggi Pack (1987) demonstrated that DCs responded to the granulocyte-macrophage colony-stimulating factor (GM-CSF) and that MHCII-rich DCs were abundant in areas of peripheral lymphoid tissues, an ideal place to initiate T cells immunity. DCs are a heterogeneous population characterized by their stellate shape with cytoplasmic expansions/emissions or dendrites (Merad *et al.*, 2013). These cells can be found in all peripheral tissues, as well as in primary (thymus and bone marrow) and secondary (lymph nodes, lymph nodes, Payer's plaques and spleen) lymphoid organs. DCs derived from hematopoietic stem cells and can have two different origins.

2.1.1. The Origin and Anatomical Location of Dendritic Cells

DCs originate from bone marrow hematopoietic stem cells, which are derived from lymphoid or myeloid precursors. In both human and murine animals, the phenotypic characteristics and anatomic settings of these two precursors give origin to myeloid and lymphoid DCs, which express high levels of CD11c, MHC-II, and co-stimulatory molecules CD40, CD80, CD83, and CD86. However, they can be differentiated based on CD1, CD8α and DEC 205 markers (Shortman *et al.*, 2010). Myeloid DCs are mainly found in the marginal zone of the spleen, while lymphoid DCs are in the T cell areas of the spleen and lymph nodes, more precisely in the periarterial lymphatic sheaths (PALS). Even though both human and murine DCs originate from the same progenitor, their subsets play different roles in regulating B cell activation and T cell differentiation into Th1 and Th2 cell subsets. $CD14^{+}/CD11C^{+}/CD1^{-}$ phenotype of myeloid DCs have high phagocytic and endocytic capacity when compared to $CD14^{-}/CD11C^{+}/CD1^{+}$ lymphoid precursors. Furthermore, both Langerhans and interstitial DCs are strong stimulators of naïve T cells,

whereas interstitial DCs also play a vital role *in vitro* activation and differentiation of naïve B cells (Zanna *et al.,* 2021).

2.1.2. Dendritic Cell Subsets

Myeloid/Conventional Dendritic Cells

Typically, myeloid DCs are classified into two subtypes: cDC1 and cDC2. The human cDC1 subset is identified by the expression of CD141 (BDCA-3), while the murine equivalent is subdivided into a CD8α splenic population and a $CD141^+$ DC non-lymphoid tissue-resident population (Bachem *et al.,* 2010, Crozat *et al.,* 2010, Haniffa *et al.,* 2012). cDC1s are characterized by producing both basic leucine zipper transcription factor (BAFT3) and interferon regulatory factor 8 (IRF8) and seem to be able to effectively induce $CD8^+$ T cell activation through antigen cross-presentation, as well as produce large amounts of IL-12p70 (den Haan *et al.,* 2000, Bedoui *et al.,* 2012).

cDC2s express both common myeloid markers such as CD11b, CD11c, CD13 and CD33, in addition to the more recently identified antigens on these cells: CD1c, CD2, FceR1 and SIRPA (Collin *et al.,* 2018). cDC2s comprise a large part of the conventional human DCs found in blood and tissues. The immune function of cDC2s is ensured by a myriad of immune receptors, including TLRs receptors (2, 4, 5, 7 and 8), C-type lectins (Dectin-1 and −2), as well as NOD and RIG-type receptors (Collin *et al.,* 2018)

Plasmacytoid Dendritic Cells

pDCs arise from the progenitor lymphoid. They are found throughout the body, circulating in the bloodstream and peripheral organs, and residing in the lymph nodes, spleen, thymus, and bone marrow. pDCs exhibit a distinct surface morphology and phenotype and have a highly developed secretory compartment. They are characterized by their round shape and plasmacytoid morphology and bear a resemblance to lymphocytes. However, when incubated with recombinant IL-3/CD40L pDCs show a microscopic appearance similar to that of moDCs (Reizis *et al.,* 2011, Reynolds *et al.,* 2015). pDCs are usually dormant cells, but upon stimulation through TLR7 and TLR9, they secrete large

amounts of IFN-γ, which plays an important role in viral and bacterial infections. However, when in an unprimed state, these DCs may have a tolerogenic potential. Since can express MHCII and costimulatory molecules, pDCs have the potential to present antigens, although their ability to phagocytose dead cells, present cell-associated antigens and exogenous antigens cross-linked to MHCI has not been established. Therefore, pDCs are known to play a vital role during inflammation compared to conventional DCs (cDCs), which play a role in sustaining the immune response.

Langerhans Cells

Langerhans cells (LCs) are a distinct population of phagocytic mononuclear cells that reside in the epidermis. After activation, LCs tend to migrate through the dermis to the nearest lymph nodes and eventually present antigens to naïve T cells. Therefore, this DC subset is categorized as "migratory DCs" and other DCs not resident in lymphoid tissues, such as dermal and interstitial DCs, also belong to this subset of DCs. LCs are characterized by MHCII expression, but mainly by the expression of Langerin type C and CD1a, conferring host immunity against many skin pathogens, such as bacteria and fungi (Ginhoux *et al.*, 2010). LCs also have cytoplasmic organelles known as "Birbeck granules".

Monocyte-Derived Dendritic Cells

Monocyte-derived DCs (moDCs) are also known as "inflammatory DCs". Human monocytes comprise two subsets of circulating cells in peripheral blood, characterized primarily by the expression of CD14 and CD16. In turn, murine monocytes are identified by the presence of Ly6C, CCR2 and CX3CR1 (Reynolds and Haniffa, 2015). In the presence of an inflammatory process, circulating monocytes invade the tissues, differentiating into moDC. *In vitro*, moDCs can be derived from peripheral blood monocytes stimulated with GM-CSF and IL-4 (Auffray *et al.*, 2007). During an infection, moDCs migrate to the site of infection, where produce TNF-α and synthesise iNOS, process and cross-present foreign antigens (Cheong *et al.*, 2010).

Phenotypically, it is difficult to distinguish moDCs from cDCs, since both cells share similar expression patterns of MHCII, CD11b and CD11c. Evidence of their monocytic past is that moDCs express CD64 and the Fc-gamma 1 receptor (FCgR1). Functionally,

moDCs are similar to cDC. Both cells are capable of processing tissue antigens and eventually migrating through lymphatic vessels to the nearest lymph nodes delivering processed antigens to naïve T lymphocytes.

None of the typical markers usually used to differentiate monocyte/MΦ from DCs in humans and mice, such as CD1c, CD11c, and CD14, allows accurate identification of canine DCs. On the other hand, canine moDCs do not lose the ability to express CD14 after cultivation, unlike human and murine moDCs (Carrasco *et al.,* 2001, Bienzle *et al.,* 2003, Ibisch *et al.,* 2005, Wijewardana *et al.,* 2006, Ricklin Gutzwiller *et al.,* 2010). Since markers specific for canine DCs were not yet clearly identified, a panel of different antibodies directed against surface molecules such as CD1a, CD11c, CD40, CD80, CD83, CD86, CD206, CD209 and TLR-3 should be used to classify canine DCs by flow cytometry (Moore *et al.,* 2006, Bund *et al.,* 2010, Ricklin Gutzwiller *et al.,* 2010, Schwens *et al.,* 2011). According to Bonnefont-Rebeix and co-workers (2007), TLR3 is abundantly expressed in dog moDCs, but not in monocytes, which is a good marker to differentiate these two cell types.

2.1.3. Dendritic Cell and Immunity

DCs play a crucial role in the immune response by phagocytoses pathogens, process foreign antigens, complex the processed antigens with MHCII and MHCI molecules, and expose these complexes at the cell surface to be presented to T lymphocytes. After being produced in the bone marrow via hematopoiesis, immature DCs migrate from the bone marrow, becoming lodged in non-lymphoid tissues of the body, where continually monitor the extracellular environment for foreign antigens (Bonetti *et al.,* 2011, Mellman and Steinman, 2001). Immature and mature DCs have different morphology and phenotypic characteristics. Immature DCs have a smooth, round surface, while mature DCs have a rough surface with multiple pseudopods (Omin.Neill *et al.,* 2004, Verdijk *et al.,* 2004, Fisher *et al.,* 2008, Xing *et al.,* 2011). In the immature state, DCs express low levels of MHCII and costimulatory molecules, such as CD80, CD86, CD83 and secrete levels of immunostimulatory cytokines, such as IL-12, IL-10 and TNF (Andreae *et al.,* 2002, Kim *et al.,* 2006). In contrast, mature DCs express high levels of costimulatory molecules and

immunostimulatory cytokines (Dudek *et al.,* 2013, Sousa *et al.,* 2006). Activation and maturation of DCs typically begin when DCs identify danger signals, called PAMPs. PAMPs are recognized by DCs through their PRRs. After stimulation by PAMPs, the concentration of intracellular calcium ions (Ca_2^+) increases. In turn, Ca_2^+ signals, activate transcription factors, such as activated T cell nuclear factor (NFAT) or NF-κB, which induce the expression of high levels of surface molecules CD80, CD86 and CD83, homing receptor (CCR7), and the secretion of immunostimulatory cytokines, such as IL-12 and TNF. Mature DCs then migrate to the lymph nodes in response to chemokines such as CCL19 and CCL21, which are secreted by the lymph nodes (Kim and Mooney, 2011). In lymph nodes, through T cell antigen receptors (TCRs), T cells recognize antigen fragments presented by MHC molecules on the surface of DCs and simultaneously the costimulatory molecules CD80/CD86 and CD28 interact (DC (Villadangos and Schnorrer, 2007, Crespo *et al.,* 2013). Consequently, naïve T cells become activated cytotoxic T cells, that leave the lymph nodes to destroy pathogens or infected cells, or helper T cells Activated naïve$CD4^+$ T cells can differentiate into different Th cells subsets by interacting with DCs, such as Th1, Th2, Th17 and helper follicular T cells (fTh cells), but the subsets of Th cells that will be differentiated depend on some factors, such as the type of antigen captured (e.g. bacterial, viral), the types of costimulatory molecules and interleukins expressed by DCs. DCs residing in lymph nodes can capture and process antigens and present them to naïve $CD4^+$ T cells to initiate and induce IL-2 secretion, which eventually leads to T cell proliferation and clonal expansion, playing a vital role in the B cells development and the establishment of humoral immune response (Kim and Kim, 2019).

Immunological tolerance

DCs that capture antigens in the absence of a local inflammatory signal remain in an immature, tolerogenic state, with low expression of MHC and costimulation molecules (Heath *et al.,* 2001; Idoyaga *et al.,* 2013; Audiger *et al.,* 2017). Antigen presentation to T cells in the absence of adequate costimulation molecules (CD80/CD86) to establish a link with T cell costimulatory CD28 molecule leads to the induction of T cell anergy. Low or no signal detected through the CD28 receptor is a pre-requirement for the induction of Treg differentiation (Semple-*et al.,* 2011). Thus, tolerogenic DCs (tolDCs) play a key role

in immune tolerance. The tolDC subset consists of naïve iDCs or, alternatively, semi-mature DCs activated by apoptotic cells or cytokines, such as IL-10 and TGF-β (Dudek *et al.,* 2013). Regulatory DCs express several immunomodulatory and immunosuppressive molecules, inhibiting pro-inflammatory immune responses and inducing immune tolerance. Indeed, the expression of Programmed death-ligand 1 (PD-L1), enhanced Inducible Costimulator Ligand (ICOS-L), thrombospondin, prostaglandins, and adenosine contributes to the induction of anergic T cells (Fucikova *et al.,* 2019). DCs also induce Tregs or regulatory B cells through the expression of PD-L molecules, inhibitory Ig-like receptors, IL-3 and IL-4, human leukocyte antigen G (HLA-G), anti-inflammatory cytokines IL-10, TGF-β, IL-27 and IL-35, retinoic acid, heme-oxygenase and indoleamine 2,3 dioxygenase (IDO) (Kushwah and Hu, 2011, Audiger *et al.,* 2017).

2.1.4. DC-mediated innate immunity

DCs recognize antigens through specialized receptors on their surface, such as PRRs. Mammalian cells express five families of PRRs, including TLRs, RIG-I-like receptors, NOD-like receptors (NLRs), lectin-like C-type receptors, and cytosolic DNA sensors (Anderson, 2000, Hardison *et al.,* 2012, Kanneganti *et al.,* 2007, Lamkanfi *et al.,* 2007, Meylan *et al.,* 2006, O'Neill *et al.,* 2010). NLRs trigger the activation of intracellular signaling cascades that lead to the activation of NF-κB or the formation of cytoplasmic multiprotein complexes known as inflammasomes. Inflammasomes mediate the activation of caspases, leading to the synthesis of the inflammatory cytokines IL-1β and IL-18. Human NLRP10 was originally identified by Wang and coworkers (2004) due to its homology to NLRP3 and APAF-1 and was initially named PYNOD. NLRP10 homologues can be found in the genomes of many species, as is the case of humans and various primates and rodents, indicating a conserved function of NLRP10 (Wang *et al.,* 2004).

In vivo, in Nlrp10$^{-/-}$ mice was observed that DCs showed incomplete maturation in contrast to *in vitro* studies where bone marrow-derived DCs (BMDCs) and splenic Nlrp10$^{-/-}$ DCs were mature and able to effectively activate T cells (Banchereau *et al.,* 1998).

Furthermore, *in vitro*, Nlrp10$^{-/-}$ DCs activated by pulsed antigens can effectively activate naïve T cells, but do not activate T cells in host lymph nodes, suggesting a defect in the interaction of DCs with T cells in Nlrp10 $^{-/-}$ mice (Eisenbarth et al., 2012).

Vaccaand collaborators (2017) generated a new line of Nlrp10$^{-/-}$ mice to investigate whether NLRP10 contributes to the induction of a CD4^{+} T type immune response. They found that Nlrp10$^{-/-}$ DCs were defective in IL-12 production both *in vitro* and *in vivo* and had flaws in NF-κB signalling. Suboptimal synthesis of IL-12 impaired IFN-γ production by CD4^{+} T cells, which decreased the ability of Nlrp10 $^{-/-}$ mice to respond to *Mycobacterium tuberculosis.* These data suggest that NLRP10 is critical for a Th1-type cellular response, mediated by DCs, against intracellular bacteria (Vacca *et al.*, 2017).

After PRRs respond to different PAMPs and DAMPs, they initiate activation of downstream signal transduction pathways. NF-κB regulates the expression of some immunological mediators, including chemokines, cytokines, adhesion molecules, and enzymes that lead to the production of secondary inflammatory mediators, such as iNOS (Karin and Lin, 2002). NF-κB makes part of conservative evolutionary elements of a group of proteins that regulates the transcription of genes involved in immune responses. The NF-κB family comprises five different members sharing the Rel homology domain (RHD), which generates the following homo- and heterodimers: RelA (p65), RelB, c-Rel, NF-κB1 (p1/p50), and NF-κB2 (p100/p52) (Hayden and Ghosh, 2012). Large inactive precursor proteins of 105 kDa (p105) and 100 kDa (p100) are synthesized and post-translationally processed in the DNA binding subunits p50 (NF-κB1) and p52 (NF-κB2), respectively (Sean and Baltimore, 1986). In the cytoplasm, these proteins are tight binding to the inhibitory protein IκB. The canonical activation of NF-κB involves the phosphorylation of IκB (NF-κB inhibitor) by the IκB kinase complex (IKK), leading to proteasome-mediated IκB degradation and the nuclear translocation of the NF-κB (Baltimore, 2011). RelA, c-Rel, and RelB are directly responsible for the induction of target genes as a consequence of their transactivation domain, while the p50 and p52 subunits exhibit DNA binding activity, but limited ability to activate gene transcription (Oeckinghaus and Ghosh, 2009). The non-canonical (or alternative) pathway of NF-κB responds selectively to a specific group of stimuli, including ligands from a subset of mem-

bers of the TNF receptor superfamily, such as the β-lymphotoxin receptor, B cell-activating factor and TNF family receptor (BAFF), and CD40 (Sun and Liu, 2011, Sun, 2012). Furthermore, non-canonical activation does not involve IκBα degradation, but rather depends on the processing of the NF-κB2 precursor protein, p100 (Sun and Liu, 2011, Sun, 2012). The central signaling molecule for this pathway is an NF-κB kinase (NIK) inducer, which functionally activates and cooperates with IKKα to mediate p100 phosphorylation. Processing of p100 involves degradation of its C-terminal IκB-like structure, resulting in the generation of mature NF-κB2 p52 and nuclear translocation of the non-canonical NF-κB p52/RelB complex (Fig. 7) (Sun, 2011, Zhang and Sun, 2015). Functionally, canonical NF-κB is involved in almost all aspects of the immune response, while the non-canonical NF-κB pathway appears to be evolved as a supplemental signaling axis that cooperates with the canonical pathway in regulating specific functions of the adaptive immune system (Liu *et al.*, 2017).

NF-κB is a master regulator of inflammation and appears to play a key role in guiding the maturation and immune functions of DCs (Rescigno et al., 1998, Kawai and Akira, 2006) as well as mediating DC protection against death caused by the release of cytokines (Radhakrishnan *et al.*, 2013). The p50 subunit of NF-kB in immature DC is essential to prevent the activation of autoreactive T cells (Dissanayake *et al.*, 2011). Larghi and colleagues (2012) demonstrated that NF-κB p50 orchestrates DC functions and survival in response to an inflammatory signal (LPS) during the maturation process, thus providing a homeostatic mechanism tuning the balance between uncontrolled activation of adaptive immunity and immune tolerance. Moreover, it is reported that DC p50 NF-κB promotes a tolerogenic phenotype, affecting both DC survival and the ability to drive the activation of effector T cells.

Among the immunosuppressive mechanisms explored by tolerogenic DC, the expression of indoleamine 2,3-dioxygenase (IDO) appears to be the most powerful. IDO-mediated immune regulation occurs through the source of tryptophan and the production of the immunoregulatory catabolites kynurenines (Grohmann et al., 2003), which in turn promote the differentiation of $FoxP3^{+}$ regulatory T cells. Thus, activation of NF-κB by the non-canonical pathway appears to induce regulatory functions in DCs, including effective induction of IDO and down-regulation of pro-inflammatory cytokine.

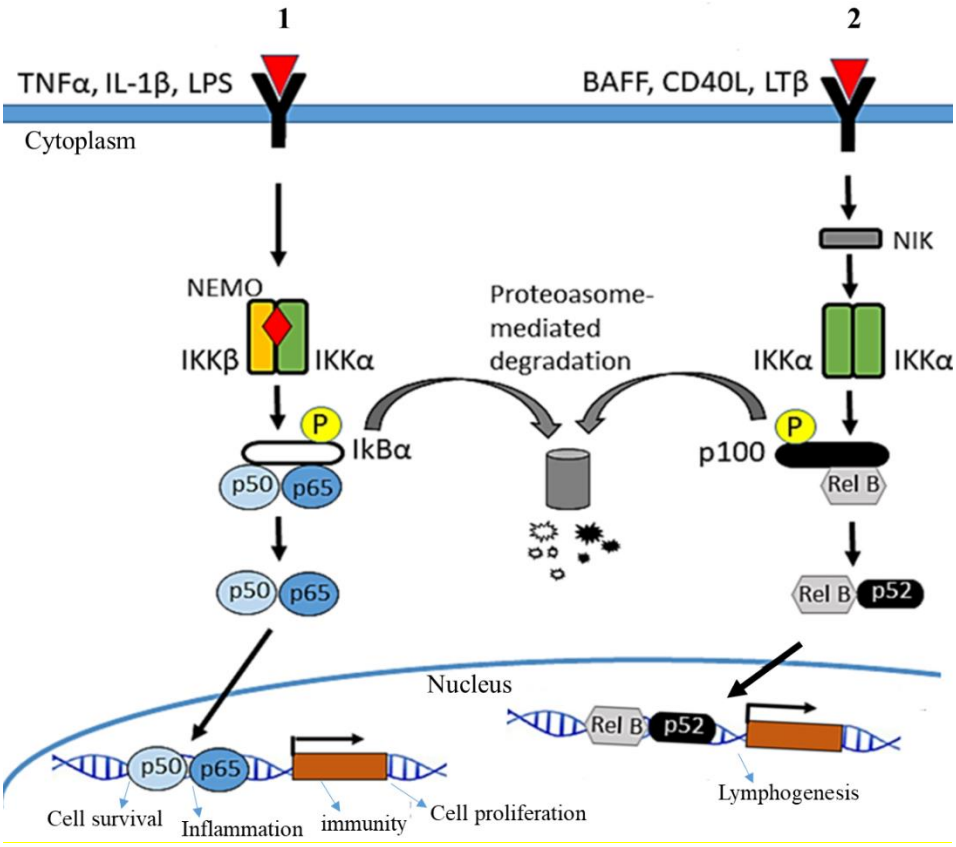


Figure 7. Schematic presentation of the main pathways of NF-κB activation. Classical pathway (1), binding to cell membrane receptors triggers the sequential recruitment of adapter proteins according to the type of stimulus and cell type. Adapter proteins recruit and activate the IKK complex, which, in turn, leads to phosphorylation and ubiquitination of IκB, followed by its degradation via the proteasome. The p50-p65 heterodimer is then released and migrates to the nucleus where it undergoes a series of post-translational modifications and binding to κB sites that allow transcription of target genes. The alternative pathway (2) activates the IKKα dimer, inducing the processing of p100 to release p52, which together with RELB translocate to the nucleus, triggering the transcription of NF-κB target genes (Adapted figure https://www.mdpi.com/2076-0817/9/10/814)

Several studies report that *Leishmania* parasites interfere with the activation of NF-κB signaling cascade (Shapira *et al.*, 2002, 2004, Reinhard *et al.*, 2012). *Leishmania* parasites can modulate the NF-κB pathway in several ways, for example after infection of human or mouse MΦ with promastigotes of several *Leishmania* species (including *L. infantum*), p65 is cleaved in p35. Then, p35 is involved in the expression of specific chemokines that

are favorable for parasite survival (Gregory *et al.,* 2008, Ranatunga *et al.,* 2020). It was also observed that mouse MΦ infected with *L. donovani* promastigotes did not alter p50 or p65 expression, resulting in an inactivation of NF-κB, mediated by hypoxia inducible factor-1α (HIF-1α) and the microRNA miR-210 (Kumar *et al.,* 2018). This leads to decreased expression of pro-inflammatory cytokines and NO by MΦ, facilitating parasite survival. Nogueira and coworkers (2020) evaluated the response of different species of *Leishmania* (*L. braziliensis*, *L. infantum* and L. *amazonensis)* and showed that only *L. amazonensis* was able to induce p65 translocation to the nucleus, activating a pro-inflammatory response. Decreased expression of IκB has also been reported, thus translocation of p65 and activation of the NF-κB pathway may occur, which may indicate pleiotropic impacts of *Leishmania* on NF-κB pathways (Baska and Norbury, 2022). These findings tend towards a pattern indicative of NF-κB pathway modulation by *Leishmania* parasites, leading to a dampening of TNF-α expression which can generate an environment that facilitates parasite survival in the host (Baska and Norbury, 2022).

NF-κB and chemokines play crucial roles in the immune response. During infections, NF-κB is translocated to the nucleus initiating innate immune responses (Naumann, 2000; Tato and Hunter, 2002) including cytokine production. Chemokines, which are chemotactic cytokines, promote immune cell trafficking during inflammation, recruiting leukocytes to the site of infection (Rot and Von Andrian, 2004).

The chemokine ligand CXCL16 is mainly expressed by MΦ and DC, but it is also produced by B and T cells, fibroblasts, and active enteric cells (Shimaoka *et al.,* 2000, Shimaoka *et al.,* 2004, Izquierdo *et al.,* 2014, Veinotte *et al.,* 2016). The structure of CXCL16 comprises a small intracellular C-terminal domain and an extracellular N-terminal chemokine domain strongly linked to the transmembrane region by glycosylated mucins (Izquierdo *et al.,* 2014). There are two forms of CXCL16 with different functions. sCXCL16 is a chemokine responsible for the chemotaxis of cells carrying the CXCR6/BONZO receptor (Shimaoka *et al.,* 2000, Rot and von Andrian, 2004), while mCXCL16 is a transmembrane protein expressed in MΦ (Shimaoka *et al.,* 2000, Tabata *et al.,* 2000, 2005) and is also present in DC found in splenic and lymph node, blood myeloid DC, and moDC (Shimaoka, *et al.,* 2003, Abel *et al.,* 2004, Baltimore, 2011). However, signal transduction of CXCR6 via mCXCL16 is not required for cell adhesion.

This property of mCXCL16 is important for the accumulation of immune cells at a site of inflammation due to increased expression of mCXCL16 in vascular walls by pro-inflammatory cytokines. Interestingly, mCXCL16 expression increases after DC maturation and in presence of pro-inflammatory factors (Matloubian *et al.,* 2000, Tabata *et al.,* 2005). In response to pro-inflammatory stimuli, such as IFN-γ and TNF, the chemokine domain of CXCL16 is cleaved by the action of the metalloproteinase ADAM10 (Abel *et al.,* 2004) and released in the extracellular space in its soluble form, acting as a chemoattractant agent for $CXCR6^+$ cells, such as NK cells, natural killer T cells (NKT), B cells, monocytes, and T cells (Abel *et al.,* 2004, Johnston *et al.,* 2003). In turn, CXCR6 expression increases in T cells during cell differentiation induced by DC (Kim *et al.,* 2001). Thus, CXCL16-CXCR6 axis plays an important role in the interaction of DCs with other immune cells (Korbecki *et al.,* 2021)

Leishmania glycocalyx consists mainly of lipophosphoglycan (LPG). LPG is one of the main glycoconjugate proteins, covering the promastigote's entire surface, including the flagellum (de Assis *et al.,* 2015). According to Chaparro and coworkers (2019), the capacity of *L. donovani* promastigotes to induce the synthesis of CXCL16 in infected MΦ depends largely on LPG (Chaparro *et al.,* 2019).

2.1.6. Dendritic cells and *Leishmania*

MΦ and DCs are both professional APCs, but they use different strategies for the uptake and internalization of *Leishmania* parasites and antigen presentation. DCs preferentially take up IgG-opsonized *Leishmania* amastigotes via FcγRI or FcγRIII surface receptors (Woelbing *et al.,* 2006). Antigen presentation and IL-12 production by DC are critical in the differentiation of $CD4^+$ Th1 and $CD8^+$ T cells to mediate a protective immune response against *Leishmania* infection (Berberich *et al.,* 2003, Bertholet *et al.,* 2006).

Several reports show a central role of DCs in orchestrating immune response against *Leishmania* infection (Gorak *et al.,* 1998, von Stebut *et al.,* 1998, Leon *et al.,* 2007). Skin contains at least three DC populations, the resident LC and two subsets of migrating dermal DCs. *In vivo* studies have shown that after *L. major* phagocytoses, LC migrate to draining lymph nodes where presents parasite antigens to T cells (Moll *et al.,* 1993¸Ritter

et al., 2004; Ng *et al.*, 2008). However, MHCII$^{-/-}$ mice control *L. major* infection similarly to wild-type mice (Lemos *et al.*, 2004), suggesting that LCs are dispensable to trigger T cell immune response during *Leishmania* infection. Furthermore, a more recent paper has shown that LCs may even play a pathogenic role during infection as they induce the expansion of Treg cells (Kautz-Neu *et al.*, 2011).

Other studies have suggested that moDC can differentiate within the sandfly bite site, phagocytose, and transport parasites to the drain LN (Leon *et al.*, 2007).

DCs can take up antigens through Fc receptors, C-type lectin receptors (CLRs), and PRRs. Veer and coworkers (2003) found that C57BL/6 MyD88$^{-/-}$ mice are more susceptible to *L. major* infection. On the other hand, a deficiency of MyD88 results in lower levels of DC activation and IL-12 production, both essential elements in the assembly of protective immunity against *L. braziliensis* (Vargas-Inchaustegui *et al.*, 2009).

In vivo neutralization of TLR2 and TLR4 reduced the expression of costimulatory molecules in *L. major* infected DCs (Komai-Koma *et al.*, 2014). However, the lack of TLR2 in mice infected with *L. braziliensis* resulted in increased DC activity and increased IL-12 production. As such, TLR2$^{-/-}$ DCs infected with *L. braziliensis* were more competent at priming naïve CD4^{+} T cells *in vitro* associated with an increase in IFN-γ production , and greater resistance to infection (Vargas-Inchaustegui *et al.*, 2009).

TLR2, TLR4, and TLR9 appear to recognize *L. major* (de Veer *et al*, 2003, Kropf *et al.*, 2004, Liese *et al.*, 2007, Schleicher *et al.*, 2007, Abou Fakher *et al.*, 2009). However, a study by Fakher and colleagues (2009) showed that only TLR9$^{-/-}$ mice are more susceptible to infection by this parasite. *In vivo* assays with *L. major* infection also confirmed the importance of TLR9 in IL-12 production by DCs (Liese *et al.*, 2007, Schleicher *et al.*, 2007).

In addition to their importance as mediators between innate and adaptive immunity, DCs is also recognized for having an efficient phagocytic activity (Savina, 2007). Although the phagocytosis capacity of DCs is not completely comparable to that of MΦ (von Stebut *et al.*, 1998). The mechanisms of pathogen capture involve specific receptor-ligand interactions, as well as the mobilization of cytoskeletal elements that promote the internaliza-

tion of parasites (Argueta-Donohué *et al.,* 2016). DC-specific ICAM-3-grabbing-non-integrin (DC-SIGN), a surface receptor found primarily on DCs, mediates a more efficient uptake rate of *L. mexicana* promastigotes after 3 h of infection *in vitro* (Argueta-Donohué *et al.,* 2016). Interestingly, DC-SIGN was nominated as the major ligand for *L. pifanoi* amastigotes (Colmenares *et al.,* 2002) and *L. mexicana* LPG (Appelmelk et al., 2003). Furthermore, DC-SIGN seems to be a receptor for both amastigotes and promastigotes of visceral (*L. infantum*) and cutaneous New World (*L. pifanoi*) *Leishmania* species, but not for metacyclic promastigotes of *L. major* (Old World). Together, these studies suggest that DC-SIGN-mediated crosstalk between *Leishmania* and DCs may have consequences for *Leishmania* infection (Brandonisio *et al.*, 2004). When activated, skin DCs upregulate CCR7 and migrate to the draining lymph node via afferent lymphatics in response to CCL19 and CCL21 (Merad *et al.*, 2013, Ohl *et al.*, 2004). The migration of monocyte-derived DCs to lymph nodes is driven by CCR2 and its ligands (Geissmann *et al.*, 2010). In VL, there is a lack of protective immune response, in part, due to an altered migration of DC to the spleen and draining lymph nodes (Ato *et al.*, 2002, 2006; Ibrahim *et al.*, 2014). Infection by *L. infantum* , but not by *L. amazonensis* or *L. braziliensis*, increases human DC (hDC) directional migration driven by chemotaxis in comparison to uninfected controls. These findings suggest that *L. infantum* infection may increase the ability of hDCs to migrate from the site of infection, which is consistent with the visceral form associated with this parasite species (Rebouças *et al.*,2021). Other authors have reported reduced DC migration following *L. amazonensis* infection (Hermida, *et al.*, 2014). Moreover, *L. donovani* infection was previously shown to increase the presence of DCs in the lymph nodes at the early stages of infection.

DCs are the main source of IL-12 in early *Leishmania* infection. Thus, DCs derived from the skin of C57BL/6 mice and splenic DCs located in the periarteriolar lymphoid sheath of BALB/c mice were recognized as the main source of IL-12p40 immediately after dermotropic infection by *L. major* or viscerotropic by *L. donovani*, respectively (Von *et al.*, 1998; Gorak *et al.*, 1998). The effect of *Leishmania* infection on IL-12 induction and DC maturation may vary according to DC subtype and *Leishmania* species. Indeed, *in vitro* infection of DCs acquired through murine marrow cells with *L. mexicana* promastigotes failed not only to induce IL-12 release but also to activate immature DCs. Additional stimuli, IFN-γ plus LPS, were required to restore DC maturation and IL-12 production in

infected cells (Bennett et al., 2001). Subsets of murine splenic DCs may vary in their ability to produce IL-12 and phagocytose *L. major* amastigotes. Resting human and murine myeloid cells, including DCs, appear to have a preformed membrane-associated stock of IL-12p70, which are released minutes after contact, *in vitro* or *in vivo*, with *L. donovani* (Quinones *et al.*, 2000).

DCs can control tryptophan catabolism through IDO, which converts tryptophan into kynurenine, as an immune regulatory mechanism. Thus, IDO produced by DCs inhibits local levels of tryptophan during the polyclonal development of T cells, blocking their proliferation. Therefore, it was shown that *Leishmania* infection induces the production of IDO by DCs derived from human monocytes, suggesting a mechanism for the parasite to escape the action of the immune system, favoring its survival (Donovan *et al.*, 2009).

2.2. Objectives

Therefore, taking into account the crucial role played by DCs in triggering the adaptative immune response, the present study aimed to investigate how *Leishmania* promastigotes can activate dog moDCs in the early phase of infection, directing dog-acquired immune response through the following specific objectives:

i. Characterize morphologically and molecularly DCs derived *in vitro* from peripheral blood mononuclear cells of healthy dogs (moDCs);

ii. Examine the interaction of dogs moDC with two species of *Leishmania* that infect the dogs, *L. infantum* and *L. amazonensis*, by evaluating the effect of these parasites on the activation of moDC effector mechanisms through the expression of MHC and co-stimulation molecules and the generation of cytokines and chemokines;

iii. Assess the effect of EVs shed by *L. infantum* and *L. amazonensis* promastigotes in modulating moDC activity;

iv. Evaluate the importance of PRR in parasite detection and, consequently, in the activation of downstream pathways that lead to APC activation.

2.3. Methods

2.3.1. Experimental design

The present study aimed to better understand the innate immune response generated by moDC when facing an infection by *Leishmania* parasites. Therefore, this section includes descriptions of the methods used to isolate, differentiate, culture and analyse moDC cells. moDC were isolated and differentiated from peripheral blood cells of healthy dogs (*Canis lupus familiaris*) belonging to the Cinotechnic Intervention Group of the Guarda Nacional Republicana (Lisbon) and the Animal Blood Bank (Port).

The experimental design used is shown in Fig. 8. Briefly, monocytes of healthy dogs were isolated and differentiated for 7 days in moDCs. Cells were exposed to *L. infantum* or *L. amazonensis* promastigotes and were stimulated with parasite soluble antigens and pro-mastigotes EVs from both parasites, for 24 h. Immune activity of moDCs were assessed by:

1. Analyze gene expression of membrane receptors, such as NOD1, NOD2, NLRP10, TLR2, TLR4 and TLR9 of anti-inflammatory (IL-10 and TGF-β) and pro-inflammatory cytokines (IL-12p35, IL-12p40, IL-8, IL-18, IL-1α and IL-1β), and of costimulation molecules (CD80 and CD86) by quantitative Real-time PCR;

2. Evaluate of MHC I and MHCII expression by multiparametric flow cytometry;

3. Examine NF-κB expression and translocation to the nucleus and assessof CXCL16 production (which exerts a chemotactic activity in lymphocytes) by ELISA;

4. Characterize the morphology of moDCs and observe the interaction of *L. infantum and L. amazonensis* promastigotes with the cell membrane by scanning electron microscopy.

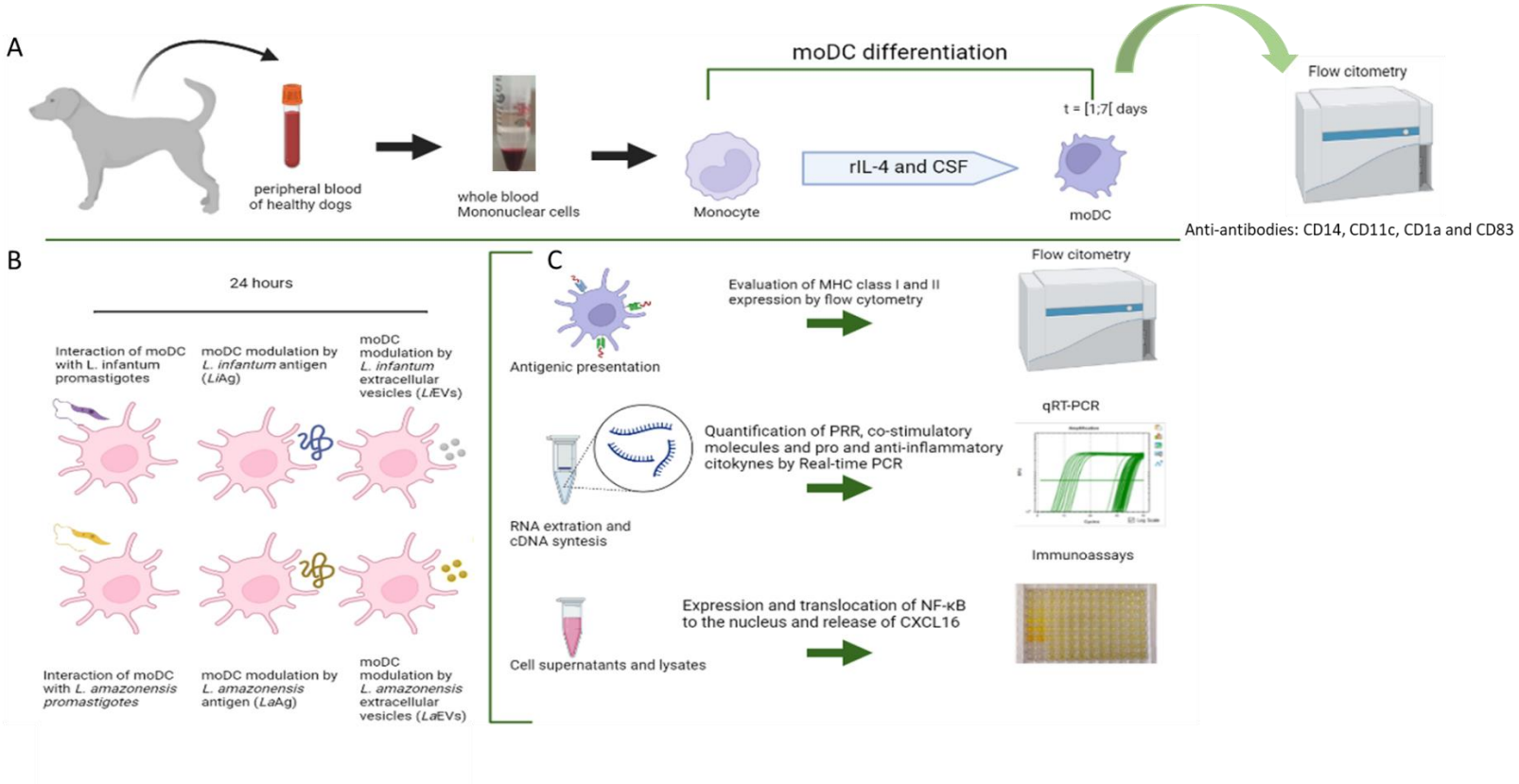


Figure 8. Schematic representation of the experimental design followed in the present study. moDC, differentiated from monocytes isolated from the blood of healthy dogs using a cocktail of immune mediators, were exposed to virulent promastigotes of *L. infantum* and *L. amazonensis* as well as soluble parasite antigens (*LiAg* and *LaAg*) and promastigotes EVs (LiEVs and *LaEVs*) of both parasites, for 24h. The immune response was evaluated through the quantification of PRRs, pro- and anti-inflammatory cytokines as well as co-stimulation molecules gene expression by qRT-PCR, evaluation of the expression of MHC molecules by flow cytometry and quantification of the chemokine CXCL16 and evaluation of NF-κB translocation to the nucleus by ELISA. Image of my own authorship, created with BioRender.com (accessed June 2022).

2.3.2 Animals and parasites

2.3.2.1 Dog

A group of 10 dogs with more than 20 kg of weight and aged between 1.5 and 5 years belonging to Grupo de Intervenção Cinotécnico da Guarda Nacional Republicana and Animal blood bank was selected. Animals were subject to a complete physical examination and blood was collected for hematology, biochemistry, ionogram and serum protein electrophoresis to assess their health status. Detection of anti-*Leishmania* antibodies was done by indirect immunofluorescence assay (Kit Leishmania Spot IF®, BioMerieux, France) with a cutoff of 1:80 and blood and lymph node samples were used to exclude *L. infantum* infection by qPCR (Helhazar et al, 2013) (Applied Biosystems 7300 RealTime PCR System). The absence of *Dirofilaria immitis* infection was confirmed by Knott test. Other hemoparasites, such as *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp. and *Rickettsia* spp. were ruled out by microscopic observation of blood smears and by qPCR, and the presence of *Mycoplasma haemocanis* was checked by microscopic observation of blood smear. Only healthy animals with negative results were included in the experiments and their owners/tutors were fully informed about the objective and nature of interventions and official consents were obtained. The study was approved by the Ethics Committee of FMV-UL.

2.3.2.2 Parasitas

2.3.3.2.1 *Leishmania* infantum promastigotes

L. infantum, MCAN/2012/IHMT0003SG (was maintained by successive passages in BALB/c mice). The spleens of infected mice were extracted and homogenized with a tissue dissociation (Medimachine, Syntec International) with a 50 μm separator screen (Medicons, Syntec International) to get a suspension of single cells. This cell suspension was added to Schneider's drosophila medium with L-glutamine (SCHN, Sigma-Aldrich) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich) and penicillin-streptomycin (Biochrom) at 100 U/ml and 100 $\mu g.ml^{-1}$ respectively

(complete SCHN medium). Spleen cells were incubated at 24 ºC until complete differentiation of promastigotes.

Only virulent parasites with less than five passages were used (Santos-Gomes and Abranches, 1996) in the present study. The concentration (C) of promastigote suspension was determined by optical microscopy in a Neubauer-improved chamber using the following equation:

$$[C] = number\ of\ parasites \times 50 \times dilution\ factor \times 10^3$$

50 and 10^3 are consideration factors for the number of squares counted and the volume of the chamber. Together they stand for counting 5 squares in 0.0001 ml of the Neubauer chamber.

2.3.3.2.2. *Leishmania infantum*-GFP

Transgenic Green Fluorescent Protein (GFP) expressing *L. infantum promastigotes* were kindly given by Professor Ana Tomás from Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto (Oporto, Portugal).

Briefly, the GFP open reading frame was inserted in the trypanosomatid expression plasmid pTEX (Kelly *et al.*, 1992) and the resulting construct was electroporated in *L. infantum* promastigotes following standard procedures. *L. infantum mutants* were selected on agar plates using Geneticin (G418 disulfate salt solution, Sigma-Aldrich) at 25 $g.ml^{-1}$.

Individual colonies were grown in culture medium using the same selection conditions, analyzed by southern blotting to confirm that the construct was structurally intact, and by fluorescence to ensure GFP expression. *L. infantum GFP* promastigotes were maintained in culture using SCHN medium supplemented with 25 $g.ml^{-1}$ of geneticin.

2.3.2.2.3. *Leishmania amazonensis*

L. amazonensis (MHOM/BR/1973/M2269) was isolated from a patient with DCL from the state of Pará, Northern Brazil. The parasites were identified using monoclonal antibodies and multilocus enzymatic electrophoresis at Instituto Evandro Chagas (Belém, State of Pará, Brazil). Parasites were maintained in BALB/c mice by subcutaneous inoculation in the animal's hind paws and later isolated and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 10 $\mu g.ml^{-1}$ gentamicin and 1000 $U.mL^{-1}$ penicillin at 25ºC (Carvalho *et al.*, 2012). These parasites were kindly provided by Dr. Filipe Passero (Institute of Biosciences, São Paulo State University (UNESP), São Vicente, SP 11330-900, Brazil).

2.3.3. Antigen preparation

L. infantum (*LiAg*) and *L. amazonenesis* (*LaAg*) soluble antigens were produced as described by Diaz and colleagues (2012). Briefly, promastigotes were centrifuged at 1800 ×g for 15 min. at 4ºC and then washed three times with phosphate-buffered saline (PBS, Lonza, Belgium))/ Ethylenediamine tetraacetic acid (ETDA, Sigma-Aldrich) at 1800 ×g for 15 min. at 4ºC. The pellet underwent six cycles of freezing and thawing (-20ºC, -70ºC and room temperature), promoting protein denaturation. The protein concentration was assessed using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific) and adjusted at 40 $\mu g.mL^{-1}$. The antigen was conserved at -20ºC until used.

2.3.4. Purification of EVs from *Leishmania infantum* and *Leishmania amazonensis*

In *Leishmania*, extracellular vesicles (EVs) have the main function of cellular communication, exerting effects on the parasite-host interaction and among parasites. In addition, *in vitro* and *in vivo* experiments demonstrate that EVs cargo is enriched with biomolecules, including virulence factors, which are fundamental in the infectious process.

The purification of EVs was carried out from cultures of *L. infantum* and *L.amazonensis* growing in SCH medium supplemented with Exosome-Depleted FBS (Gibco™, Thermofisher Scientific) at 72 h. Normal FBS cannot be used in these cases as it contains extremely high levels of exosomes that will contaminate the parasite-derived exosomes (Fig. 9).

After 72 h of incubation, cultures were centrifuged at 2000 × g for 30 min. to remove dead parasites and debris. The supernatant containing the parasite-free culture medium was transferred to a new tube without touching the pellet, 0.5 volumes of the total exosome isolation reagent were added, mixed and incubated at 4ºC, overnight. After incubation, the samples were centrifuged at 10,000 × g for 1 h at 4 º C and the supernatant was discarded. The EVs are in the pellet at the bottom of the tube (not visible in most cases). The pellet was resuspended in a convenient volume of 1× PBS. The protein concentration was assessed using a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, USA) and the EVs were cryopreserved at -20ºC until further use.

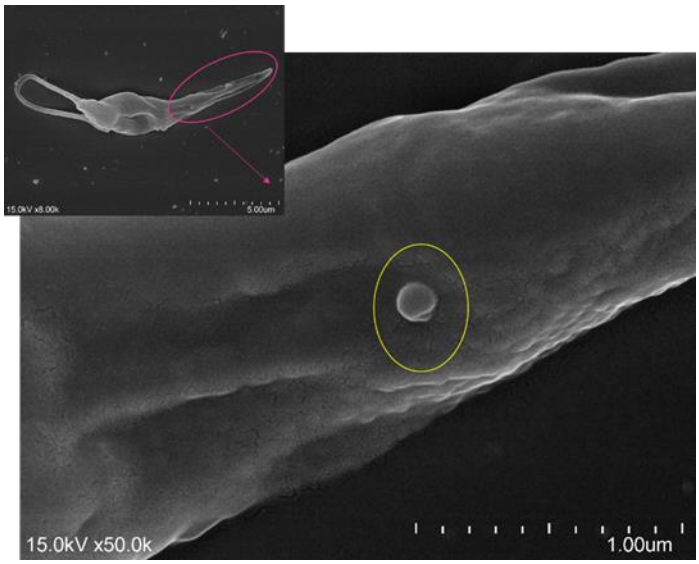


Figure 9. EVs of *Leishmania* promastigotes. Cultured *Leishmania* promastigote budding a nanovesicle were observed by scanning electron microscope and images were acquired. The extracellular vesicle is surrounded by the yellow circle.

2.3.5. Monocyte-Derived Dendritic Cells (moDC)

2.3.5.1. Isolation and purification of peripheral blood mononuclear cells

The blood collection was made according to the veterinary norms with therapeutic shearing and antisepsis of the venipuncture site with povidone-iodine and ethanol. Each time, 20 mL of blood was collected using citrate phosphate dextrose adenine (CPDA)-1 solution, which is an anticoagulant (Kawasumi, Germany). Isolation of mononuclear cells was achieved by using a Histopaque gradient, according to the technique described by Strasser et al (1998). The opaque band between plasma and Histopaque-1077 (Sigma-Aldrich) containing leukocytes was collected and washed with 0.9% (w/v) sodium chloride (NaCl). To lyse the contaminant red blood cells, a swift passage through deionized sterile water for 1 min. was followed by the addition of a solution of NaCl 1.8% (w/v). Cells were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich).

Cell yield and viability were estimated under an optical microscope, by counting in a Neubauer chamber after staining with trypan blue. Trypan blue is a vital dye that allows the assessment of cell viability. Viable cells with an integral cell membrane can exclude the dye, while non-viable cells stay blue-stained.

2.3.5.2. Differentiation of dendritic cells (DC) derived from monocytes

Colony stimulating factor (CSF) was obtained from the L929 cell line, which has fibroblast characteristics (Campos-Neto *et al.,* 1995). L929 cell line secretes a factor that promotes the differentiation of peripheral blood mononuclear cells into MΦ and monocytes. The conditioned medium of L929 cells is frequently used as a crude source of murine CSF (Fisher *et al.,* 1988). L929 cells also secrete a chemotactic factor specific for monocytes and an uncharacterized factor that promotes the growth of a large variety of cell lines. L929 cells, at a concentration of 10^6 cells.mL^{-1}, were cultured in complete RPMI medium, at 37 ºC in a humid atmosphere of 5% CO_2. Cell adhesion was confirmed under an inverted microscope (Olympus, Japan). After three days of incubation, supernatants were collected, centrifuged at 500×g for 10 min, filtered through a 70 μm cell filter (BD

Biosciences, USA) and stored at -20ºC until further use. After trypsinization, cells were detached, counted in a Neubauer's chamber using trypan blue dye, to confirm cell viability and inoculated in new T-flasks, at the same cell concentration, repeating the production cycle.

The mononuclear cell suspension was cultured in 6-well plates at 37ºC, in a humidified atmosphere containing 5% CO_2. After 24 h and 96 h of incubation, the culture medium was discharged to remove non-adherent cells, and a fresh medium (complete RPMI supplemented with 10% CSF and 100 ng/mL of canine recombinant IL-4 (rIL-4) (Ibisch *et al.*, 2005, Wang *et al.*, 2007) previously heated to 37ºC was added in order to maintain the conditions to which the cells were originally subjected. Monocytes obtained from dog peripheral blood strongly adhere to the plaque surface. Medium replacement allows the elimination of non-adherent mononuclear cells (lymphocytes) and other contaminating cells. After 7 days of differentiation, the monocytes were compatible with moDCs.

The MΦ obtained by differentiating canine monocytes was identical to the method-logy used for moDCs. In this case the medium was enriched with 10% CSF and 10% FBS. After 5 days, the monocytes were completely differentiated into MΦ (Pereira *et al.*, 2019).

2.3.6. moDC immunophenotyping

moDC and MΦ were immunophenotyped by flow cytometry analysis. moDC and MΦ were washed two times with cold 1× PBS (300 ×*g*, 10 min., 4ºC), fixed with 2% paraformaldehyde (Sigma-Aldrich) (w/v) for 20 min. at room temperature and resuspended in PBS.

moDC and MΦ populations were incubated with the following monoclonal antibodies directly conjugated with fluorescent dyes (Table 5):

Table 5. Monoclonal antibodies to the cell surface markers CD14, CD11c, CD1a, CD83 and their specifications

Laser	488 nm		640 nm	
Emission filters	525/40	585/42	665/20	660/20
Flurochrome	FITC	PE	APC	Alexa Fluor® 647
Biomarker	CD14	CD83	CD11c	CD1a
Monoclonal antibody	Anti-human CD14 monoclonal antibody	Anti-human CD83 monoclonal antibody	CD11c monoclonal antibody	CD1a monoclonal antibody
Clone	TuK4	HB15e	BU15	NA1/34-HLK,
Company	ThermoFisher Scientific	Invitrogen	ThermoFisher Scientific	Bio-Rad

Controls for calibrating the cytometer, identifying the background fluorescence and adjusting channel spillover to ensure the correct reading of the samples, were: fixed and unstained cells, cells individually labelled with each of the antibodies used and cells labelled with multiple combinations of antibodies.

Cell acquisition was performed on a CytoFLEX system cell analyzer (Beckman Coulter), and Flowjo_V10 analyzed data. The FSC-H vs SSC-H gate was used to remove debris and pyknotic cells as well as the very large debris. The singlet gate was used to define the non-clumping cells based on pulse geometry FSC-H vs FSC-A. Data were analysed using theFlowjo_V10 software package (Tree Star Inc., USA).

2.3.7. moDC *in vitro* infection

Dog moDCs (1×10^5 cells/well) were seeded in 96-well plates with *L.infantum* promastigotes and *L. amazonensis* promastigotes at a ratio parasite-DC of 5:1 in 300 µL of RPMI supplemented with 10% heat-inactivated FBS (v/v). moDCs were also stimulated by *Leishmania* soluble antigen (*LiAg*, *LaAg* - 40 µg ml^{-1}), and by *L. infantum* (*LiEVs*) and *L. amazonensis* (*LaEVs*) EVs (10 µg.mL^{-1}). Plates were incubated at 37ºC in a humidified atmosphere containing 5% of CO_2 for 24 h. In parallel, resting-moDC and

phorbol myristate acetate (PMA) stimulated-moDC were used as negative and positive controls, respectively.

After the incubation period, supernatants were used to determine CXCL16 production and cells were used to examine the expression of NF-kB and to quantify the gene expression of PRR and cytokines.

2.3.8. moDC viability after infection or stimulation

Viable, pre-apoptotic and apoptotic cells were assessed by flow cytometry analysis. Resting-moDC (negative control), moDC exposed to *L. infantum* and *L. amazonensis* promastigotes and moDC stimulated by *Leishmania* soluble antigen (*LiAg*, *LaAg*) and EVs isolated from both parasite species were incubated for 24 h.

After incubation, cultures were washed one time with 200 mL of cold 1× PBS (300 ×*g*, 10 min., 4ºC) and incubated with the commercial kit TACS^{TM} Annexin V FITC (R&D Systems, USA), according to the manufacturer's instructions. Before flow cytometry acquisition, cells were treated with 1 μL of propidium iodide (PI) (R&D Systems). FL1-H (Annexin V FITC) *vs* FL5-H (PI) gate on untreated-moDC was used to delimit annexin V FITC^{-}/PI^{-} population (viable cells), annexin V FITC^{+}/PI^{-} (pre-apoptotic cells) and annexin V FITC^{+} or $^{-}$ /PI^{+} cells (apoptotic cells).

2.3.9. Microscopy

2.3.9.1. Scanning electron microscopy (SEM)

The morphology and interaction of moDC with *Leishmania* were evaluated by SEM in moDC incubated for 1.5 and 5 h with *L. infantum promastigotes* and *L. amazonensis* promastigotes.

Sample preparation for SEM was performed on a 24-well plate. Sterilized round glass coverslips of 22 mm were placed in each well and 3 × 10^{5} moDC were added. Then, cells

were incubated with *L. infantum* and *L.amazonensis* in the proportion of 1 moDC: 3 promastigotes for 1.5 and 5 h at 37ºC in a humid atmosphere of 5% CO_2.

After the incubation, cover slides were fixed inside the well with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer solution pH 7.4, for 2 h at 4ºC. The coverslips were washed with 0.1M sodium cacodylate buffer pH 7.4 to remove fixative (glutaraldehyde) and incubated for 15 min. at room temperature. This procedure was repeated twice. Then, the samples were dehydrated using a battery of ethanol solutions with increasing concentration, starting in ethanol at 70º until absolute ethanol:

1. Ethanol at 70º, 15 min. at room temperature;

2. Ethanol at 90º, 15 min. at room temperature;

3. Ethanol at 95º, 15 min. at room temperature;

4. Ethanol at 100º, 3 × 15 min. at room temperature.

The sample was then subjected to the critical point and metallized, observed by SEM (WITHOUT Hitachi, SU8010) and images were acquired.

2.3.9.2. Confocal microscopy

Dog moDC were cultured on round coverslips in 24-well plates, at 37 °C in a humidified atmosphere of 5% CO_2. *L. infantum* GFP-promastigotes were added to the cultured cells at a 3:1 ratio. After 5 h of incubation, coverslips adherent cells were washed with 1× PBS and fixed with 2% paraformaldehyde in PBS for 20 min. on ice.

After fixation, the coverslips were incubated with Dil Stain (1, 1'.-Dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine Perchlorate ('DiI'; DiIC18 (3)) (Invitrogen™ D282) for 2h at ambient temperature. DiI is a lipophilic fluorescent dye that stains cell membrane of orange/red and diffuses laterally to stain the entire cell. It is a weakly fluorescent compound until incorporate into cell membranes.

After 2 h, the coverslips were washed two times with PBS. For examination under fluorescent microscopy, cells were stained with DAPI (4′,6-diamidino-2-phenylindole,

VWR). DAPI is a blue-fluorescent dye that binds to adenine–timine regions of double-strand DNA, increasing about 20-fold fluorescence intensity. Sincecan cross-intact cell membranes this dye is commonly used as a nuclear counterstain.

The cover slides were observed under Leica TCS SP2 Laser Scanning Confocal Microscope and digital images were acquired.

2.3.10. Immunoassays

To investigate the activation of sensing pathways after the recognition of the parasite's PAMPs, the expression and translocation of NF-κB, as well as, DC ability in recruiting other cells, were measured by ELISA.

ELISAs (Enzyme-Linked Immunosorbent Assays) are a type of immunoassay used to quantify the levels of a specific target within a sample.

There are four types of ELISAs (Direct ELISA, Indirect ELISA, Sandwich ELISA and Competitive ELISA) and two different types were used in this work (Fig. 10).

Sandwich ELISAs are the most common type of ELISA. In this method, the Ab raised against a specific antigen, called the capture Ab, is initially adsorbed in the plate wells. Then, the sample with the antigen is added and binds to Ab. Soon after; another Ab specific to the antigen is added. Finally, a third Ab linked to an enzyme is added. This enzyme will react with the added substrate, generating color. The intensity of the reaction (weaker or stronger color) is proportional to the amount of antigen present in the evaluated sample (Fig. 10).

Competitive ELISAs are commonly used for small molecules when the protein of interest is too small to efficiently sandwich within two Ab. In this method, the presence of Ab in a given sample is revealed by competition with a specific Ab (mono or polyclonal) directed against the antigen. Likewise, the result is given by the addition of a conjugate, but the color will appear in the wells where there were no antibodies.

Quantitative determination of Canine NF-kB and

CXCL-16

To quantify NF-κB activation and CXCL16 synthesis, $1x10^5$ moDCcell lysates and culture supernatants, respectively, incubated for 24 h with *L. infantum* , *L. amazonensis* promastigotes, *LiAg*, *LaAg*, *LiEVs* , *LaEVs* were used. In parallel, resting moDC and moDC incubated with 0.2 $\mu g.mL^{-1}$ of PMA were used as a negative and positive control, respectively.

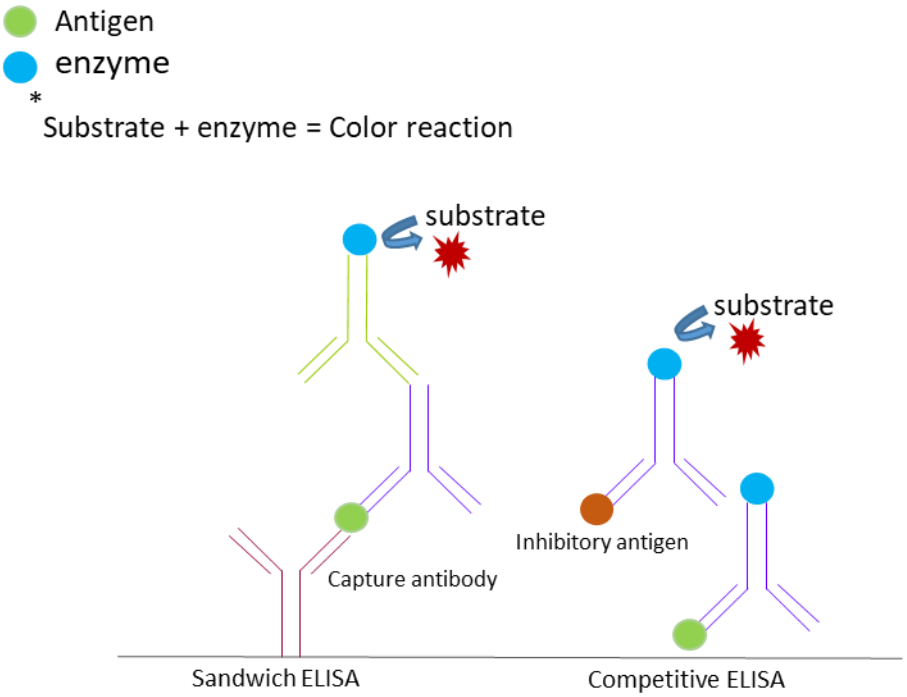


Figure 10. Two types of ELISA. Sandwich ELISA It is called a "sandwich" because the antigens are placed between two layers of antibodies. - Competitive ELISA uses two specific antibodies, an enzyme-conjugated antibody and another antibody. Combining the two antibodies in the wells will allow competition for antigen binding. Image of my own authorship.

2.3.10.1. Activation of nuclear factor-kappa B

After the recognition of antigens, TLRs trigger the release of NF-κB that is translocated to the nucleus, promoting transcription and synthesis of pro-inflammatory cytokines (Barton and Medzhitov, 2003). The release of NF-κB is mediated by MyD88, an adapter molecule present in most TLRs (Tuon *et al.,* 2008).

The Canine Nuclear Factor Kappa B (NFkB) ELISA kit (BlueGene Biotech CO., LTD, Shanghai) was used to evaluate the release of NF-κB by moDC exposed to parasites and stimulated by parasite soluble antigens, recombinant proteins and EVs.

The plate was previously coated with a monoclonal Ab specific for NF-κB. Then, 50 µl of the standards (concentrations of NF-κB ranging between 0 ng - 25 ng), 50 µl of moDC samples and 50 µl of PBS (blank) were added to the plate. NF-κB present in the samples should bind to the monoclonal Ab used to coat the plate wells. Afterwards, 5 µl of balance solution followed by 100 µl of the conjugate was added to all wells, except the blank. The plate was incubated for 1 h at 37ºC and then washed five times with the wash solution to remove all unbound components. Substrate A (50 µl) and substrate B (50 µl) were added to each well and the plate was incubated again at 37ºC for 15 min. The enzyme-substrate reaction was terminated by adding the "STOP" solution and the resulting color (absorbance) was measured at 450 nm using a plate reader (Fig. 11), (TRIADTM 1065, DYNEX Technologies, USA). The concentration of NF-κB was subsequently calculated by regression analysis.

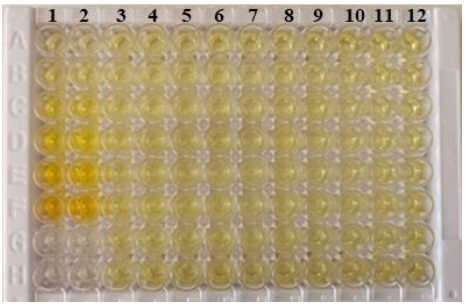


Figure 11. Image representative of a microplate from the commercial ELISA Canine Nuclear Factor Kappa B. Dog moDC samples were evaluated. Standards (Column 1 and 2 (A-F), blank (Column 1 and 2 (G and H)), samples (Column 3 to 12).

2.3.10.2. Release of the chemokine ligand CXCL16

The chemokine ligand CXCL16 is mainly expressed by MΦ and DC, but it is also produced by B and T cells, fibroblasts, and active enteric cells (Shimaoka *et al.*, 2000, 2004, Izquierdo *et al.*, 2016, Veinotte *et al.*, 2016). In response to pro-inflammatory stimuli, such IFN-γ and TNF, the chemokine domain of CXCL16 is cleaved by the action of metallo proteinase ADAM10 (Abel *et al.*, 2004), being released in its soluble form into the extracellular space.

For this case, it was used a commercial competitive ELISA, the Canine CXC Chemokine Ligand 16 (CXCL16) ELISA Kit (MyBioSource, San Diego, USA).

In order to estimate the CXCL16 concentration in the moDC samples, a standard curve was constructed using the standards with known concentrations of chemokine (0 pg to 1000 pg).

A 96-well plate was coated with anti-CXCL16 antibody and 100 µl of standards, 100 µl of samples and 100 µl of PBS (blank) were placed. Then, 10 µl of Balance solution was added to the samples, and 50 µl of the conjugate was added to all wells except the blank. The plate was incubated for 1 h at 37ºC. After the incubation period, five washes were performed with the wash solution to remove all unbound elements. Afterwards, substrate A (50 µl) and substrate B (50 µl) were added to all wells (Fig. 12A).

The plate was incubated at 37ºC for 15 min. and 50 µl of STOP solution was added to the standards, samples and blank, to stop the reaction (Fig. 12B). The absorbance was read at 450 nm through a plate reader. The CXCL16 concentration was subsequently calculated by regression analysis.

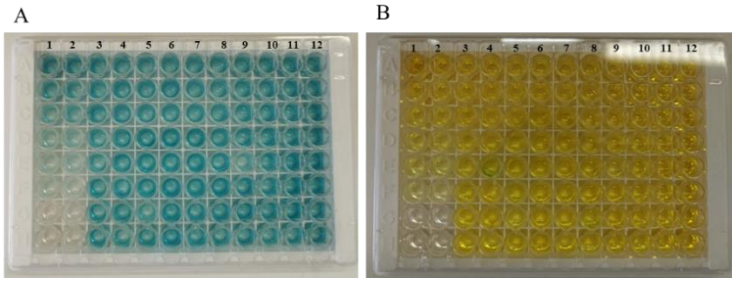


Figure 12. **Image representative of commercial ELISA Canine CXC Chemokine Ligant 16 (CXCL16).** moDC samples incubated at 37ºC for 15 min. (A) and after the reaction be stopped (B). Standards - Column 1 and 2 (A-F), blank - Column 1 and 2 (G and H), Samples (Column 3 to 12).

2.3.11. Expression of MHC molecules by moDC

moDC were incubated for 24 h with promastigotes of *L. infantum* and *L. amazonensis* in the proportion of 1 moDC:3 promastigotes, stimulated with *LiAg* and *LaAg* and EVs of both species. moDC and moDC incubated with 0.2 μg.mL^{-1} of PMA were used as a negative and positive control, respectively. Cells were harvested and washed with 100 μL of 1× PSB at 500×g for 10 min. to remove traces of proteins present in the culture medium. Samples were fixed with 2% paraformaldehyde (Sigma-Aldrich) (w/v) for 20 min. at 4ºC and incubated with the following monoclonal Abs (Table 6):

Cells were incubated on ice for 30 min. and washed with 1× PBS at 500×g for 10 min. in order to eliminate antibodies that did not bind specifically. The sediments were resuspended in 100 μL of PBS 2% FBS.

The controls for cytometer calibration and fluorescence adjustment, ensuring the correct reading of the samples, were: cells individually labelled with each of the Ab used and cells labelled with both Ab. Cell acquisition was performed on a CytoFLEX system cell analyzer , and Flowjo_V10 were used to analyze the data. The FSC-H *vs* SSC-H gate was used to remove debris and pyknotic cells as well as the very large debris. A singlet gate was used to define the non-clumping cells based on pulse geometry FSC-H *vs* FSC-A.

Table 6 - Monoclonal antibodies anti-MHC I and anti-MHCII and their specifications

Laser	488 nm	
Emission filters	525/40	585/42
Flurochrome	FITC	PE
Biomarker	MHC II	HLA ABC
Monoclonal antibody	Rat anti Dog MHC Class II	Mouse anti Human HLA ABC
Clone	YKIX334.2	W6/32
Company	Bio-Rad	Bio-Rad

2.3.12 Gene expression of PRR, pro- and anti-inflammatory cytokines and co-stimulatory molecules

2.3.12.1 RNA extraction and cDNA synthesis

moDCs samples were stored in lysis buffer (NR buffer supplied with the NZY Total RNA Isolation kit, Nzytech - Genes & Enzymes) supplemented with β-mercaptoethanol (Sigma-Aldrich) which preserves total RNA. Samples were conserved at -80 ºC until further processing. After thawed, RNA extraction was performed using NZY Total RNA Isolation kit according to manufacturer's instructions followed by digestion of contaminant DNA with DNAse to maximize the purity of extracted RNA. The purity level of total RNA was confirmed by Nanodrop® 1000 spectrophotometer at the absorbance ratio of 260/280 nm. Values ≈2 indicate pure RNA.

Extracted RNA samples were additionally processed into cDNA synthesis using NZY First-strand cDNA Synthesis Kit (Nzytech- Genes & Enzymes) according to the manu-

facturer's instructions. Complementary DNA (cDNA) consists of a DNA copy synthesized from RNA using a short primer complementary to mRNA 3'end. cDNA strands can be used directly as a template for a posterior PCR. Degradation of the cDNA:RNA hybrids formed after the first-stranded cDNA synthesis is ensured by the addition of RNase H. This step increases RT-PCR sensitivity, as primers will bind more easily to cDNA.

2.3.12.2. Selection of target genes and primers

Gene expression of innate immune receptors NOD1, NOD2, TLR2, TLR4 and TLR9, as well as pro- and anti-inflammatory cytokines and costimulation molecules were analyzed by qRT-PCR.

The 5'-3' (forward) and 3'-5' (reverse) primers of NLRP10, TLR4, IL-1β, IL-12p35, IL-13, IL-3, and CCL3 were designed using Primer3 software (Untergasser A *et al.*, 2012). The NOD1, NOD2, and TLR9 primers had been previously designed by our group, and the remaining primers were already described by several authors, as shown in Table 7.

To correct possible flaws in the reverse transcription reaction, the quantification of gene expression was regulated by a constitutive expressing gene (housekeeping gene) that must be expressed at the same level in different tissues and at different stages of the development of an organism. Therefore, β-actin was used as endogenous control.

For each pair of primers (Stabvida, Portugal), PCR conditions were optimized by changing the annealing temperature (TAN) and duration of cycles until the desired fragment was the only DNA amplified in each sample. TAN of each gene fragment was calculated by subtracting 5 ºC from the primer melting temperature specified by primer's manufacturer. To establish the PCR conditions, TAN was gradually increased by 0.5 ºC until the required amplification specifications were set. For all the pairs of primers used in this study, it was confirmed the absence of cDNA amplification in dog, mouse and *L. infantum* samples, evidence high specificity of the primers.

The PCR product was electrophoresed on 3% (w/v) agarose gel (Sigma-Aldrich) to which 6 μL of GelRed (0.1 $\mu L.mL^{-1}$ of 10000 × GelRed Nucleic Acid Stain Biotium, USA was

added) in 1 × TAE buffer (0.04 M Tris-HCl pH 8, 0.02 M sodium acetate and 0.00 2M EDTA) for about 120 min. at 80 V. The presence of the PCR product was confirmed in the gel by visualizing the fluorescence emission of the GelRed intercalated with the DNA in a transilluminator under ultraviolet light.

2.3.12.3. Purification and cloning of the amplified product in plasmid vectors

The PCR products were purified using a commercial kit, INPREP DOUBLE pure kit (Analytik Jena AG, Germany) following the manufacture's instructions. The purified product was inserted into a plasmid vector, pGEM®-T Easy Vector (Promega, USA) which has T-terminal's at its ends. The BIOTAQ DNA polymerase used has proofreading activity by adding poly-A tails to the amplified product, which allows its binding to the plasmid. For each ligation reaction was used 5 µL of 2 × binding buffer, 1 µl of the enzyme T4 ligase, 1 µl of the vector, and an adequate amount of insert (purified DNA) in a final volume of 10 µl. The solution was then incubated for 1 h at room temperature (RT) and then 8 h at 4ºC.

The amount of insert used in the reaction was determined by the following formula:

$$\text{Insert}\ (\text{ng}) = \frac{[\text{vector (ng) x insert size 0.5 (kb) x Proportion (insert:vector)}]}{\text{vector size (kb)}}$$

The plasmid was used to transform competent NZY5α bacteria (Nzytech- Genes & Enzymes). Competent cells were placed with the plasmid suspension. The mixture was incubated for 30 min. on ice, subjected to a thermal shock at 42ºC for 60 s and placed again on ice for 2 min. Super optimal broth with catabolite repression (SOC, Invitrogen, USA) was added to each reaction, followed by incubation at 37ºC, with shaking at 250 rpm for 3 h. 250 µl of the culture was placed on plates containing Luria-Broth medium (LB, Sigma-Aldrich) with 1.5% (w/v) agar (Sigma-Aldrich) supplemented with 100 $\mu g.mL^{-1}$ of ampicillin (Sigma-Aldrich), 80 $\mu g.ml^{-1}$ of X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside).

Table 7. List of forward (FW) and reverse (RW) primers, base-pair number (bp) of amplified fragments and primer annealing temperature (TAN) for each gene studied.

Gene	Primer	Reference	Fragment size (bp)	TA (°C)
β-Actin	FW-5'ACGGAGCGTGGCTACAGC3' RW-5'TCCTTGATGTCACGCACGA3'	Sauter *et al., 2005*	61	60.5
TLR2	FW-5'AATCCCCCGTTCAAGTTGTG3' RW-5'ATGGTTTTGCGGCTCTTCTC 3'	Ishii *et al.,2006*	101	61
TLR4	FW-5'AGAGGATTTCCCCATTGGAC3' RW-5'ACGCAGGTAGCTTGAAGGAA3'	*	86	56
TLR9	FW-5'ACCACATCATCACCCTGGCACCT3' RW-5' CGGCGACAGTTCCCACCCAC 3'	Rodrigues *et al* ., 2017	82	64
NOD1	FW-5' CCTTGGCTGTCGGAGATTGGCT 3' RW-5' ACCTGCTTACTGGGTCGGTGT 3'	Rodrigues *et al* ., 2017	82	61
NOD2	FW-5' TGGCGTGGGAGCAGGGTTTC 3' RW-5' CGCTGGGAGGATGTGAAGATGG 3'	Rodrigues *et al* ., 2017	76	66
NLRP10	FW-5'CCATGAGTGTGCGTGGATAC3' RW-5'TGTGCAAGGGTGTGTTTCAT3'	*	73	57
CD80	FW-5'GCAGCAGAAGCCATGGATTAC3' RW-5'CACCAAGAGCTGAGAGACCTTGA3'	Yasunaga et al., 2002	84	60
CD86	FW-5'CGAAACCCACCCCTGATG3' RV-5'CACAAAATGACCAACATTACAAGCA3'	Yasunaga *et al* ., 2002	70	60
TGF-β	FW-5'CAGAATGGCTGTCCTTTGATGTC3' RW-5'AGGCGAAAGCCCTCGACTT3'	Huang, 2008	79	60
IL-10	FW-5'CAAGCCCTGTCGGAGATGAT3' RW-5'CTTGATGTCTGGGTCGTGGTT3'	Do-Hyeon *et al.,* 2010	78	54
IL-12p35	FW-5'ATGACGGTCCTGTGCCTTAG3' RW-5'CTGCCTCTTGGGATCCATTA3'	*	102	57
IL-12p40	FW-5'CAGCAGAGAGGGTCAGAGTGG3' RW-5'ACGACCTCGATGGGTAGGC3'□	Peters *et al.,* 2005	109	58
IL-1α	FW-5'GTTGTTCATTGCCACACAGG3' RW-5'CAAGGCTGGGTTTCCAGTAA3'	*	93	56
IL-1β	Fw-5'CCATGAGTGTGCGTGGATAC3' Rv-5'TGTGCAAGGGTGTGTTTCAT3'	*	80	60.5
IL-8	FW-5'CACTCCACACCTTTCCATCC3' RW-5'GTCCAGGCACACCTCATTTC3'	Harman *et al.* , 2014	120	60
IL-18	FW-5'CTCTCCTGTAAGAACAAAACTATTTCCTT3' RW-5'GAACACTTCTCTGAAAGAATATGATGTCA3'	Argyle *et al.,* 1999	100	56
CCL3 (MIP-1α)	FW-5'CCAGGTCTTCTCTGCACCAT 3' RW-5'AACTTGCGTGGAATCTGCTT3'	*	84	59
CCL4 (MIP-1β)	FW-5'TCCTACTGCCTGCTGCTT3' RW-5'GCTGGTCTCAAAGTAATCTGC3'	Menezes-Souza *et al* ., 2012	76	58
IFN-γ	FW-5' TCAACCCCTTCTCGCCACT 3' RW-5' GCTGCCTACTTGGTCCCTGA 3'	D. Menezes-Souza et al., 2011	113	60
TNF-α	FW-5' AATCATCTTCTCGAACCCCAAGT 3' RW-5' GGAGCTGCCCCTCAGCTT 3'□	Sauter *et al* ., 2005	75	57
IL-13	FW-5'CCCAGTGACTCGGGTTTAGA3' RW-5'GCAGCATCTCTGACCCTTTC3'	*	116	60

* indicates the primers designed with Primer3 software

(Sigma-Aldrich) and 0.5 mM IPTG (sopropyl-beta-D -thiogalactopyranoside) (Sigma-Aldrich), to allow the identification of recombinants through the differentiation of white and blue colonies. The plates were incubated at 37ºC for 18 h. The white colonies [resulting from the interruption of the gene sequence encoding β-galactosidase (lacZ) due to the presence of the recombinant plasmid with the cloned insert] were picked and placed in LB medium supplemented with 100 $\mu g.mL^{-1}$ ampicillin and incubated at 37ºC with shaking at 250 rpm for 18 h.

Plasmid DNA (pDNA) was extracted using the innuPREP Plasmid Mini Kit (Analytik Jena AG, Germany) according to the manufacturer's instructions. Briefly, alkaline lysis of the bacterial suspension was performed and the resulting supernatant was centrifuged in order to eliminate impurities. The binding of pDNA to the column was established, followed by washing and elution. The pDNA was analyzed using conventional PCR and electrophoresis on 3% (w /v) agarose gel to confirm the presence of the insert in the plasmid.

2.3.13. Real-time PCR

For the absolute quantification of DNA copies, calibration curves were established for each gene using the plasmids with the insert fragment. pDNA was quantified and, for each gene, 1:2 serial dilutions were prepared, starting from 625 $pg.\mu l^{-1}$ to 9.77 $pg.\mu l^{-1}$ in ultrapure water. Samples were analyzed in triplicate for each gene. For each sample, 20 µl of the real-time PCR reaction mixture was prepared with 2 µl of pDNA, 10 µl of SsoAdvanced Univ SYBR Green Supermix (Bio-Rad), 0.15 µl of 20 $pmol.\mu l^{-1}$ forward primer solution, 0.15 µl of 20 $pmol.\mu l^{-1}$ reverse primer solution, and 7.7 µl of ultra-pure water. Amplification was done in Bio-Rad CFX Maestro PCR System thermal cycler (Bio-Rad, UK) under the following conditions: 5 min. at 95ºC for complete DNA denaturation, 40 cycles of 30 s at 95ºC and 30 s at primer/gene-specific TAN (Table 1) for annealing and extension, and 90 cycles of 10 s at starting temperature of 50ºC with an increment of 0.5ºC for each cycle. The fluorescence levels of each sample were analyzed in real-time by the thermal cycle and the amount of DNA amplified was calculated by comparing it with the calibration curves. Then, the number of gene copies was calculated

as follows, where $9.1x10^{11}$ is the amount of DNA base pairs in 1 μg of DNA, and 3015 is the length in base pairs of the plasmid used:

$$Gene\ copy\ number = \frac{9.1 \times 10^{11} \times quantity\ (\mu g)}{3015 + \text{insert length (bp)}}$$

2.3.14. Statistical analysis

Assays were realized in, at least, 10 different dogs, except SEM, and immunofluorescent assays that were performed on samples from three dogs. Each sample was analyzed in duplicate. The Wilcoxon test for paired samples was used to perform the statistical analysis as is the non-parametric equivalent of the paired samples t-test. It should be used when the sample data are not normally distributed, as is the case of the present study.

Differences were considered significant with a 5% significance level (p<0.05). GraphPad Prism version 9 for Windows (GraphPad Software, USA) was used for statistical analysis and graphical representation of data.

2.4. Results

2.4.1. Monocyte differentiated dendritic cells (moDCs) exhibit characteristic morphology

The implemented methodology to achieve differentiated DCs derived from blood monocytes showed that 7 days is the optimal time to obtain canine differentiated moDCs. After 24 h and 72 h of incubation, cells exhibited a uniform round shape. However, after 7 days of differentiation, cells showed a dramatic change in morphology, presenting an elongated shape and irregular surface, and showing filiform cytoplasmic projections compatible with the conventional DC description (Fig. 13 d, e and f, Fig. 14D). Moreover, when compared with *in vitro* monocyte-derived macrophages (MDM), using a protocol distinct from the protocol used to differentiate DCs, it is possible to verify that both cells present distinct topography (Fig. 13 a, b and c). MDM present a round shape and irregular surface (Fig. 14A, B and C) while moDCs are elongated, showing cytoplasmatic projections and a less irregular surface.

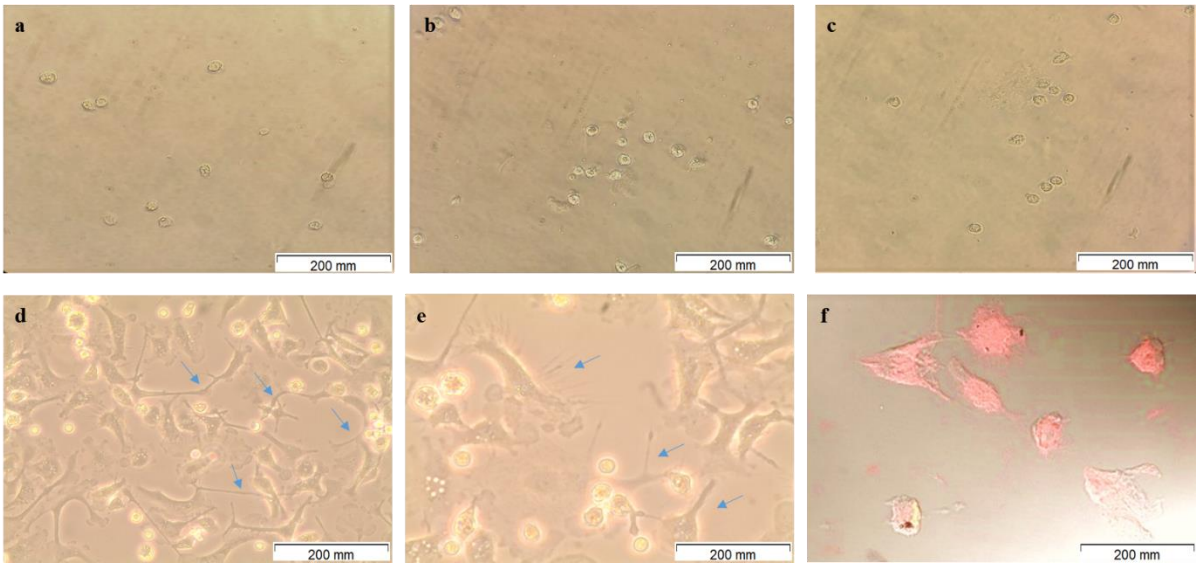


Figure 13. Following the morphological differentiation of monocyte-derived dendritic cells. The morphology changes of dog mononuclear cells incubated in the appropriate conditions for differentiation of moDCs were followed by microscopy and images were acquired. After 24 h (a-d) and 7 days of differentiation (d and c), cells were observed under an inverted light microscope (magnification ×40). After 7 days of differentiation, cells were also observed by confocal microscopy (f). Blue arrows indicate cell cytoplasmic projections.

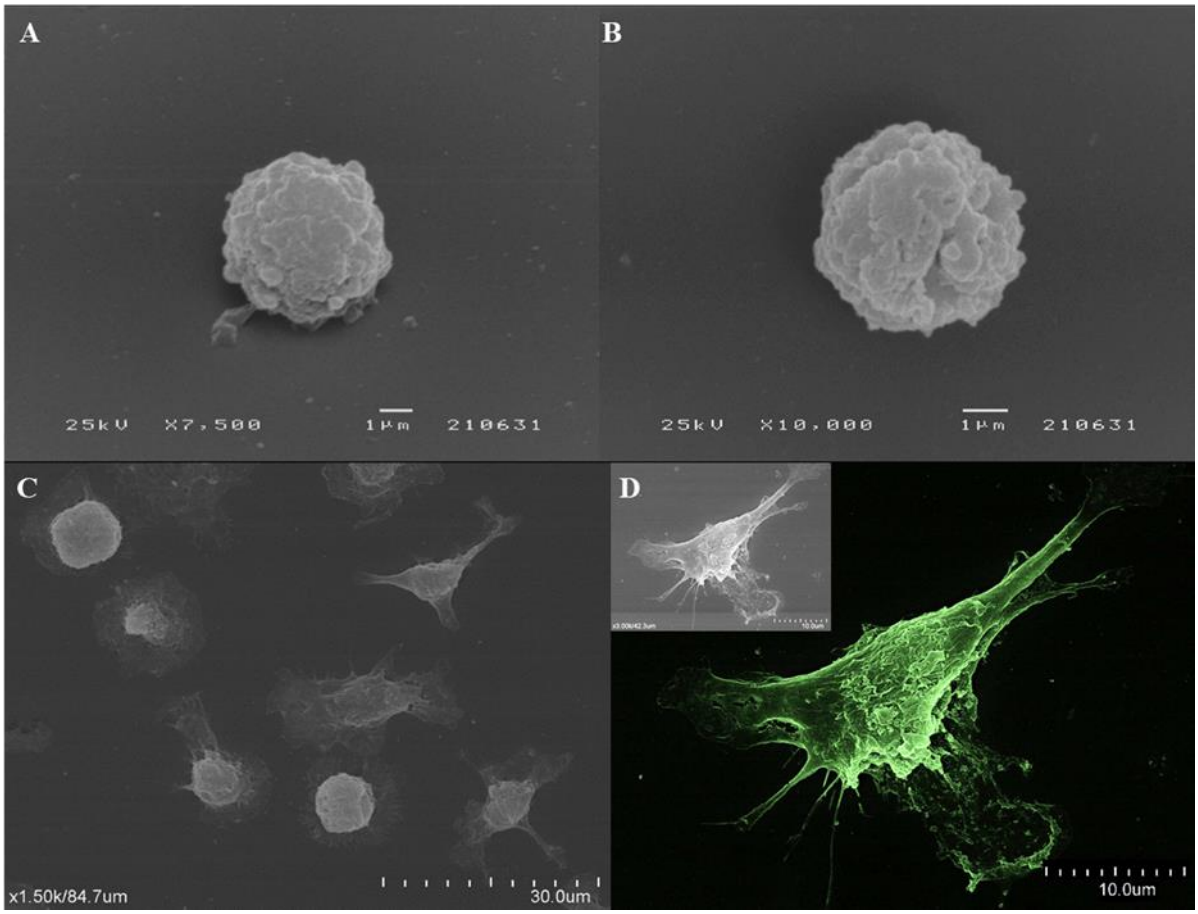


Figure 14. Topography of dog MDM and moDCs. The topography of dog mononuclear-derived macrophages and mononuclear-derived DCs were observed by scanning electron microscopy and images were acquired. MΦ with 5 days of differentiation (A, B) and moDC with 7 days (C and D) can be observed.

Taken together these results indicate that the protocol implemented was successful in differentiating cells with morphology compatible with DC.

2.4.2. moDCs exhibit a specific molecular signature

To verify the molecular signature of *in vitro* differentiated canine moDC, the expression of CD1a, CD11c, CD14 and CD83 surface molecules were analyzed by multiparametric flow cytometry. An identical analysis was also performed on MDM.

Most moDC exhibited a $CD11c^+CD1a^+CD83^+$ phenotype (Fig 15a) and lower frequency of cells expressing CD14. In turn, the majority of MDM showed a $CD14^+CD11c^+CD1a^+$ phenotype while a reduced proportion of cells express CD83 molecules (Fig. 15b). The frequency of cells that express CD14 and CD83 molecules were significative different in moDCs (P = 0.0005) and MDM (P = 0.0313).

When comparing the frequency of $CD14^+$, $CD11c^+$, $CD1a^+$ and $CD83^+$ moDCs with $CD14^+$, $CD11c^+$, $CD1a^+$ and $CD83^+$ MDM, moDC population showed a twofold increase of cells expressing CD83 (Fig. 13c).

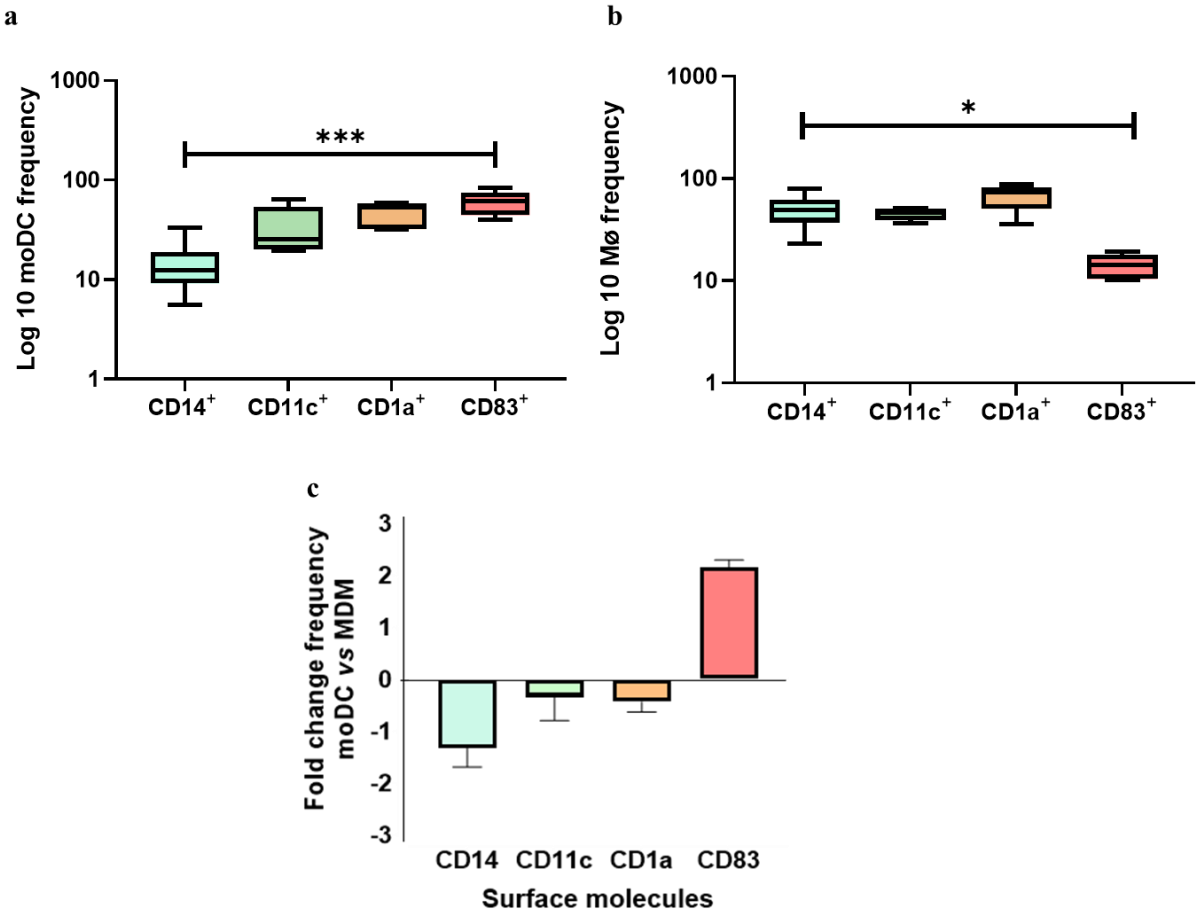


Figure 15. Phenotypic profile of MDM and moDCs. After being labelled with monoclonal antibodies anti-CD1a, CD11c, CD14 and CD83 directly conjugated, moDC (a) and MDM (b) were evaluated by multiparametric flow cytometry, Results of at least five dogs performed in duplicate are represented by box plots and whiskers (minimum to maximum). The non-parametric Wilcoxon test was used for statistical comparisons. *(P < 0.05) and *** (P < 0.001) indicate statistical significance differences. The fold change of the frequency of $CD1a^+$, $CD11c^+$, $CD14^+$ and $CD83^+$ moDC were compared with MDM (c). Data mean and SEM are represented by column bars graph.

However, MDM presents the higher surface density of CD14, CD1a molecules when compared with moDC, but CD11c had a low density in both cells (Fig. 16).

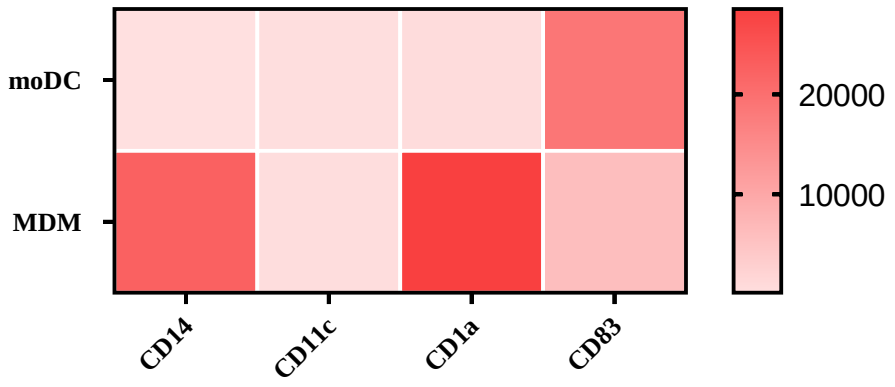


Figure 16. Density of CD1a, CD11c, CD14 and CD83 molecules at moDCs surface. moDC and MDM labelled with monoclonal antibodies anti-CD1a, CD11c, CD14 and CD83 were evaluated by multiparametric flow cytometry and the fluorescence intensity of each fluorometer was analyzed. The mean density of at least five dogs performed in duplicate is represented by the heat map.

Despite both cells being derived from the same dog cell (monocytes), during the differentiation process, there was a shift in the expression of the evaluated surface molecules. In contrast with MDM, the moDC population showed a predomination of $CD83^{+}$ cells but a lower density of the evaluated surface molecules, with exception of CD11c.

2.4.3. moDC binds and internalizes *L. infantum* and *L. amazonensis* parasites

To examine if moDCs are functional and can recognize and internalize pathogenic agents the interaction of moDC with *Leishmania* parasites was observed by SEM. After 5 h of incubation (Fig.17a), promastigotes seem to establish contact with dendrites. moDC exposed to *L. amazonensis* and *L.infantum* GFP promastigotes for 24 h observed by optical (Fig. 17b and c), confocal (Fig. 17f) and fluorescence (Fig.17d and e) microscopy show intracellular amastigote forms.

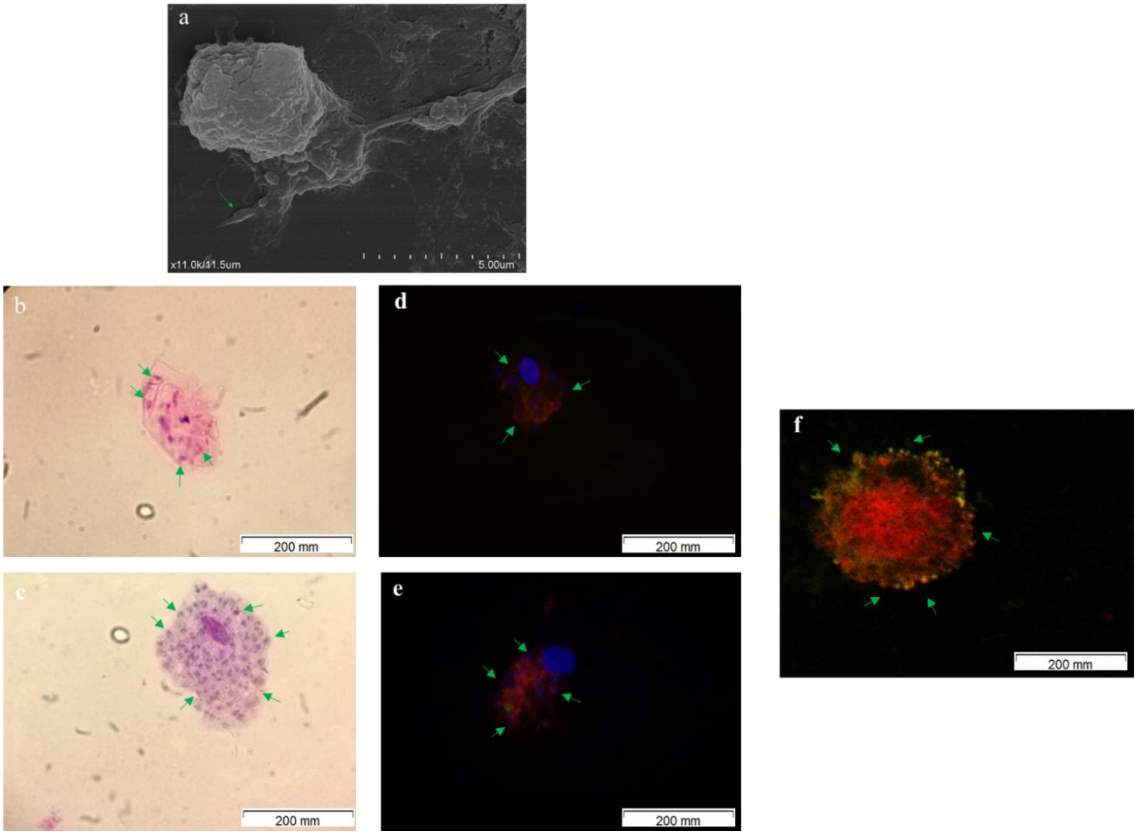


Figure 17. *L. infantum* and *L. amazonenesis* promastigotes bind to moDC and are uptake. The interaction of *Leishmania* promastigotes with moDC was observed by SEM (a). Arrow points to a promastigote. Cytospin slides from moDCs incubated with *L. amazonensis* promastigotes for 24 h, stained with Giemsa (b and c) were observed by optical microscopy (×1000 magnification). Arrows indicate intracellular amastigote forms. Cytospin slides from moDCs incubated with *L. infantum* -GFP promastigotes for 24 h and stained with DAPI were observed by fluorescence (×1000 magnification) (d and e) and confocal microscopy (f). The nucleus of moDCs and *Leishmania* can be seen in blue (d and e). moDC cytoplasm is red and the parasite internalized forms present a yellow/orange colour (f). Green arrows indicate the intracellular parasites

Therefore, dog moDCs seem to be functional. These cells differentiated *in vitro* recognize *Leishmania* promastigotes of two different species, internalize the parasites and support the differentiation into amastigotes.

2.4.4. *Leishmania* infection causes a slight increase of viable moDC

To evaluate the effect of internalized parasites on moDC, cell viability was assessed by multiparametric flow cytometry. In comparison with resting moDCs, which represent the natural cell progression, *L.infantum* or *L.amazonensis* infected moDCs showed a slight increase in the proportion of viable cells ($P_{Li \text{ and } La}$ = 0.0313) and a slight decrease of apoptotic cells. Furthermore, moDCs exposed to *L.amazonensis* antigen (P= 0.0313) also increase the proportion of viable cells (Fig. 18).

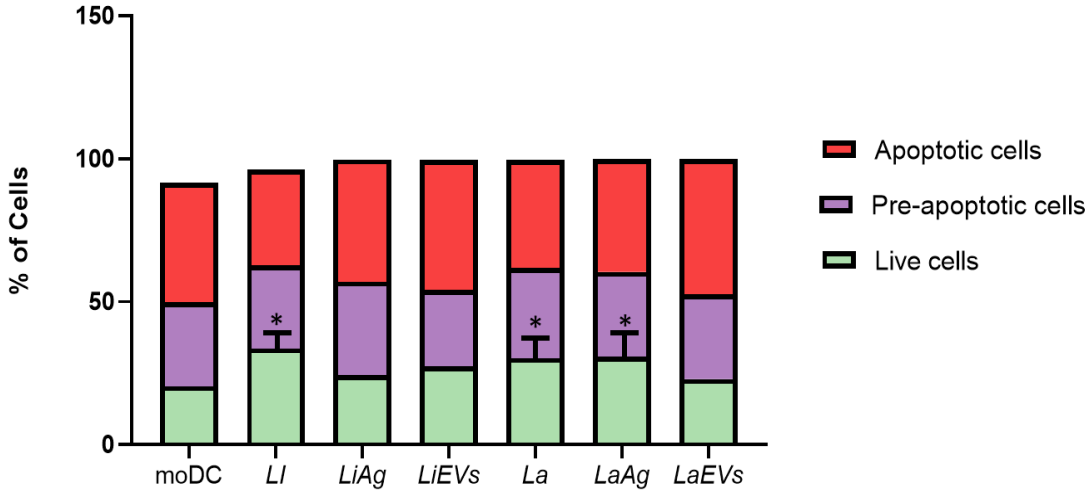


Figure 18. Viability of *L.infantum* and *L. amazonensis* infected moDCs. The viability of *L.infantum* (Li) and *L. amazonensis* (La) infected moDCs and moDCs stimulated by *L. infantum* (*LiAg*) and *L. amazonensis* (LaAg) antigens or by *L. infantum* (*LiEVs*) and *L. amazonensis* (*LaEVs*) EVs was assessed by multiparametric flow cytometry, using annexin-V and propidium iodide (A). The median of 10 dogs and two replicates per sample are represented by stacked bars. * (P < 0.05) indicate statistical significance differences.

Thus, both species of *Leishmania* parasites, as well as *L. amazonensis* antigen favour moDC viability and slightly increase moDCs lifetime.

2.4.5. *L. infantum* infection causes the expansion of MHCI$^+$ moDC subset

To indirectly evaluate the ability of infected moDC and moDCs exposed to parasite antigens (*LiAg* and *LaAg*) and EVs (*LiEVs* and *LaEVs*) presenting antigens through MHC, the surface expression of class I and class II molecules of MHC was evaluated by multiparametric flow cytometry. In parallel, MHC surface expression was also assessed in resting moDCs and moDCs stimulated by PMA.

When compared with resting moDCs, PMA stimulation caused a significant increase of MHCI$^+$ moDCs (P = 0.0078), indicating that these cells were viable and able to present antigens to T lymphocytes.

L. infantum infection caused a significant increase in the frequency of MHCI$^+$ moDCs (P = 0.0313). Moreover, EVs of both *Leishmania* species also promoted a high level of MHCI$^+$ moDCs (P_{LiEVs} = 0.0156, P_{LaEVs} = 0.0078) (Fig. 17a).

In contrast, it was not found any increase in MHCII$^+$moCDs (Fig. 19b) or MHCI$^+$MHCII$^+$ moDCs (Fig. 19c) regardless of being infected or exposed to parasite antigens or EVs. A marked decrease in the frequency of MHCII$^+$ moCDs or MHCI$^+$MHCII$^+$ moDCs was found in *L. infantum* infected moDCs (P=0.0313) as well as in moDCs exposed to *LiEVs* (P=0.0313). Antigens (P_{LiAg}=0.0313, P_{LaAg}=0.0313) also promoted a significant decrease of MHCI$^+$MHCII$^+$ moDCs.

Taking together, these results suggest that an elevated proportion of *L. infantum* infected moDCs can present parasite antigens to CD8$^+$ T cells through MHCI molecules. Furthermore, *L. infantum* EVs seem to enhance a considerable proportion of MHCI$^+$ moDCs that can present parasite antigens as well.

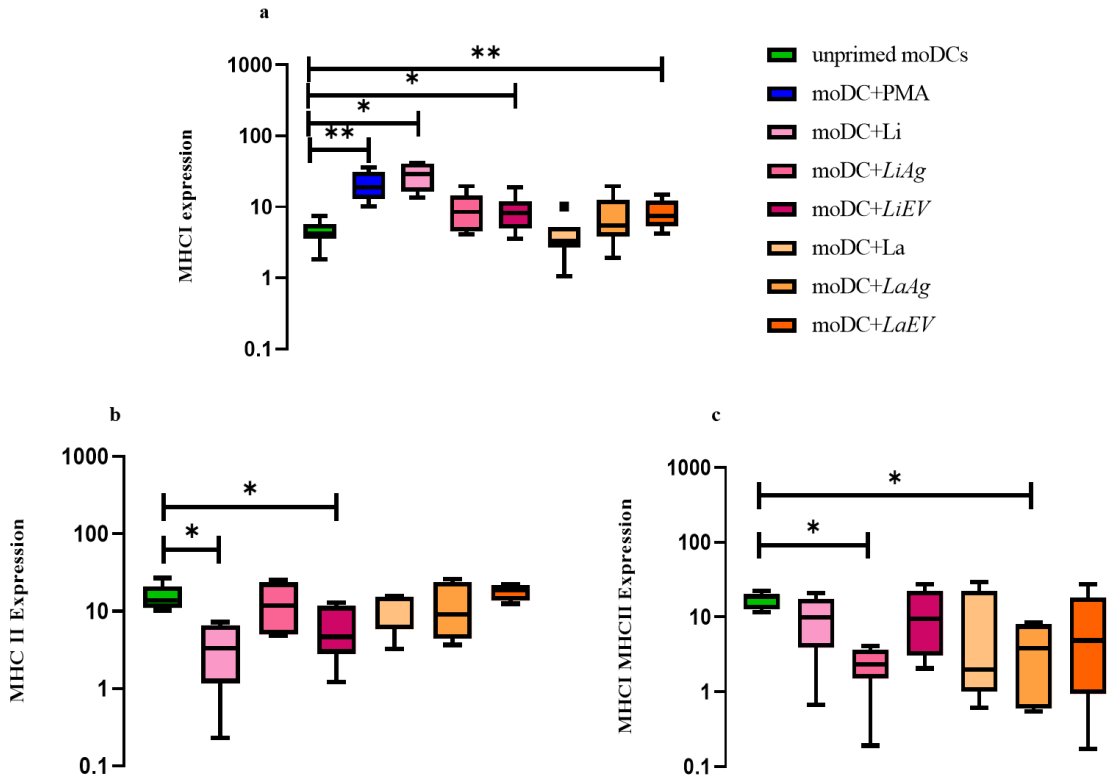


Figure 19. Frequency of MHCI⁺ moDCs infected by *Leishmania* parasites. The proportion of MHCI⁺(a), MHCII⁺(b) and MHCI⁺MHCII⁺(c) moDCs was evaluated in infected moDCs (Li and La) and moDCs exposed to *L. infantum* (*LiAg*) and *L. amazonensis* (*LaAg*) antigens and parasite EVs (*LiEVs* and *LaEVs*) by multiparametric flow cytometry. In parallel, resting and PMA-stimulated moDCs were also assessed. The results of 10 dogs performed in duplicate are represented by Tukey box plots, including the median, interquartile ranges and minimum and maximum values. Black squares are indicative of outliers. Nonparametric Wilcoxon's test was used for statistical comparisons. * ($P<0.05$) and ** ($p<0.01$) indicate significant differences.

2.4.6. *L. amazonensis* upregulates the gene expression of CD80 and CD86 costimulatory molecules in infected moDCs

The expression of CD80 and CD86 was evaluated by real-time PCR in unprimed moDCs, *L. infantum* or *L. amazonensis* infected moDCs, and moDCs stimulated by parasite antigens (*LiAg* and *LaAg*) or Evs.

EVs of both species of *Leishmania* directed the upregulation of CD80 and CD86 ($P_{La\ and\ Li\ CD80}$=0.0156; P_{LaCD86}=0.0117; P_{LiCD86}=0.0010). However, only *L. amazonensis* parasites (P_{CD80} = 0.0156, P_{CD86} = 0.0195) and *L. infantum* antigens (P_{CD80} = 0.0156; P_{CD86}=0.0020) stimulate moDCs to increase the gene expression of these co-stimulatory molecules (Fig. 20a). *L.infantum* infected moDCs (P = 0.0020) exhibited a significantly high accumulation of CD86 mRNA when compared with resting moDCs (Fig. 20b)

The increased gene expression of these co-stimulation molecules is an indication that the generation of CD80/CD86 by moDCs can be modulated by parasites of the cutaneous species of *Leishmania*, by EVs of both species, and by *L. infantum* soluble antigens. Therefore, moDCs expressing both CD80/CD86 can mediate the initial steps of T cell activation. *L.infantum* parasites only upregulate CD86.

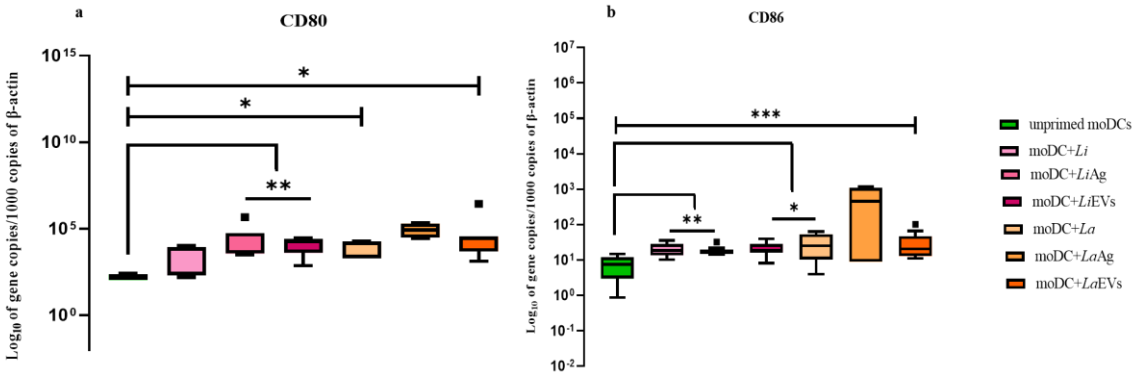


Figure 20. Gene expression of co-stimulatory molecules in *L. infantum* and *L. amazonensis* infected moDCs. RNA extracted from infected moDCs (Li and La) and moDCs exposed to *L. infantum* (*LiAg*) and *L. amazonensis* (*LaAg*) antigens and parasite EVs (*LiEVs* and *LaEVs*) were used to evaluate the gene expression of CD80 (a) and CD86 (b). In parallel, unstimulated moDC was also assessed. The results of 10 dogs performed in duplicate are represented by Tukey box plots, including the median, interquartile ranges, and minimum and maximum values. Black squares are indicative of outliers. Nonparametric Wilcoxon's test was used for statistical comparisons. * (P<0.05), ** (P<0.01) and *** (P<0.001) indicate statistically significant differences.

2.4.7. *L. infantum* infected moDCs generate IL-1β and IL-18

The gene expression of pro- and anti-inflammatory cytokines by *Leishmania* infected moDCs and moDCs exposed to parasite antigens (*LiAg* and *LaAg*) and extracellular vesicles (*LiEVs* and *LaEVs)* were quantified by real-time PCR.

Gene expression of the anti-inflammatory cytokine IL-10 was significantly increased in *L.amazonensis* infected moDCs (P=0.0049) and in moDCs exposed to *LiAg* (P=0.0134) in comparison with resting moDCs (Fig. 21a). On the contrary, *L. amazonensis* -infected moDCs significantly inhibited TGF-β gene expression (P=0.0295) (Fig. 21b).

When compared to resting moDCs, moDCs infected with *L.infantum* or stimulated by parasite antigens showed a significant accumulation of IL-1β (P_{Li}=0.007; P_{LiAg}=0.0007) (Fig 22d) and IL-18 (P_{Li} =0.0391) mRNA (Fig. 22e). On the other hand, *L.infantum* promoted downregulation of IL-1α (P = 0.0353) (Fig. 22c).

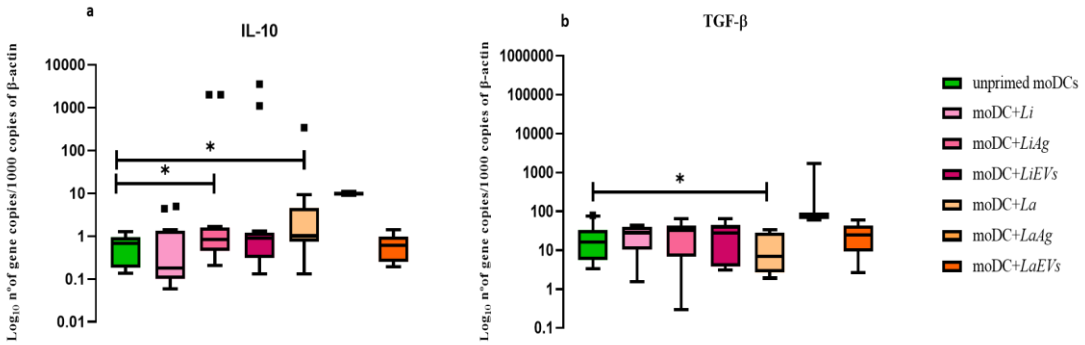
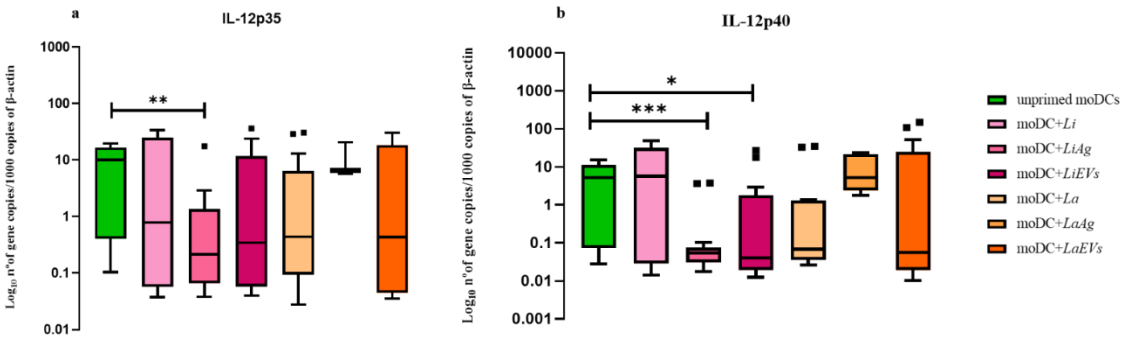


Figure 21. Gene expression of anti-inflammatory cytokines by *Leishmania* infected moDCs.RNA. of infected moDCs (Li and La) and moDCs exposed to *L. infantum* (*LiAg*) and *L. amazonensis* (*LaAg*) antigens and parasite EVs (*LiEVs* and *LaEVs*) were used to evaluate IL-10 (a) and TGF-β (b) gene expression by real-time PCR. In parallel cytokines of unprimed moDCs were also assessed. The results of 10 dogs performed in duplicate are represented by Tukey box plots, including the median, interquartile ranges and minimum and maximum values. Black squares are indicative of outliers. Nonparametric Wilcoxon's test was used for statistical comparisons and *(P<0.05) indicates significant differences.

Dog moDCs infected with *L. amazonensis* parasites upregulated IL-18 (P=0.0049), but significantly inhibited the generation of IL-1α (P=0.0353). In addition, *L. infantum* antigens led to a significant downregulation of IL-12p35 (P=0.0067) (Fig. 22a) and IL-12p40 (P=0.0001) gene expression (Fig. 22b). Moreover, EVs shed by *L. infantum* also reduced 12p40 gene expression (P=0.0386). Whereas *L. amazonensis* EVs increased the expression of IL-1β (P=0.0012).

L. amazonensis induces canine moDCs to generate anti-inflammatory IL-10, in contrast, *L. infantum* parasites selectively induce moDCs to generate pro-inflammatory IL-1β and IL-18, which may be associated with Th cell differentiation.



2.4.8. *L. infantum* and *L. amazonensis* trigger the release of the chemokine CXCL16 from moDCs surface

Supernatants and RNA of *L. infantum* or *L. amazonensis* infected moDCs and moDCs exposed to parasite antigens (*LiAg* or *LaAg)* and EVs (*LiEVs* and *LaEVs)* for 24 h were used to analyse the concentration of CXCL16 chemokine by ELISA and IL-8 (CXCL8) gene expression by qRT-PCR respectively.

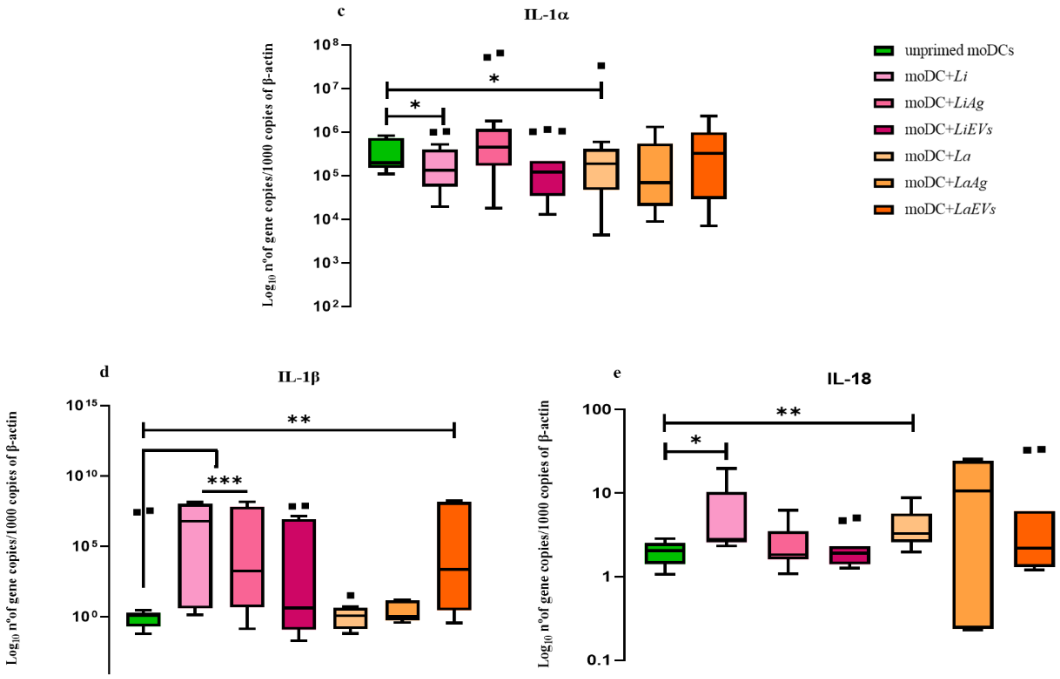


Figure 22. Gene expression of pro-inflammatory cytokines by *Leishmania* infected moDCs. Infected moDCs (Li and La) and moDCs exposed to *L. infantum* (*LiAg*) and *L. amazonensis* (*LaAg*) antigens and parasite EVs (*LiEVs* and *LaEVs*) were used to evaluate IL-12p35 (a), IL-12p40 (b), IL-1β (c), IL-1β (d), and IL-18 (e) gene expression by real-time PCR. In parallel, cytokines of resting moDCs were also assessed. The results of 10 dogs performed in duplicate are represented by Tukey box plots, including the median, interquartile ranges and minimum and maximum values. Black squares are indicative of outliers. Nonparametric Wilcoxon's test was used for statistical comparisons. *(P<0.05), ** (P<0.01) and ***(P<0.001) indicate significant differences.

Overall, there was increase in CXCL16 concentration in infected moDCs (P_{Li}= 0.0273, P_{LA}= 0.0137) and EVs stimulated moDCs (P_{LiEVs} = 0.0137 and P_{LaEVs} = 0.0098) compared to unprimed moDCs (Fig. 23b). In turn, downregulation of IL-8 gene expression (P = 0.0182) was observed in moDCs infected with *L. infantum (*Fig. 23a).

Therefore, these data indicate that *L. infantum* and *L. amazonensis parasites*, as well as EVs regulate moDCs to release chemotactic CXCL16, which can promote immune cell migration to microenvironments enriched into CXCL16. The induction of CXCL16 release indicates that moDCs are ready to attract naïve and memory T cells. In contrast, *L.*

infantum restrains IL-8 gene expression by moDCs, inhibiting other leukocytes from being attracted.

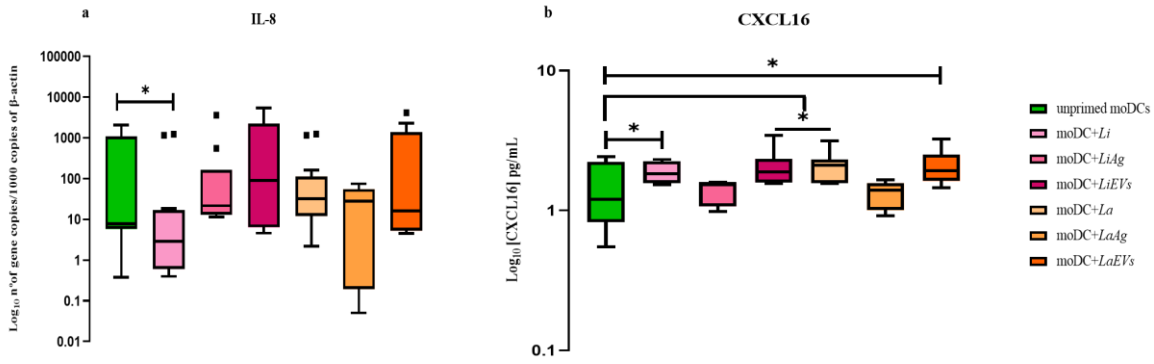


Figure 23. Release of CXCL16 and generation of CXCL8 in *Leishmania* infected moDCs. Supernatants and RNA of infected moDCs (Li and La) and moDCs exposed to *L. infantum* (*LiAg*) and *L. amazonensis (LaAg*) antigens and parasite EVs (*LiEVs* and *LaEVs)* were analyzed by ELISA and real-time PCR. In parallel resting moDCs were also evaluated. The results of 10 dogs performed in duplicate are represented by Tukey box plots, including the median, interquartile ranges, and minimum and maximum values. Black squares are indicative of outliers. Nonparametric Wilcoxon's test was used for statistical comparisons. * ($P<0.05$) indicates statistically significant differences

2.4.9. TLR4 of dog moDC senses *L. infantum* parasites

To investigate the ability of moDCs to recognize parasite antigens through innate sensors, the gene expression of endocytic (TLR9), cytoplasmic (NOD1, NOD2, and NLRP10) and membrane (TLR2 and TLR4) innate receptors was quantified by real-time PCR in *L. infantum* and *L. amazonensis* infected moDCs and in moDCs exposed to parasite antigens (*LiAg* and *LaAg*), and parasite EVs (*LiEVs* and *LaEVs*). In parallel, gene expression of innate sensors of unprimed moDCs (negative control) was also analysed.

In comparison with unprimed moDC, TLR2 gene expression registered a significant increase in moDCs exposed to parasite antigens (P_{LiAg}= 0.0078, P_{LaAg}= 0.0313) and *L. amazonensis* EVs (P_{LaEVs} =0.0020), but decreases when primed by *L.infantum* EVs (P_{LiEVs} = 0.0098) (Fig. 24a).

L. infantum infected moDCs and moDCs exposed to *L. amazonensis* antigens showed a higher accumulation of TLR4 mRNA (P=0.0035) when compared with unprimed moDCs (Fig. 24b).

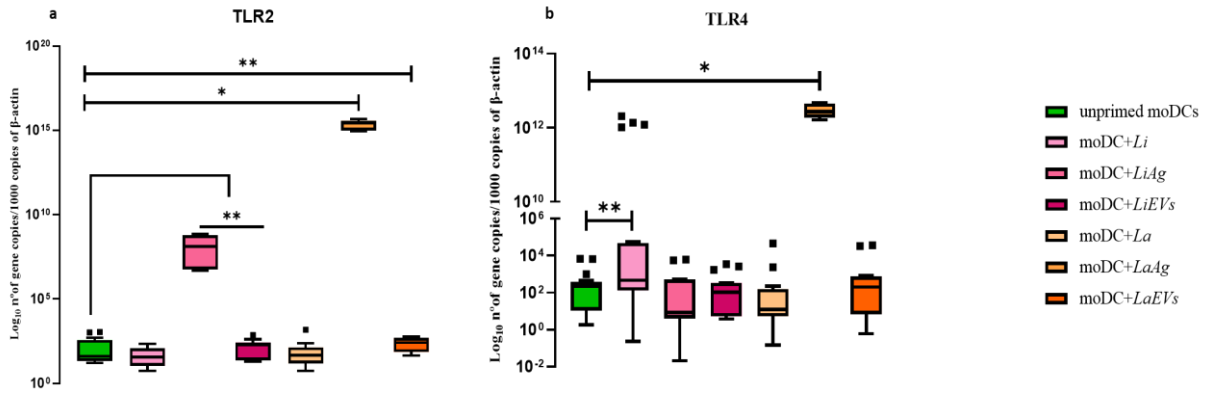


Figure 24. Gene expression of TLR2 and TLR4 by *L. infantum* and *L. amazonensis* infected moDCs. RNA extracted from infected moDCs (Li and La) and moDCs exposed to *L. infantum* (*LiAg*) and *L. amazonensis* (*LaAg*) antigens and parasite EVs (*LiEVs* and *LaEVs*) were used to evaluate the gene expression of TLR2 (a) and TLR4 (b). In parallel, unprimed moDCs were also assessed. The results of 10 dogs performed in duplicate are represented by Tukey box plots, including the median, interquartile ranges and minimum and maximum values. Black squares are indicative of outliers. Nonparametric Wilcoxon's test was used for statistical comparisons, and * (P<0.05) and ** (P<0.001) indicate statistically significant differences.

In contrast, gene expression of NOD1 significantly decreases when exposed to *LiEVs* (P = 0.0385) in comparison to unstimulated moDC (Fig. 25a). In turn, NOD2 does not show significant differences regarding infected moDCs or moDCs stimulated by parasite antigens or EVs (Fig. 25b).

L. infantum infected moDCs showed a marked decrease (P = 0.0068) in NLRP10 gene expression when compared with unstimulated moDC (Fig. 25c). Moreover, the accumulation of TLR9 mRNA significantly decreased in *L. amazonensis* infected moDCs (P=0.0052), and in moDCs exposed to *L. infantum* EVs (P=0.0034) (Fig. 25d) and *L. amazonensis* antigen (P=0.0313) as well.

Thus, *L. infantum* and *L. amazonensis* parasites show different effects on moDCs sensors. *L. amazonensis* seemed to downregulate TLR9 gene expression and *L. infantum* inhibited the accumulation of NLRP10 mRNA. However, *L. infantum* and *L amazonensis* soluble antigens and *LaEVs* may signalize moDCs through TLR2. TLR4 also seems to sense *L.amazonensis* antigens and *L. infantum* parasites. *L. amazonensis* parasites do not seem to signalize moDCs through the PRRs evaluated in this study.

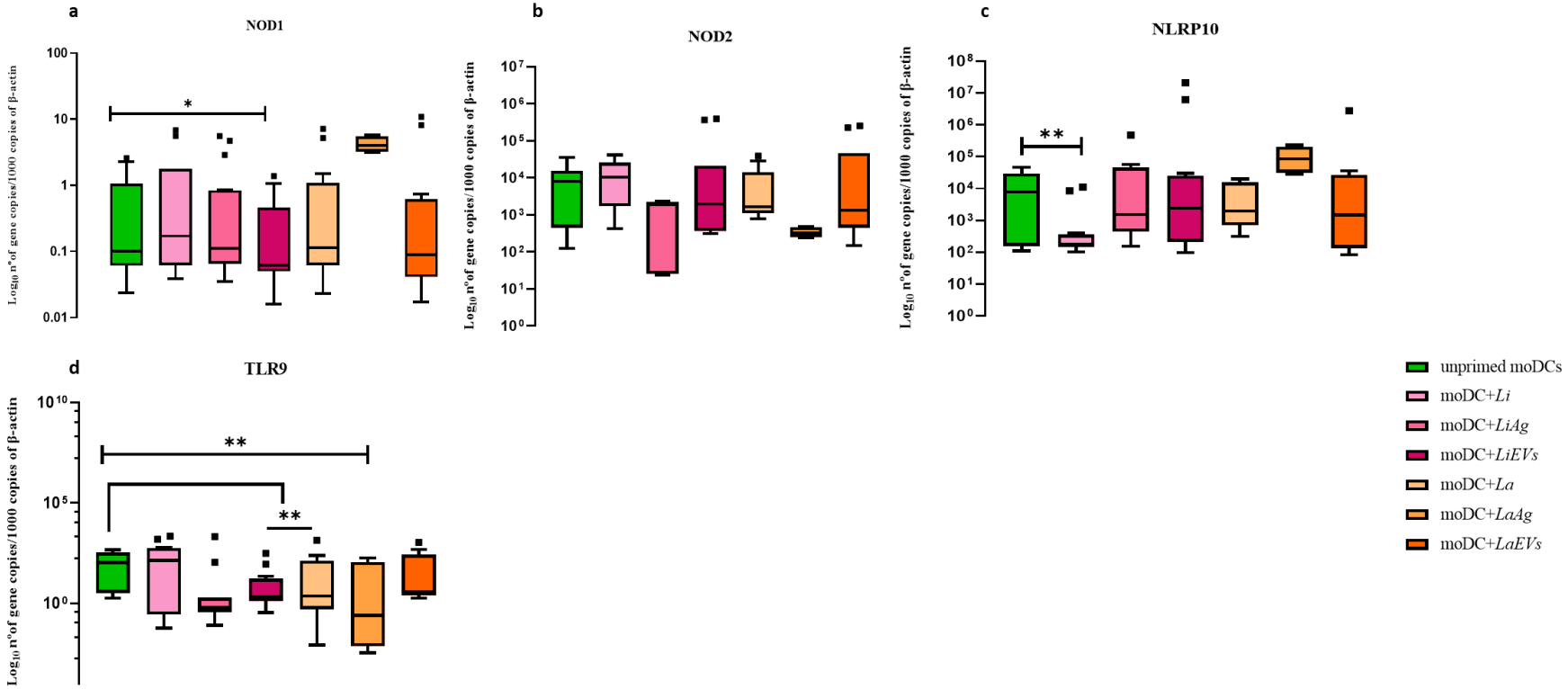


Figure 25. Gene expression of endocytic innate receptors of moDC infected by *L. infantum* and *L. amazonensis* parasites. RNA extracted from infected moDCs (Li and La) and moDCs exposed to *L. infantum* (*LiAg*) and *L. amazonensis* (*LaAg*) antigens and parasite EVs (*LiEVs* and *LaEVs*) were used to evaluate the gene expression of NOD1 (a), NOD2 (b), NLRP10 (c) and TLR9 (d). In parallel unstimulated moDC were also assessed. The results of 10 dogs performed in duplicate are represented by Tukey box plots, including the median, interquartile ranges and minimum and maximum values. Black squares are indicative of outliers. Nonparametric Wilcoxon's test was used for statistical comparisons. * ($P<0.05$) and ** ($P<0.001$) indicate statistically significant differences.

2.4.10. *L.infantum* infected moDCs increase the expression and translocation of NF-κB to the nucleus

NF-κB expression and translocation to the nucleus were analyzed in *L. infantum* and *L. amazonensis infected* moDCs, moDCs incubated with parasite antigens and EVs of both parasite species and in resting moDCs as well.

L. infantum infected moDCs exhibited a significantly high expression and translocation of NF-κB to the nucleus (P=0.0078) when compared to unstimulated moDCs Furthermore, moDC exposed to *LiAg* (P=0.0391) and *LaEVs* (P=0.0078) also showed a significant increase of NF-κB (Fig. 26).

These results point toward the activation and translocation of NF-κB to the nucleus of *L.infantum* infected moDCs, directing the transcription of genes coding for chemokines, pro-inflammatory cytokines and costimulatory molecules. Moreover, *L.infantum* antigens also promote the translocation of this transcription factor to moDC nucleus. Curiously, *L. amazonensis* parasites do not seem to affect the activation NF-κB of moDC but in contrast, L. *amazonensis* EVs play a role in activating this protein complex that regulates nuclear gene transcription.

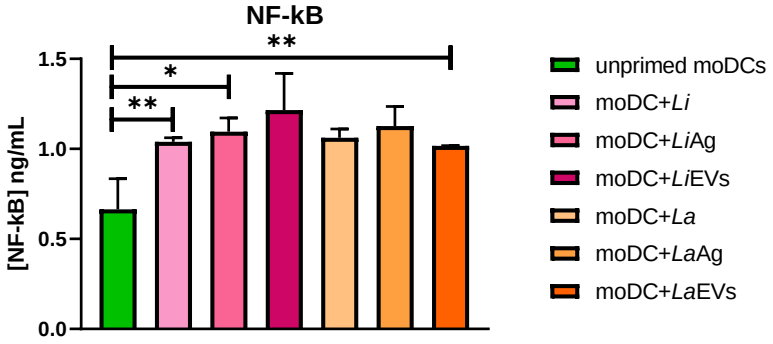


Figure 26. Expression and translocation of NF-κB to the nucleus in *Leishmania* infected moDCs. Lysates of infected moDCs (Li and La) and moDCs exposed to *L. infantum* (*LiAg*) and *L. amazonensis (LaAg*) antigens and parasite EVs (*LiEVs* and *LaEVs)* were analyzed by ELISA. In parallel resting moDCs were also evaluated. The mean and standard error of 10 dogs performed in duplicate are represented by column bar graph. Wilcoxon's nonparametric test was used for statistical comparisons. *($P<0.05$) and ** ($p<0.001$) indicate significant differences.

These results point toward the activation and translocation of NF-κB to the nucleus of *L.infantum* infected moDCs, directing the transcription of genes coding for chemokines, pro-inflammatory cytokines and costimulatory molecules. Moreover, *L.infantum* antigens also promote the translocation of this transcription factor to moDC nucleus. Curiously, *L. amazonensis* parasites do not seem to affect the activation NF-κB of moDC but in contrast, *L. amazonensis* EVs play a role in activating this protein complex that regulates nuclear gene transcription.

2.5. Discussion

DCs are the only APCs capable of attracting and activating naïve $CD4^+$ and $CD8^+$ T cells. They are also considered the most efficient APCs, as the decrease in proteolytic activity and the acidity of their endocytic compartments reduces the rate of antigen digestion and consequently increases the availability of partially processed peptides for presentation via the MHC (Liu, 2016). The balance of T cell immunity mechanisms relies greatly on the activity of DC subtypes found in inflammation sites, such as moDCs that act as inducers of Th1, Th2, and Th17 immune responses (Giza and Bozzacco, 2021). The biological cycle of *Leishmania* is initiated when parasite promastigote forms are inoculated into the skin of a mammalian host by female phlebotomine sandflies during blood feeding. Inside the vertebrate host, MΦ and DC take up parasites, which can then survive inside MΦ, the definite *Leishmania* host cell (Arango Duque and Descoteaux, 2015). According to the literature, it seems that DCs are also key elements in the early interaction with *Leishmania* parasites, which are believed to be important for the outcome of the infection (Liu *et al.*, 2012). Thus, the complex interactions that occur between DC and parasites can lead to the establishment of an effective immune response against this pathogen.

In the early 1990s, the observation that human monocytes cultured *in vitro* with GM-CSF and IL-4 generate DCs (Sallusto-and Lanzavecchia, 1994) paved the way for the description of protocols that allow the production of these cells. Indeed, the *in vitro* differentiation and maturation of moDCs represent an attractive alternative to complicated and poorly reproducible methods involved in isolating DCs from tissues (Cabeza-Cabrerizo *et al.,* 2021). For this reason, moDCs become commonly used to study human DC biology (Da Silva Simoneti *et al.,* 2014, Chometon *et al.,* 2020) as well as in other mammal species, such as the dog.

After seven days in culture in the presence of rIL-4 and GM-CSF cocktail, monocyte-derived DCs showed a typical DC-like morphology with long cytoplasmic projections compatible with dendrites. Cytoplasmic projections are the most characteristic morphological features of DCs, distinguishing them from MΦ. In the present study moDCs, changed their morphology over time, they began to be more stretched and to present cytoplasmic projections in agreement with dendrites. In addition to their characteristic morphology, dog moDCs exhibit certain species-specific properties. Contrary to human and

mouse DCs, canine moDCs do not lose the ability to express CD14 after *in vitro* maturation (Carrasco *et al.,* 2001, Wijewardana *et al.,* 2001, Bienzle *et al.,* 2003, Ibisch *et al.,* 2005, Gutzwiller *et al.,* 2019). After seven days in culture, canine moDCs express high levels of MHCII and CD11c (Ibisch et al., 2005, de Carvalho et al., 2006). Moreover, these cells show increased expression of CD1a, CD40 and CD83, as well as the co-stimulatory molecules, CD80 and CD86 (Wang et al., 2007, Gutzwiller et al., 2010). The family of CD1 molecules, which facilitate the presentation of non-protein antigens, are highly expressed in canine DCs (Affolter and Moore, 2002). Consequently, none of the typical markers that are used to differentiate monocyte/macrophage DCs in humans and mice, such as CD1c, CD11c, and CD14, allow for accurate identification of canine DCs. In the current study, the percentage of gated cells was determined to characterize the phenotype of MΦ and moDCs. Both MDM (5 days of differentiation) and moDCs (7 days of differentiation and maturation) revealed a predominant frequency of CD14, CD11c, CD1a and CD83 (except in MDM). According to several studies, these surface markers are present on canine MΦs/monocytes but at a lower density when compared to canine moDC (Affolter *et al.,* 2002, Ibisch *et al.,* 2005, Wang *et al.,* 2007, Goto-Koshino *et al.,* 2011). In the current study, there is a lower frequency of $CD14^+$ moDC and a high frequency of $CD83^+$ moDC, being the exact opposite in MDM. CD83 is a member of the immunoglobulin (Ig) superfamily and can be detected on a variety of activated immune cells, although it is higher and more stable on mature DC. DCs are not the only cells that express CD83, which is also found transiently on a wide range of leukocytes (Ju *et al.,* 2016, Tanaka *et al.,* 2015). While CD83 expression on activated DCs was sustainable, its expression on macrophages was transient. During the *in vitro* generation of moDCs, IL-4 has been considered essential for the stable expression of surface CD83 (Cao *et al.,* 2005).

In the present study, the moDCs have a completely different molecular signature from the MDMs. Our dog moDCs express large amounts of $CD83^+$moDCs, which plays a relevant role in the regulation of antigenic presentation and is recognized as a mature CD marker. They also express CD1a necessary for the presentation of lipid antigen and CD11 which is the complement factor 4 receptor α chain important in phagocytosis, as well as CD14 which is the coreceptor of TLR4 essential in the recognition of antigenic patterns.

Leishmania parasites are obligate intracellular parasites that reside mainly in mammalian host MΦs, but are also phagocytosed by other cells, such as DCs. In addition to being important mediators between innate and adaptive immunity, DCs are also recognized for their highly efficient phagocytic power (Collin *et al.*, 2018). Normally, for parasite internalization to occur, mobilization of cytoskeletal elements as well as specific receptor-ligand interactions have to occur (Jaumouillé and Grinstein, 2017). The uptake of *Leishmania* by DCs depends on the life form of the parasite, promastigote or amastigote, as DCs preferentially phagocytosis IgG-coated amastigotes. GP63 mediates the conversion of complement factors (iC3b binds to CR3) resulting in *Leishmania* adherence to the surface membrane of DCs (Woelbing *et al.*, 2006). Then, the internalization of amastigotes involves the participation of the receptors FcgRI and FcgRIII (Woelbing *et al.*, 2006). Argueta-Donohué and colleagues (2016) demonstrated that DC-SIGN, a surface receptor found primarily on DCs, efficiently mediates high internalization rates of *L. mexicana* promastigotes after 3 h of in *vitro* infection. *In vitro* infection studies have collectively indicated that mDCs (but not LCs) can efficiently engulf promastigotes. In the present work, moDC phagocytoses both *L.infantum* and *L.amazonensis* promastigotes after 24 h of contact and allow the intracellular differentiation of promastigotes into amastigotes-like forms. Margaroni and collaborators (2022) showed that almost 50% of bone marrow-derived dendritic cells (BMDCs) were effectively infected by *L. infantum* promastigotes, after 24 h, demonstrating successful transformation into amastigotes. These data are in agreement with previous studies on the infection rate of BMDCs exposed to *L. infantum* (Neves *et al.,* 2010, Falcao *et al.,* 2016). In turn, cexposure of BMDCs with promastigote forms of dermotropic *Leishmania* species, such as *L. major*, *L. amazonensis* and *L. braziliensis* resulted in similar infectivity rates with parasites being able to survive and multiply within BMDCs (Prina *et al.,* 2004, Falcao *et al.,* 2016, Von Stebut *et al.,* 2018).

Moreover, internalized *L. infantum* and *L. amazonensis* parasites seem to prolong the lifespan of infected dog moDCs, as found in the present study. Curiously, *L. amazonensis* soluble antigens also seem to favour the extension of moDCs lifespan.

DCs recognize foreign antigens through specialized receptors such as TLR, CLR and intracellular helicases such as the retinoic acid-inducible gene I (RIGI). Receptors detect and bind different types of surface components of pathogens, which are distinct from the

host such as LPS, lipopeptides or nucleic acids, such as RNA or DNA. This triggers the receptors and eventually results in the intake of the foreign particle. TLR recognition is often associated with the production of pro-inflammatory cytokines and the generation of effector molecules, which promote differentiation of Th1 cells, leading to an inflammatory response. The specific response initiated by individual TLRs depends on the recruitment of a single or a specific combination of TIR-domain-containing the adaptor protein (e.g. MyD88, TIRAP, TRIF, TRAM) (Kawai and Akira, 2010). MyD88, which is used by all TLRs, except TLR3, and members of the IL-1 receptor family transmit the antigenic signals through the downstream pathway, culminating in NF-κB and MAP kinase activation and transcription of inflammatory cytokines, such as IL-1β, IL-12, TNF-α, and IL-6. TLR2 and TLR4 use TIRAP as an additional adaptor to recruit MyD88. In the current study, moDCs-TLR2 sense parasite antigens (*LaAg* and *LiAg*) and EVs from *L. amazonensis,* but not from *L. infantum* whereas TLR4 sense *L.infantum* parasites and *LiAg*. Interesting, *L. amazonensis* parasites do not seem to be recognized by the innate surface receptors evaluated in this study. Moreover, endocytic and cytoplasmatic PRR also do not seem to sense the parasites, antigens or EVs.

Bamigbola and Ali (2022) reported that the absence of TLR2 affects DC maturation consequently affecting Th1 and Th17 protective immune response against *L. infantum* infection. TLR2 absence also impaired the recruitment and activation of neutrophils for the production of NOS, NO and TNF-α, while IL-10 production is upregulated. From these observations, it is evident that TLR2 signalling is crucial to confer enhanced protective adaptive immune response as well as neutrophils. Anti-parasitic function coordinated by DC production of CXCL1. On the other hand, *in vitro* infectivity index of *L. amazonensis* was much higher in BMDM of TLR2$^{-/-}$ C57BL/6 mice when compared to WT mice (Muxel *et al.*, 2018). The detection of *L. amazonensis* mediated by TLR2 and TLR4 confers resistance to MΦ by upregulating the expression of NOS2, resulting in the conversion of L-arginine to NO, which leads to the death of the parasite. Therefore, the deficiency of these TLRs induces the production of polyamines that favor the replication of the parasite (Muxel *et al.*, 2018). This observation is indicative that TLR2 can be determinant in the outcome of *Leishmania* infection.

More recently, TLR4 was implicated in the mechanism underlying the inhibition of IL-12 production in LPS-treated MΦ infected with *L. mexicana*. Metacyclic promastigotes were found to greatly increase the phosphorylation of the three major MAP kinases, ERK, p38, and JNK, in a manner dependent on TLR4 but not on TLR2 (Shweash *et al.*, 2011).

TLR9 is expressed intracellularly, within endosomal compartments, and is usually activated by unmethylated CpG sequences. Once activated, TLR9 moves from the endoplasmic reticulum to the Golgi apparatus and lysosomes, where it interacts with MyD88, the primary protein in its signaling pathway (Martínez-Campos *et al.*, 2017). TLR9 signaling leads to cell activation initiating pro-inflammatory reactions that result in the production of cytokines such as type I interferon, IL-6, TNF, and IL-12. In this study, *L. amazonensis* infected moDCs and *LiEVs* , and *LaAg* primed moDCs seem to inhibit TLR9 expression. The recognition of *L. major* DNA by TLR9 promotes DC activation, which induces a Th1-dominant response that resolves the lesions (Abou *et al.*, 2009). In addition, TLR9 has been reported to be related to draining lymph node hypertrophy, indicating that TLR9 is associated with cell recruitment (Carvalho *et al.*, 2012). During *L.braziliensis* infection, TLR9 is important for the early control of lesion development and parasite load, but it is unnecessary for the differentiation of a Th1 response (Weinkopff *et al.*, 2013), suggesting that there are other mechanisms related to the protection of TLR9. According to a previous study, *L. infantum* infection modulates the expression of TLR9 on the surface of DCs, which indicates that this receptor may be involved in parasite recognition and thus initiate a protective immune response against the parasites (Sacramento *et al.*, 2015). Therefore, an impaired or failed expression of TLR9 could favor the persistence of the parasites in host cells. DC activation requires the recognition of *L. infantum* by TLR9 leading to the production of IL-12 (Schleicher et al., 2007). *In vivo*, $TLR9^{-/-}$ mice are more susceptible to *L. amazonensis* infection than WT mice, failing to activate DC in both models. CL susceptibility is marked by larger lesions and increased parasite burden during chronic infection due to decreased synthesis of IFN-γ in infected tissue as well as high IgG production (Pratti *et al.*, 2019). However, TLR9 signaling can promote the progression of cutaneous lesions, facilitating intracellular survival of *L. amazonensis* by inducing the expression of CD200, a ligand known for suppressing the production of pro-inflammatory cytokines by MΦ (Sauter *et al.*, 2019)

Following the pathogen's internalization by phagocytosis or through membrane pores, NODs that are intracellular innate receptors able to recognize PAMPs (Ranjeet *et al.,* 2013). NLRs and TLRs share similar structures and cooperate in triggering host responses against invaders. NOD1 and NOD2 detect pathogens that invade and multiply intracellularly. Once stimulated, these receptors induce the activation of NF-κB and MAPKs, which lead to the transcription of genes involved in inflammation and immune responses (Scaccabarozzi *et al.,* 2019). Our findings indicated that *LiEVs* downregulate NOD1 of dog moDCs while gene expression of NOD2 remains unchanged. Only *L. infantum* parasites seem to slight induce the upregulation of NOD1 when compared to *L. amazonensis* infection. Although NOD2 is known as the innate receptor that recognizes structures present in the bacterial cell wall (Girardin *et al.,* 2003), the findings of the current study suggest that NOD2 also can recognize protozoan structures. However, the current study reinforce the non-involvement of NOD1 or NOD2 in moDCs immune response against visceral and cutaneous species of *Leishmania*.

NLRP10 is widely expressed in myeloid cells, epithelial cells, and keratinocytes. It is the only NLR protein without a leucine-rich repeat (putative ligand-binding) domain, leading investigators to hypothesize that it acts as a dominant-negative inhibitor of inflammasomes. As such, the anti-inflammatory properties of NLRP10 have been attributed to the inhibition of inflammasome activation via ASC binding and inhibition of ASC-mediated NF-κB activation. NLRP10 has been implicated in adaptive immune responses through the regulation of DC migration. NLRP10/DOCK8-deficient mice harbored lower parasite loads at the cutaneous site of inoculation compared to WT controls, while mice and controls deficient in NLRP10 only had similar parasite loads, suggesting that DOCK8 promotes local growth of parasites in the skin, while NLRP10 does not. $NLRP10^{-/-}$ mice developed adaptive immune responses, indicating that there was no global defect in the development of specific cytokine production. The anti-inflammatory role of NLRP10 was mediated by NLRP10 expressed in skin-resident cells and not by bone marrow-derived cells. These data suggest a new role for NLRP10 in determining local inflammatory responses during *L. major* infection. Our findings show that *L. infantum* negatively modulates NLRP10 expression while antigens and vesicles, as well as *L. amazonensis* did not affect this receptor, indicating that moDCs cannot migrate to inflamed tissues.

TLRs initiate signaling cascades that induce nuclear translocation of NF-κB (Barton and Medzhitov, 2003). Most importantly, NF-κB acts as a central regulator of immune response and inflammation by upregulating many chemokines (CXCL1, CXCL2 and CXCL3, among others) and cytokines (TNF-α, IL-1β, IL-6 among others) (Hayden and Ghosh, 2011), as well as DCs costimulatory molecules that are essential for T cell activation. The family of NF-κB transcription factors is crucial for immune defense against pathogens. Several studies have reported that *Leishmania* and other parasites, such as *Toxoplasma gondii,* interfere with the activation of NF-kB signaling pathways. MyD88-dependent pathways lead to NF-κB activation via TRAF6. After recruitment by IRAK proteins, TRAF6 binds to the TAB/TAK1 complex, leading to the TAK-1-mediated activation of IKK2, which is implicated in the classical pathway of NF-κB activation. When we analyzed the activation and translocation of NF-κB, we found that *L.infantum* parasites, *LiAg* and *LaEVs* act positively on this protein transcription factor, producing pro-inflammatory cytokines such as IL-1β and IL-18. The central role of NF-κB in the regulation of the immune response includes bea regulator of the expression of MΦ genes (Grigoriadis *et al.,* 1996), which are the cells of excellence for *Leishmania* invasion, meaning that NF-κB offers an attractive target for parasitic manipulation. In this case, a study by Nogueira *et al.,* (2020) on THP-1 monocytes showed that EVs from *L. amazonensis* when compared to *L. infantum* and *L. braziliensis* EVs, activate TLR4/TLR2 and induce the nuclear translocation of NF-κB p65.

Human monocytes infected with *L. major* amastigotes preferentially induce NF-κB transcriptional repressor homodimer (p50/p50) (Guizani-Tabbane *et al.,* 2004) associated with IL-10 (Driessler *et al.,* 2004). Infected monocytes release increased concentrations of IL-10 and TNF-α (Guizani-Tabbane *et al.,* 2004). While IL-10 is expected to be beneficial to the parasite and implicated in the survival of *Leishmania,* TNF-α is involved in inflammation and is expected to be protective. However, most studies reveal that it is difficult to draw a clear conclusion about the processes and roles of NF-κB modulation during *Leishmania* infection, but there seems to be a tendency towards negative modulation of NF-κB by *Leishmania*. The generation of a permissive environment is also evident in DC from mice infected with *L. amazonensis* . The infection leads to pleiotropic inhibition of TLR/NF-κB/NLRP3 pathways, promoting transcriptional activation of the alternative NF-κB pathway which is proposed to lead to MHCI-restricted antigen presentation

while delaying DC maturation (Lecoeur *et al.,* 2020). Delayed mouse DC maturation is also evident upon infection by *L. infantum* promastigotes (Neves *et al.,* 2010). Parasite impairs NF-κB through cleavage of p65, although no impact on IkBα expression was reported. Cleavage of p65 to p35 has also been reported following DC infection by *L. infantum* promastigotes (Neves *et al.,* 2010).

Cytokines play vital roles in cell propagation and differentiation toward defense against pathogens. However, the balance of pro- and anti-inflammatory cytokines is crucial to prevent immunopathological disorders. Some cytokines play a protective role or help in disease progression (e.g. IL-3, IL-5, IL-7, IL-13) (Bogdan *et al.,* 1993, Matthews *et al.,* 2000), while others have a dual role in the disease, as is the case of IFN-γ, TNF-α, TGF-β, IL-10, IL-27, and IL-22. For example, although TNF-α and IFN-γ are two important cytokines for inducing protective immune responses in leishmaniasis, their overproduction may cause pathological effects and severe damage in host tissues (Ribeiro-de-Jesus *et al.,* 1993). Our study findings indicated that TGF-β, IL-1α, IL-8 and IL-12 (p35 and p40) were inhibited or showed no changes. On the other hand, *L. infantum* induced the generationof IL-1β and IL-18 while *L.amazonensis* had a positive effect on IL-10 and IL-18. *L. infantum* antigen and *L. amazonensis* vesicles also lead to IL-1β generation. Suggesting that *L. infantum* parasites promote an inflammatory response while *L. amazonensis* induces a balance between Th1 and Th2 cytokines

The classic Th1/Th2 dichotomy of the immune response to CanL still holds true today, in the sense that a predominant Th1 response appears to confer protection against *Leishmania*, while a strict Th2 response does not. However, Santos and colleagues (2019) found that dogs with CanL can remain sick even when they show a characteristic Th1 response, but a mixed profile between Th1/Th2 and Th1/Treg was more often observed. Mana and coworkers (2006) showed that asymptomatic dogs that were naturally infected, and that later developed symptoms, did not show significant expression of cytokines other than IL-18. However, six months later onthe onset of clinical signs, their cytokine profile shifted to a Th1 /Th2 type cytokines, 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 long-term asymptomatic dogs showed a Th1 response mediated by IL-2, IFN-γ and IL-18. Six months later these dogs presented additional expression of IL-4 and IL-10

(Manna et al. 2006). 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. Interestingly, IL-4, a type 2 cytokine, is required for the optimal induction of Th1 responses by DCs as it inhibits IL-10 production.

Although IL-10 is known to be a potent immunoregulatory cytokine, it also suppresses MΦ activation and DC maturation. Despite the production of IL-10 by Treg cells limits immune responses against intracellular parasitic infections, such as *L. major* and *Toxoplasma gondii* (O'Garra *et al.,* 2007) this cytokine is associated with susceptibility to leishmaniasis and persistence of the parasite at the site of infection (Belkaid *et al.,* 2001). IL-10 directly blocks MΦ and DC-derived IL-12 production, thereby impairing Th1 cell proliferation and IFN-γ production (Liu *et al.,* 2012). Paradoxically, IL-10 had little effect on lesions caused by *L. mexicana* and *L. amazonensis* , as infected IL-$10^{-/-}$ BALB/c mice failed to control the infection (de Veer *et al.,* 2003). Later on, Buxbaum and Scott (2005) revealed that C57BL/6 IL-$10^{-/-}$ mice cured the lesions of *L. mexicana* infection, but failed to resolve lesions caused by *L. amazonensis*. Therefore, IL-10 does not appear to play a vital role in determining disease progression in *L. amazonensis* infections (Campos *et al.,* 2018). *L. donovani* chronic VL is linked to $CD4^{+}$ T cells that express both IL-10 and IFN-γ (Aguirre-Garcia *et al.,* 2018). Overall, IL-10 is an important immune deactivating cytokine in VL.

Nylén *and* collaborators (2011) reported that plasma of VL patients with, increaseIL-10 led to the replication of the parasite in MΦ and that blocking IL-10 the parasite growth is reduced. Thus, IL-10 is associated with the most severe clinical manifestations, as it inhibits the production of other immune mediators, as well as the anti-leishmanial action generated by MΦ.

IL-18 together with IL-12 induce Th1 cell activation. On the other hand, IL-18 is also capable of inducing Th2 responses. Monteforte and collaborators (2000) found that C57BL/6 mice deficient in the IL-18 gene (IL-$18^{-/-}$) developed strong Th1 responses and therefore high resistance to *L. major* infection. Other studies reported that *L. major* in-

fected BALB/c mice treated with recombinant IL-18 promoted Th2 responses in the absence of IL-4, leading to disease exacerbation when compared to untreated mice (Xu *et al.,* 2000). It is probably that due to the lack of persistent IL-12 stimulation, IL-18 contributes to an enhancement of Th2 (Wei *et al.*, 2004). Indeed, during infection, IL-18 appears to carry out a Th1 or Th2 response action based on the generated cytokine environment and the genetic background of the host.

Resende and colleagues reported a dichotomous response between *L. infantum* -infected and uninfected DCs. In this study, the authors observed higher levels of IL-12p40 and costimulatory molecules in uninfected DCs, which allowed DCs to activate $CD4^+$ T cells, while infected DCs express lower levels of costimulatory molecules and high production of IL-10 (Resende *et al.,* 2020). Currently, the role of IL-1β during *Leishmania* infection is considered controversial (Curry *et al.,* 1992). IL-1β is produced as a propeptide that is processed upon assembly and activation of the inflammasome, a molecular complex composed of proteins, such as the NOD, LRR and pyrin domain-containing 3 (NRLP3) complex, and caspase-1, which mediates IL-1β cleavage (Lima-Junior *et al.*, 2013). It has been hypothesized that when parasites are phagocytized by cells of the innate immune system through C-type lectin receptors, *Leishmania* can indirectly activate the inflammasome and induce ROS production (Scott and Novais *et al.,* 2016, Zamboni *et al.,* 2019) or by activating a non-canonical pathway, which involves the participation of LPG (Zamboni *et al.*, 2019). It has been shown that IL-1β can promote the expansion of IL-12-mediated Th1 cells, and stimulates NO and TNF production, which contributes to parasite elimination (Alexander *et al.,* 2005, Lima-Junior *et al.,* 2013). However, a study performed in *L. mexicana*-infected patients found that elevated IL-1β expression was associated with disease severity (Fernández-Figueroa *et al.,* 2012). Therefore, the mechanisms responsible for resistance or susceptibility also depend on the infecting species causing CL. As mentioned in previous sections, *L. major* can block IL-12 production by MΦs (Ramer et al., 2006; Ashok and Acha-Orbea, 2014). IL-1α acts together with IL-12 and promotes Th1 differentiation and prevents disease progression in BALB/c mice susceptible to *L. ma*jor (Maurer et al., 2009, Ritter *et al.,* 2008). Experimental studies have suggested that IL-12 production is needed for the maintenance of the Th1 response in *Leishmania* infection (Mark *et al.,* 2004, Rivera-Fernández *et al.,* 2019, Gutiérrez-Kobeh *et al.,* 2020). Splenic DCs are the critical source of early IL-12 production after *L. donovani*

infection, and DC activation is crucial for optimal induction of immunity in the liver during the early phase of VL infection (Mollinedo *et al.,* 2010, De Menezes *et al.,* 2016; Oulha *et al.,* 2019). A study in mice infected with *L. infantum* showed that the TLR9 receptor and the release of IL-12 by myeloid DCs are functionally linked to the activation of IFN-γ producing NK cells and control the disease (Gabriel *et al.,* 2010).

For the initiation of adaptive immune responses, DCs present antigenic peptides complexed with MHCII to naïve $CD4^+$ T lymphocytes. MHC molecules direct antigen specificity of adaptive immunity toward invading pathogens. MHCI molecules on DCs predominantly help eliminate infected cells through the activation of antigen-specific $CD8^+$ (cytotoxic) T cells. However, the MHCI-driven killing of infected cells by cytotoxic T cells, also requires MHCII-dependent activation of $CD4^+$ T cells via DCs. MHCII molecules of DCs induce humoral immune response and instruct regulatory T cells and memory T cells. Reiner and collaborators (1987) described that *L. donovani* parasites decrease the expression of MHCI and MHCII molecules in MΦs. Downregulation of MHC molecules in *L. major* infected DCs has also been reported (Muraille *et al.,* 2003), which may be mediated by direct internalization of the parasite (Antoine *et al.,* 1999; Bennett *et al.,* 2004; Prina *et al.,* 2004). The findings of our study also indicate thar *L. infantum* parasites inhibited the expression of MHCII molecules in moDCs, as well as their EVs, showing that this parasite, as well as their EVs do not favor antigenic presentation and further activation of $CD4^+$ T lymphocytes. *L. donovani* EVs have been shown to inhibit MHCII expression in human moDCs (Silverman *et al.,* 2010). On the other hand, our study showed that *L. infantum* parasites and EVs from *L. infantum* and *L. amazonensis* favor the predomination of $MHCI^+$ moDCs molecule, being able to activate cytotoxic T cells.

In vitro studies have shown that, depending on the culture conditions, all major types of APCs are capable of cross-presenting exogenous Ags (Norbury *et al.,* 1995; Ke *et al.,* 1996, Rock-*et al.,* 1996, Norbury *et al.,* 1997, Albert *et al.,* 1998). Previous *in vitro* findings have also indicated that DCs are more potent in cross-presenting exogenous antigens to $CD8^+$ T cells than MΦ or B cells (Albert *et al.,* 1998, Regnault *et al.,* 1999, Rodriguez *et al.,* 1999)

Furthermore, the skin may be especially well suited for driving $CD8^+$ T cells in response to the intradermal challenge. Langerhans cells have been shown to effectively prime $CD8^+$ T cells, and immature DC can induce cytotoxic T lymphocytes via the presentation of Ag through an exogenous pathway (Shen *et al.,* 1997, Yewdell-*et al.,* 1999) or via the presentation of peptides acquired from the uptake of apoptotic cells (Albert *et al.,* 1998). These findings are consistent with previous studies indicating that, in contrast to MΦ, DC can efficiently process exogenously delivered Ag for presentation by MHC I molecules (Yewdell *et al.,* 1999, Rodriguez *et al.,* 1990), and that, in contrast to infected MΦ, *L. major*-infected DCs up-regulate MHCI and MHCII molecules, costimulatory molecules, and IL-12 (Von Stebut *et al.,* 1998, Marovich *et al.,* 2000), and effectively prime T cells *in vivo* (Moll *et al.,* 1993, Von Stebut *et al.,* 1998). DCs surface costimulation molecules, CD80 and CD86 on DCs bind to CD28 expressed on T cells (Bluestone *et al.,* 1999). Although both CD80 and CD86 bind CD28 with low affinity, these molecules exhibit different expression patterns. Thus, CD86 is constitutively expressed at low levels in immature DC (Inaba-*et al.,* 1994), resting B cells (Azuma *et al.,* 1993), and resting monocytes (Azuma *et al.,* 1993). In contrast, CD80 is only expressed on activated APCs (Freedman *et al.,* 1991). While CD80 and CD86 are upregulated in APC activation (Inaba *et al.,* 1994, Larsen *et al.,* 1994), CD86 is induced more rapidly than CD80 (Lenschow *et al.,* 1994, Boussiotis *et al.,* 1993). Because of these findings, it was proposed that CD86 was more important than CD80 in initiating the T cell response (Freeman *et al.,* 1993).

Julia and colleagues (1996) reported that differentiation of the Th2 subset requires interaction with the co-stimulatory molecule CD86. However, the costimulatory molecule CD80 does not appear to play an important role in the generation of Th1 cells in the context of *L. major* infection. CD40 and CD86 played the biggest role in the production of anti-*Leishmania* antibodies in humans, *in vitro*. The relatively small influence of CD80 on this system is an interesting finding in light of the marked upregulation of CD80 in MΦ cultured with peripheral blood lymphocytes and *L. major* (Brodskyn *et al.,* 2001)

Neves and coworkers (2010) supported that *L. infantum* promastigotes do not induce upregulation of CD40 and CD86 expression. However, in the case of human DCs, *L. infantum* and *L. braziliensis*, as well as *L. amazonensis* enhance CD86 expression (Falcao *et*

al., 2016). Interestingly, the fixed parasites induced strong expression of all co-stimulatory molecules tested, implying that active infection impaired DC activation.

CXCL16 is expressed on the surface of APC cells (B cells, MΦ and DCs in T cell zones of lymphoid organs) and by cells in the splenic red pulp. The CXCL16 receptor (CXCR6) is preferentially expressed on memory T cells and subsets of activated Th1 and type 1 cytotoxic T cells (Tc1). The exact biological role of the CXCL16/CXCR6 axis is unknown. However, reasonable hypotheses include attracting subsets of activated T lymphocytes during inflammation, facilitating immune responses via cell-cell contact, and directing T cell traffic in the splenic red pulp (Murphy *et al.*, 2003, Oghumu *et al.*, 2010, Hurrell *et al.*, 2017). Although *L. donovani* upregulates CXCL16, this regulation is not exclusive to species causing visceral leishmaniasis. A similar phenotype was detected in MΦ infected with *Leishmania spp*. from different geographic origins (i.e. Old World and New World species), including two that cause cutaneous leishmaniasis (i.e., *L. major* and *L. mexicana*). Importantly, CXCR6, the cognate receptor of CXCL16, is expressed by monocyte/macrophage subsets (Huang *et al.,* 2008, Linke *et al.,* 2017) and neutrophils (Steffen *et al.,* 2018). Therefore, it is tempting to speculate that CXCL16 induced by *Leishmania spp*. helps to attract naïve immune cells that later become infected to facilitate pathogen spread. In a recent report, Murray and colleagues (2017) used cxcr6 KO mice infected with *L.donovani* and found that the CXCL16-CXCR6 axis is dispensable for infection control, as granuloma formation and elimination of the parasite in the liver were not affected by the absence of CXCR6. However, VL pathology of encompasses different tissues and organs (Kumar and Nylen 2012). In VL, disruption of the spleen microarchitecture is associated with local production of TNF (Engwerda *et al.,* 2009). The expression of CXCL16 is upregulated by pro-inflammatory cytokines such as TNF and IFN (Abel *et al.,* 2004), thus the recruitment of TNF and IFN-producing cells by this chemokine could contribute to inflammatory activity in the spleen.

By analyzing the production of this chemokine in the current study, we found that only the antigens of both *Leishmania* species do not have a positive effect on CXCL16 production. Bahr and colleagues (1993) discovered a new function for *L. donovani* LPG through the induction of CXCL16, although *L. donovani* amastigotes, which are not rich in LPG, are also able to increase CXCL16 expression in MΦ , although to a lesser extent

than promastigotes, which are entirely covered by LPG (glycocalyx). During the differentiation of *L. donovani* promastigotes into amastigotes, a drastic reduction in GLP levels occurs (Naderer *et al.,* 1993) but it appears that amastigotes retain a glycocalyx of glycosilinositol phospholipids (Naderer and McConville, 2008) which may be responsible, at least in part, for the induction of CXCL16. Despite these findings, further studies are needed to elucidate the mechanism of CXCL16 upregulation by *Leishmania* and define its role during leishmaniasis. Indeed, within the region upstream of the CXCL16 promoter, binding sites for several transcription factors were predicted, including sites for CREB, SMAD, GATA, IRF, NF-κB and AP-1, the latter being the main driver of IL-18-induced CXCL16 expression (Chandrasekar *et al.,* 2001). LPG from *L. major* was able to induce NF-κB activation in 293T cells (de Veer *et al.,* 2003). Thus, it seems that LPG can promote macrophages to increase CXCL16 gene expression via AP-1 and/or NF-κB dependent transcriptional activity.Therefore, taking together the findings of this study indicates that Canine moDCs differentiated *in vitro* from peripheral blood monocytes can recognize parasitic antigens, become immune activated, present the antigens to $CD8^{+}$T lymphocytes, and initiate the acquired immune response.

2.6. Conclusion

DCs play a key role in bridging the innate and acquired immune response through antigenic presentation to T cells, directing the activation of cellular immunity. The current study investigated *in vitro* the activation of dog DCs when exposed to *L. infantum and L. amazonensis parasites*, using peripheral blood monocyte differentiated DCs (moDCs). For comparison, moDCs were also exposed to *L. infantum* and *L. amazonensis* EVs and primed by L. *infantum* and *L. amazonensis soluble* antigen. After evaluating the morphology and molecular signature, and confirming the functionality of moDCs, it was assessed the viability of infected cells. To examine the activation of moDCs, the gene expression of innate immune receptors, the expression and translocation of NF-κB and the generation of cytokines were analysed. Moreover, to indirectly assess the ability of moDCs to activate T cells, the generation of chemokines and the surface expression of MHC and co-stimulatory molecules were evaluated.

The findings of this study led to the following main conclusions:

- The methodology was successfully used to differentiate functional dog moDCs with the expected molecular signature and morphology;

- *L.infantum* parasites seem to signal moDCs through TLR4, leading to the activation of downstream pathways and activation and translocation of NF-κB to the nucleus. This transcription factor is involved in multiple aspects of innate and adaptive immune functions, including the expression of immune mediators as is the case of pro-inflammatory cytokines and chemokines. Activated *L.infantum* infected moDCs generate the pro-inflammatory cytokines IL-1β and IL-18, which are type 1 cytokines and can be involved in the activation of Th1 and NK cells;

- *L.infantum* infected moDCs release the chemokine CXCL16, which is chemotactic for T cells, and $MHCI^{+}$ moDCs expand leading to recruitment and activation of cytotoxic ($CD8^{+}$) T cells that can induce the apoptosis of infected cells. However, infected moDCs only induce the gene expression of the co-stimulatory CD86 molecule which can lead to suboptimal T cell activation;

- On the other hand, *L. amazonensis* does not seem to signalize moDCs through the PRRs analysed in the current study. Even so, cells become activated, generating the regulatory cytokine IL-10 and also IL-18, promoting a balance between regulation and inflammation, which can lead to immunological tolerance to parasite infection;

- *L. amazonensis* infected moDCs do not seem to promote MHC expression, compromising the antigenic presentation, but release the chemokine CXCL16 which can attract other leukocytes, such as MΦ that can favor the dissemination of infection;

- In contrast to *L. infantum* parasites, *L.infantum* soluble Ag was highly recognized by TLR2, which seems to activate downstream pathways, leading to the activation and translocation of NF-κB to the nucleus and generation of IL-1β and IL-10, favoring immune tolerance. Although Ag-primed moDCs upregulate co-stimulatory molecules the low expression of MHC molecules associated with the reduced chemokine levels may impair antigen presentation;

- Despite TLR2 and TLR4 can sense *L. amazonensis* Ag, moDCs do not seem to be activated, pointing throughout the induction of a state of moDC anergy, which can facilitate parasite infection;

- *L. infantum* EVs promote the expansion of $MHCI^+$ moDCs and the upregulation of CD86, associated with the release of CXCL16, which can lead to the recruitment and activation of cytotoxic ($CD8^+$) T cells that can drive some control in parasite dispersion;

- *L. amazonensis* EVs seem to signalize moDCs through TLR2, leading to activation and translocation of NF-κB to the nucleus associated with IL-1β generation, the release of CXCL16, expansion o $MHCI^+$ moDCs and upregulation of both

costimulatory molecules which is indicative of a pro-inflammatory immune response that includes leukocytes recruitment and induction of a cytotoxic T cell response.

3|Chapter 3: Crosstalk between NK cells and *L. infantum* and *L. amazonensis* infected moDCs

3| Chapter 3: Crosstalk between NK cells and *L. infantum* and *L. amazonensis* infected moDCs

3.1. NK cells

NK cells, which originate from the bone marrow, are a subtype of granular lymphocytes characterized by the loss of CD3 expression (Cooper *et al.*, 2001). They represent about 10 to 15% of peripheral blood lymphocytes and are crucial in the innate immune response due to their ability to rapidly produce cytokines and lyse target cells without prior activation (Cooper *et al.*, 2001). Are known as null cells since do not have surface receptors like B or T cells. Their functions are regulated by the integration of signals from activating and inhibitory receptors that recognize ligands on the surface of target cells. Upregulation of ligands for activating receptors in NK cells (e.g. NKG2D) or downregulation of ligands for inhibitory receptors (e.g. CD94-NKG2A) represent relevant stimuli for the activation of these cells (Lanier, 2008, Yokoyama, 2008a, 2008b). Many of the inhibitory receptors expressed by NK cells, such as KIR (Killer Immunoglobulin-like Receptors), recognize MHCI molecules. Thus, the alteration or absence of MHCI molecules, usually seen in infected cells (and in malignant cells), prevents the binding of the inhibitory signal, resulting in the activation of NK cells (Lanier, 2001, 2008, Held *et al.*, 2011). Once activated, NK cells acquire the ability to exert the following effector functions:

1) Production of cytokines and chemokines, mainly IFN-γ and TNF, which contribute to the development of a Th1 response and activation of MΦ for the implementation of antimicrobial mechanisms (Bogdan, 2001, Laouar *et al.*, 2005), but NK cells are also capable of producing the cytokines GM-CSF, IL-5, IL-13, and the chemokines macrophage inflammatory protein 1 (MIP-1) and RANTES (regulated upon activation, normal T cell expressed and presumably secreted) (Loza *et al.*, 2002, Robertson, 2002, Dorner *et al.*, 2004). In addition, NK cells acquire the ability to produce IL-10 during systemic infections, acting as regulators of the immune response through the inhibition of IL-12 (Maroof *et al.*, 2008);

2) Induce theexpression of costimulatory ligands (such as CD40L and OX40L) on T or B lymphocytes, linking innate and adaptive immunity. APCs stimulate NK

cells, which in turn provide co-stimulatory signals to lymphocytes (Blanca *et al.*, 2001, Zingoni *et al.*, 2004);

3) Cytotoxic mechanisms, the main one being via granule exocytosis. These cells also can direct cell lysis via Fas/Fas ligand (FasL), tumor necrosis factor (TNF) or TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), but in a more slowly manner and presenting lower efficacy (Orange and Ballas, 2006).

In addition to cytotoxic mechanisms, NK cells also can direct the lyse of Ab-coated cells, through a mechanism called Antibody-dependent cell cytotoxicity (ADCC), by the activation of the CD16 receptor (Chan *et al.*, 2012).

NK cells can be identified by the expression of CD16 and CD56 as do not contain CD3. They are divided into two distinct subtypes based on CD56 expression density: more than 90% of NK cells belong to the CD56 dim subtype whose main function is cytotoxicity, whereas the CD56 bright subtype, which is responsible for the production of cytokines, is rare in the bloodstream (≈10%) (Otani *et al.*, 2008). Most NK cells express CD16, which is a Fc receptor (type III Fcγ) for the Fc portion of IgG (Robertson *et al.*, 1990).

The main families of NK cell receptors are (i) the superfamily of C-type lectin-like receptors, (ii) natural cytotoxicity receptors (NCR) and the killer immunoglobulin-like receptor superfamily (KIR).

The first family of receptors is structurally characterized by extracellular domains similar to C-type lectin and its receptors are expressed as heterodimers composed of a common subunit, CD94. In turn, the second family of receptors is known as NCR, represented by NKp46, NKp44 and NKp30. NKp46 and NKp30 are constitutively expressed in peripheral blood NK cells, while NKp44 is only expressed in activated NK cells. NKp30 are very important in the interaction with DC (Farag *et al.*, 2002, Zingoni *et al.*, 2005). The KIR receptors are the most studied, and the KIR2DL4 receptor is very important because it is constitutively expressed on the surface of CD56 bright NK cells, which are characterized by high levels of regulatory cytokines and chemokines and low cytotoxic activity. KIR2DL4 in resting NK cells induces only IFN-δ secretion, although in active NK cells it not only enhances IFN-γ expression but also cytotoxic activity. The mechanism of recognition of target cells by NK cells is inversely proportional to the expression of MHC

molecules in the target cell. In this way, NK can recognize and attack cells that have low levels of MHCI and not attack normal autologous cells, which is called missing self (Ljunggren *et al.*, 1990). Immunosurveillance in the loss or alteration of MHCI expression induces a rapid response in absence of memory, making these cells considered innate immune cells acting on the failures committed by adaptive immunity.

The cytotoxic activity of NK cells is the result of a balance between positive and negative signals transmitted by activating and inhibitory receptors, respectively, in a consequence of diverse intracellular signaling and activation of adapter proteins.

Activation normally occurs through two mechanisms: by cell-to-cell contact, which may be DC, lymphocytes or the target cell; or through cytokines present in the microenvironment, namely IFN-γ, IL-12, and IL-15.

Mature NK cells have cytotoxic activity as their main function, this cytotoxicity is fast and very potent, so the death of target cells can occur in minutes, thus hindering the performance of resistance mechanisms. NK-mediated cell death occurs through granule exocytosis and the induction of apoptosis through signaling via members of the TNF cell death receptor family (Smyth *et al.*, 2005). Adhesion is the main step towards cytotoxicity, integrins and other adhesion molecules play a crucial role in the formation of the conjugate between NK cells and target cells. One of the main integrins is LFA-1 (lymphoid function associated with antigen-1) which has the function of inducing actin polymerization, cytoskeletal rearrangement, and agglomeration of lipid vesicles. The signals given by LFA-1 alone can be sufficient to activate cell cytotoxicity (Ljunggren *et al.*, 1990). The adhesion process induces changes in morphology, the lytic granules are moved towards the site of interaction with the target cell and the outer membrane of the granule fuses with the cytoplasmic membrane of the target cell and the granules are released into the synaptic space. The granules contain molecules of perforin and granzyme (Smyth *et al.*, 2005, Tassi *et al.*, 2006). Perforins are proteins that form pores in the target cell membrane, changing cell permeability and leading to osmotic lysis while granzymes belong to a family of serine proteases.

3.1.2. NK cells and their cytotoxic power

NK cells are cytotoxic innate lymphocytes that can lyse cancerous or infected cells. Perforin is found within the granules as a soluble monomer and is released by cytotoxic cell degranulation at the immunological synapse formed with the target cell. (Peters *et al.*, 1991). Once released, perfortin anchors to the outer membrane of the target cell and initiates polymerization in the presence of calcium (Ca_2^+) to form transmembrane pores with an internal diameter of 5 to 20 nm (Sauer *et al.*, 1991, Liu *et al.*, 1995, Voskoboinik *et al.*, 2005). The perforin pores are used as passive conductors of granzymes and granulysin across the target cell membrane, inducing ion exchange that generates an imbalance (Voskoboinik *et al.*, 2006). In addition, perforin induces invaginations in the cell membrane, allowing granzymes to be delivered by endocytosis (Trapani *et al.*, 2003, Veugelers *et al.*, 2004, Praper *et al.*, 2011). Granzymes can adhere to the surface of the target cell by electrostatic binding while the pore formed by perforin induces the flow of $Ca2^+$ from the extracellular environment to the intracellular environment. This shift of $Ca2^+$ causes target cell activation that tries to repair the cell membrane to avoid necrosis. Thus granzymes are internalized along with perforin, being released into the cytoplasm of the target cell (Keefe *et al.*, 2005). However, studies indicate that, even though perforin is not essential for the entry of proteases into the target cell, its presence is necessary for cytolysis (Chávez-Galán *et al.*, 2009). Granzymes are intragranular proteins of the serine protease family. To date, five different granzymes have been described in humans (A, B, H, K and M). Granzymes A (GZMA) and B (GZMB) are the most abundant constituents of granules and have been extensively studied due to their important role in eliminating transformed cells. On the contrary, the role of GZMH, GZMK and GZMM remains poorly understood (Wensink *et al.*, 2015; Voskoboinik *et al.*, 2015; Bovenschen *et al.*, 2010). GZMA induces cell death via a caspase-independent pathway. This serine protease alters the mitochondrial inner membrane potential, leading to the release of ROS (Martinvalet *et al.*, 2008). ROS production prompts translocation of the endoplasmic reticulum (ER)-associated SET complex to the nucleus, where SET is cleaved by GZMA, releasing DNA damage-associated nucleases, such as NM23-H1 DNase which, together with thioredoxin 1(TRX1), degrades DNA and leads to cell death (Fan *et al.*, 2003, Martinvalet *et al.*, 2005, Chowdhury *et al.*, 2006). Granzyme B can cleave proteins at aspartate residues followed by the activation of pro-caspase-10 in caspase-10 that can cleave ICAD, among other

factors. CAD transpose nuclear membrane and can cleave DNA leading to cell death (Tittarelli *et al.*, 2015). Other evidence also supports that granzyme B can induce cell death by directly activating caspase 3 or induce cell death after cleaving the BH3 protein into Bid, resulting in a truncated form, tBid, which moves to the mitochondria, where it interacts with the pro-apoptotic proteins Bax and/or Bak. This results in a conformational change of the mitochondrial membrane may cause the release of apoptotic factors, such as smac/DIABLO, cytochrome c, apoptosis-inducing factor (AIF), and Omi/HtrA2, leading to cell apoptosis (Wensink *et al.*, 2015; Voskoboinik *et al.*, 2015). However, caspase-3 can also be activated by granzyme B (Martin *et al.*, 1996). Once activated, caspase 3 induces apoptosis (Bovenschen *et al.*, 2010).

Present in the lytic granules of NK cells and cytotoxic T lymphocytes, granulysin, a membrane destabilizing protein belonging to the saposin-like protein family that is effective in microorganisms that have membranes with low cholesterol content, as is the case of bacteria, fungi, and parasites, is delivered along with granzymes in a perforin-dependent manner (Martinvalet *et al.*, 2005). Furthermore, granulysin delivered by NK cells triggered cellular processes that lead to caspase-7 activation triggering endoplasmic reticulum stress in target cells, interfering with the normal cell functions.

On the surface of the lytic granules of NK cells are expressed lysosomal-associated membrane proteins (LAMPs), including CD107a (LAMP-1), CD107b (LAMP-2) and CD63 (LAMP-3). These proteins are on the inner membrane of granules and are exposed on the surface of NK cells in the degranulation process, which is a prerequisite for granule target cells (Peters *et al.*, 1991, Betts *et al.*, 2003). It has been suggested that LAMPs and cathepsin B, which are highly glycosylated, form a matrix that may prevent the self-destruction of cytotoxic cells by keeping the proteases (granzymes) in an inactive state (Fukuda, 1991, Kannan *et al.*, 1996, Balaji *et al.*, 2002, Lopez *et al.*, 2012). Thus, increased CD107a expression has been correlated with cytokine secretion, loss of perforin by cytotoxic cells, and death of target cells (Alter *et al.*, 2004, Aktas *et al.*, 2009).

3.1.3. NK and dendritic cells crosstalk

DCs and NK cells play a crucial role in modulating innate and adaptive immune responses through complex cell-to-cell crosstalk. Interactions between DCs and NK cells can result in cellular activation, maturation, and cytokine production by both cells (Ferlazzo and Morandi, 2014). DC-NK cells crosstalk can occur in lymph nodes (Celli *et al.*, 2008, Bajénoff *et al.*, 2006), inflammation sites, and peripheric tissues, such as skin and mucosas (Buentke*et al.*, 2002) and solid tumor microenvironments (Mocikat *et al.*, 2004). DC mediate NK cell activation mainly through the release of soluble factors, although cell-to-cell contact plays an important role during the NK-DC interaction. DCs induce cytokine production by NK cells (mainly TNF and IFN-γ) and promote the proliferation of cytolytic NK cells. For an effective immune response by NK cells to occur, it is crucial that become pre-activated by DCs (Andrews *et al.*, 2003, Kassim *et al.*, 2006, Lucas *et al.*, 2007). IL-12, mainly secreted by mDCs, stimulates NK cells to efficiently produce IFN-γ. IL-18 can potentiate the effect of IL-12 by inducing the expression of IL-12R in NK cells. Moreover, the synergism between IL-18 and IL-12 increases the cytolytic activity of NK cells (Walzer *et al.*, 2005). IL-15 is another cytokine produced by DC that is relevant for NK cell development, stimulating their proliferation and initiating a protective response. On the other hand, NK cells also have a fundamental role in DC maturation, through the release of large amounts of TNF and IFN-γ. TNF increases the expression of DC co-stimulatory molecules and when in the presence of IFN-γ, contribute to the production of IL-12 by DCs (Gerosa *et al.*, 2002, Mailliard *et al.*, 2003). In addition, exposure of NK cells to cytokines, such as IL-12 and IL-18 (both released by mDCs) can promote Th1 polarization, since NK cells can promote the priming of $CD4^+$ T cells by secreting IFN-γ (Agaugue *et al.*, 2006, Morandi *et al.*, 2006, Wu et al., 2007). Membrane-bound IL-15 of DCs can be induced by IFN-γ, supporting T cell survival and activation of NK cells (Fig. 27). Still, the ability of NK cells to induce DC activation is not the only mechanism by which NK cells can influence DC functions. Once activated by DCs, NK cells acquire the ability to kill immature mDCs, but not mature DCs. Mature and activated DCs upregulate MHCI expression and would be protected from lysis by NK cells. On the other hand, immature DCs, expressing lower levels of MHCI molecules, are more susceptible to death by NK. DCs expressing insufficient amounts of MHCI do not adequately

induce low-affinity T cells, resulting in Th2 response or induction of tolerance. DC mediated - the killing of targeting cells by NK cells may represent a DC selection mechanism for the control of adaptive immune response (Moretta *et al.*, 2008). According to some reports, NK cells may play a role in promoting the cross-presentation of antigens by DCs. DCs have been shown to absorb cell death by NK cells and present them through MHCI.

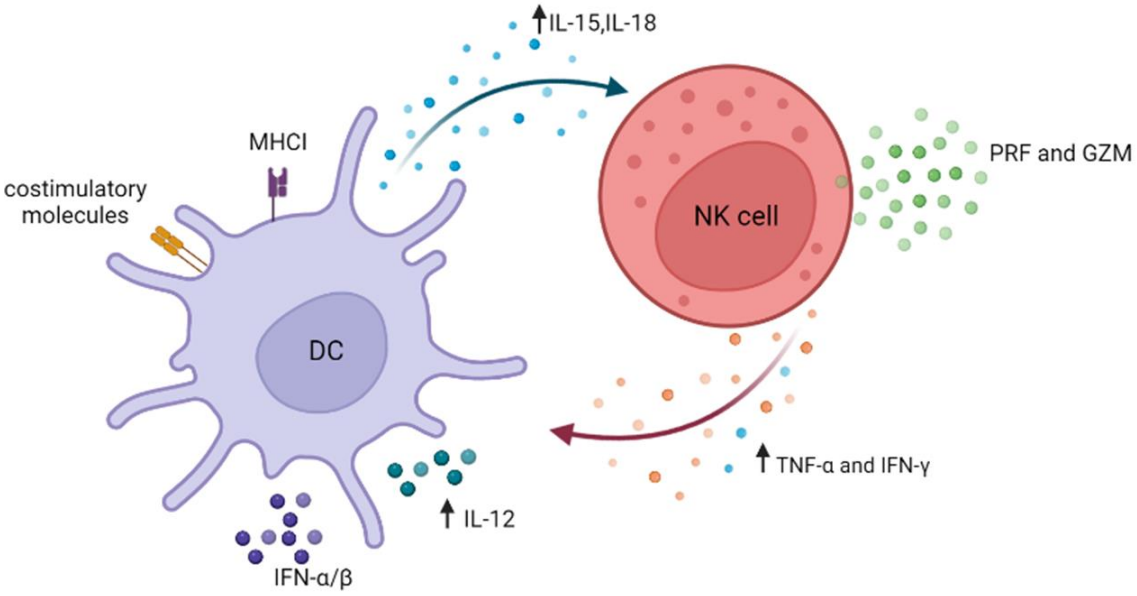


Figure 27. NK-DC crosstalk. Furthermore, direct NK-DC contact is required for optimal NK cell activation by DC. After exposure to various stimuli recognized by a wide variety of PRRs expressed by DCs, there is a release of different cytokines, such as IL-12, IL-15, IL-18, and IFN-γ. IL-12 and IL-18 stimulate NK cells to produce IFN-γ and increase their cytotoxicity. Activated NK cells produce IFN-γ and TNF-α, which promote DC maturation. (PRF - Perforin; GZM- Granzyme B). Image of my own authorship, created with BioRender.com (accessed August 2022).

3.1.4. Interplay of NK cells and *Leishmania* parasites

Along with phagocytic cells, NK cells represent the first line of defense against pathogens using two different mechanisms of action: cytolytic destruction of infected cells and secretion of pro-inflammatory cytokines, such as IFN-γ and TNF-α (Nylén and Gautam,

2010). Previous studies have shown that the production of IFN-γ by these cells is important for the development of a protective immune response and control of *Leishmania* infection, which can delay the disease onset, but is not necessary for a Th1 response to occur (Bogdan, 2012). In the early stage of infection in murine models for VL (*L. donovani*) and CL (*L. major* and *L. braziliensis*), it was verified that TLR9 is involved in the activation of NK to control parasite replication (Vargas-Inchaustegui *et al.,* 2009). NK cells from cured patients and healthy individuals living in *Leishmania* endemic areas are capable of more pronounced proliferation after *in vitro* stimulation with *Leishmania* antigens when compared to patients with active CL (Maasho *et al.,* 1998). Other *in vitro* experiments demonstrate the importance of NK cells in lysing *Leishmania* infected MΦ and inducing the death of extracellular promastigotes as a consequence of their cytotoxic action as well as directing the immune response to a Th1 profile that leads to the activation of MΦ microbicide mechanisms through cytokine production (Aranha *et al.,* 2005, Bajénoff *et al.,* 2006, Lieke *et al.,* 2011). In experimental models of spontaneously healing of *L. major* CL, it was possible to detect cytotoxic activity and IFN-γ production NK cells in peripheral lymph nodes on the first day of infection (Bajénoff *et al.,* 2006). However, some studies suggest that the cytotoxic role of NK cells in CL is not protective, contributing to lesion severity (Brodskyn *et al.,* 1997, Machado *et al.,* 2002).

3.1.5. DC-NK cells crosstalk in *Leishmania* infection

There are not many studies on the interaction of DC-NK cells in the context of infection by *Leishmania* parasites (Sanabria *et al.,* 2008). So far the published studies reported that *Leishmania*-infected mDCs and pDCs may be essential for the *in vivo* activation of NK cells due to their ability to release large amounts of IL-12 and IFN-γ (Liese *et al.,* 2008). Moreover, studies using the mouse model showed that *L. major* genomic DNA and *L. braziliensis* promastigotes activate cDC and pDC to release IL-12 and IFN α/β, which in turn led to an increase in IFN-δ and cytotoxic activity of NK cells (Liese *et al.,* 2007, Schleicher *et al.,* 2007).

NK cells and DC interact in the paracortex of lymph nodes, modulating a localized $CD4^+$ T cell response. According to Bajénoff and collaborators (2006), NK cells of *L. major*

infected mice form a "network" in the lymph nodes where they interact with DCs, inducing the production of cytokines by DC and influencing the establishment of an adaptive immune response. NK cells are usually associated with disease protection or cure (Bajenoff *et al.,* 2006, Bogdan, 2012). On the other hand, previous studies reported that *L. tropica*, *L. amazonensis* and *L. mexicana* amastigotes are poor inducers of IL-12 producing DCs, thus explaining the limited response of NK cells (Soong, 2008, Xin *et al.,* 2008). Moreover, DCs and IL-12 seem not to be sufficient to activate NK cells, requiring IL-2 producing $CD4^+$ T cells (Scharton and Scott, 1993, Liese *et al.,* 2007). Interestingly, in absence of specific $CD4^+$ T cells *in vivo* and *in vitro,* it was verified that DCs were not able to activate NK cells after infection with *L. major* (Bihl *et al.,* 2010).

Taken together, these studies highlight the concept that stimulation of the adaptive immune response by DC-NK cells- crosstalk enhances NK cell activation and cytotoxicity promoting an effective immune response against *Leishmania* parasites.

3.2. Canine NK Cells

The NK gene complex (NKC) in dogs is restricted to a single region on chromosome 27 (Hao *et al.,* 2006). In addition to the type C lectin genes in NKC, the dog genome encodes proteins of group V that function as NK cell receptors for MHCI molecules, as well a proteins of group II that do not seem to be expressed in NK cells. Human, mouse and dog NKC have 2.8 Mb, 8.7 Mb and 2.4 of length, respectively. When compared to humans (29 genes) and mice (57 genes), the dog has a reduced number of NKC genes (22 genes). Despite these differences, based on comparative genomic analysis, killer cell lectin-like receptor (KLR) orthologs, particularly KLRD1 (CD94), appear to be highly conserved between humans, mice and dogs (Brown *et al.,* 2006, Graves *et al.,* 2019). However, it is important to note that NKG2A, which forms a heterodimer with CD94 in humans and mice to recognize MHCI, has not been studied in dogs and was not reported in the CanFam3.1 canine genome analysis (Kent *et al.,* 2002, Gingrich *et al.,* 2019).

The Ly49 and KIR genes in the dog genome have also only been partially studied. Using the Southern blot technique, Gagnier and collaborators (2003) demonstrated that dogs

have a copy of the Ly49 gene which seems to be located in the NKC region of chromosome 27. Furthermore, analysis of Ly49 protein revealed that contains the immune receptor tyrosine inhibitory motif domain (ITIM). However, it was also observed by the same authors that in the dog, the Ly49 gene shows at position 168 the conversion of cysteine into tyrosine. Within the Ly49 gene, there are normally six highly conserved cysteine residues. Thus this change can interfere with Ly49 function and still is unclear if the gene codes for a functional protein.

In mammals, the KIR gene is found in a highly variable region. However, the KIR gene appeared to be absent from the canine genome while the gene that encodes for the Fc fragment of the IgA receptor (FCAR) seems to be prematurely truncated at the 5' end, coding for the transmembrane receptor FcαRI, also known as CD89 (Kent *et al.,* 2002, Hammond *et al.,* 2009). Based on this evidence, dogs may lack a functional KIR gene (Hammond *et al.,* 2009). However, it is possible that a further in-depth analysis reveals a functional KIR in dogs, which has crucial implications for the immune activation of NK cells.

3.2.1. Surface markers for phenotypic identification of canine NK cells

CTAC, a canine thyroid adenocarcinoma cell line established in 1964 (Kasza *et al.,* 1964), has been a valuable tool to study the cytotoxicity of NK cells in the dog (Krakowka, 1983; Knapp *et al.,* 1993, Guenther *et al.,* 1994), since this cell line is a susceptible target for NK cell, probably due to the lack of MHCI. Morphologically, canine NK cells are medium to large-sized lymphocytes with intracytoplasmic granules containing granzyme B and perforin (McDonough *et al.,* 2000). In agreement with the typical molecular markers of NK cells, dog NK cells are $CD4^{-}CD20^{-}$, which are phenotypic markers for T and B cells, respectively (McDonough *et al.,* 2000). In 1985, dog cells with NK cell activity against CTAC were characterized as Ig^{-} and Thy-1^{+} ($CD90^{+}$) cells (Ringler and Krakowka, 1985). Cells with natural cytotoxic activity against CTAC and morphological characteristics consistent with NK cells have been described as $CD5^{dim}$ and $CD8^{+}$ (Huang. *et al.,* 2008; Lin *et al.,* 2010). $CD5^{dim}$ cells comprised about 15% of isolated PBMCs and were morphologically larger than $CD5^{bright}$ cells (Huang *et al.,* 2008). Following exposure

to IL-2, $CD5^{dim}$ cells contained more cytoplasmic granules and demonstrated antigen-independent cytotoxicity. PCR revealed elevated levels of mRNA for several NK receptors and activation markers including NKp30, NKp44, CD16 (FcγRIII), and CD94 (NKG2A) (Huang *et al.,* 2008). However, in these previous studies, the cells described also expressed CD3 which is not expected in NK cells. Shin and collaborators (2013) found that the majority of the cytotoxic large granular lymphocytes (CLGLs) lack the T-cell receptor (TCRαβ or TCRγδ) but express $CD3^{+}$. Thus, despite the presence of T cell marker CD3, the cells exhibiting the immune phenotype$CD5^{dim}CD3^{+}CD8^{+}$ $TCR\alpha\beta^{-}TCR\gamma\delta^{-}$ $CD4^{-}CD21^{-}CD11c^{+/-}$ $CD11d^{+/-}$ $CD44^{+}$ were considered NK cells and not NKT cells. Other authors recognize that dog NK cells could express CD3 at certain stages of maturation (Yasuda *et al.,* 2007, Lee *et al.,* 2018), or could be constitutively expressed by a small subset of lymphocytesnot belong to NK cell lineage that has persisted in culture, such as lymphokine-activated killer (LAK) cells. However, it may also be a technical issue or an antibody-binding artefact. Therefore, the expression of CD3 by canine NK cells is not yet entirely clarified.

IL-2 stimulated LAK cultures to exhibit natural cytotoxicity, but the cultures turn out to be a mixture of T cells and NK cells, which indicates that LAK cultures cannot be considered NK cell cultures (Takei, 2011, Michael *et al.,* 2013). Grondahl-Rosado and collaborators (2016) examined the cell population of $CD3^{-}GranzymeB^{+}$, which were Natural cytotoxicity triggering receptor 1^{+} ($NCR1^{+}$) and $NCR1^{-}$. The authors concluded that the NK cell population include both $CD3^{-}GranzymeB^{+}NCR1^{-}$ and $CD3^{-}GranzymeB^{+}NCR1^{+}$ cell subsets, which represents an activated cell population. Based on this evidence, it appears that NCR1 may be a good phenotypic marker to define resting canine NK cells (Grondahl-Rosado *et al.,* 2016).

CD16 and NKG2D are considered potent mediators of NK cytotoxicity (Biassoni *et al.,* 2001, Chiossone *et al.,* 2017). Also known as FcγRIIIa and being encoded by the FCGR3A gene, the CD16 molecule contains a constant region of the Fc receptor. However to date studies have not conclusively demonstrated whether CD16 is expressed on dog NK cells, and notably, the FCGR3A gene was not annotated on the CanFam3.1 dog genome (Kent *et al.,* 2002). However, these protein are normally expressed at low levels on the cell surface, but when cells are infected, transformed, and senescent (as well as rapidly proliferating cells), the expression of this proteins is upregulated. Although the

KLRK1 gene has been identified on chromosome 27 of dogs and presents a high homology to humans and mice (Hao *et al.*, 2006, Hammond *et al.*, 2009), the expression of this protein has not been formally detected on canine NK cells. So far, the presence of these receptors in canine NK cells has not been fully conclusive. Therefore, CD16 and NKG2D may be absent in dog NK cells or the methodology used prevent their detection.

Thus, further studies are needed fully identified cell surface markers capable of a clear phenotypic identification for canine NK cells.

3.3. Objetives

The objective of this study was to investigate the interaction between NK cells and moDC infected with *Leishmania* promastigotes, and stimulated by EVs shed by *Leishmania* parasites.

To accomplish the objective, the study includes the following specific goals:

1. Isolate NK cells from PBMC of healthy dogs and immunophenotyping;
2. Establish co-cultures of NK cells and moDCs (*L. infantum* and *L.amazonensis* infected or EVs stimulated moDC):
    2.1. Analyse NK cell activity by studying cytokine profile and cytotoxic activity;
    2.2. Examine the viability of moDC intracellular parasites;
    2.3. Evaluate the apoptotic status of moDCs.
3. Assess moDCs activity by examining cytokine generation as well as the surface expression of MHC and costimulation molecules.

3.4. Methods

3.4.1. Experimental design

To investigate the interplay of NK cells with moDC infected with *Leishmania* promastigotes or stimulated by EVs (Fig. 28), NK cells were isolated from dog peripheral blood and DCs differentiated from peripheral blood monocytes as previously described. NK cells were immunophenotyped by flow cytometry.

Then cocultures were established. To evaluate the activity of NK cells, the supernatant was used to estimate the release of perforin and granzyme B through ELISA and not adherent cells were utilized to examine the gene expression of CCL3/MIP-1α, CCL4/MIP-1β, IFN-γ, IL-13 and IL-8, which was quantified by Real-time PCR.

To assess the activity state of adherent DCs, RNA was extracted to analyse pro-inflammatory (IL-IL-12p40) and anti-inflammatory cytokines (IL-13 and IL-10) and co-stimulatory molecules (CD80/CD86) by Real-time PCR and evaluated the presence of surface MHC by flow cytometry.

To determine the effect of NK cells on moDC viability, the apoptosis/necrosis levels were evaluated by flow cytometry. To evaluate the effect of the interplay between NK cells and moDC on intracellular parasite survival resuspended moDCs were transferred for Schneider's drosophila medium and incubated at 24ºC. Free-moving promastigotes were observed and quantified under an optical microscope.

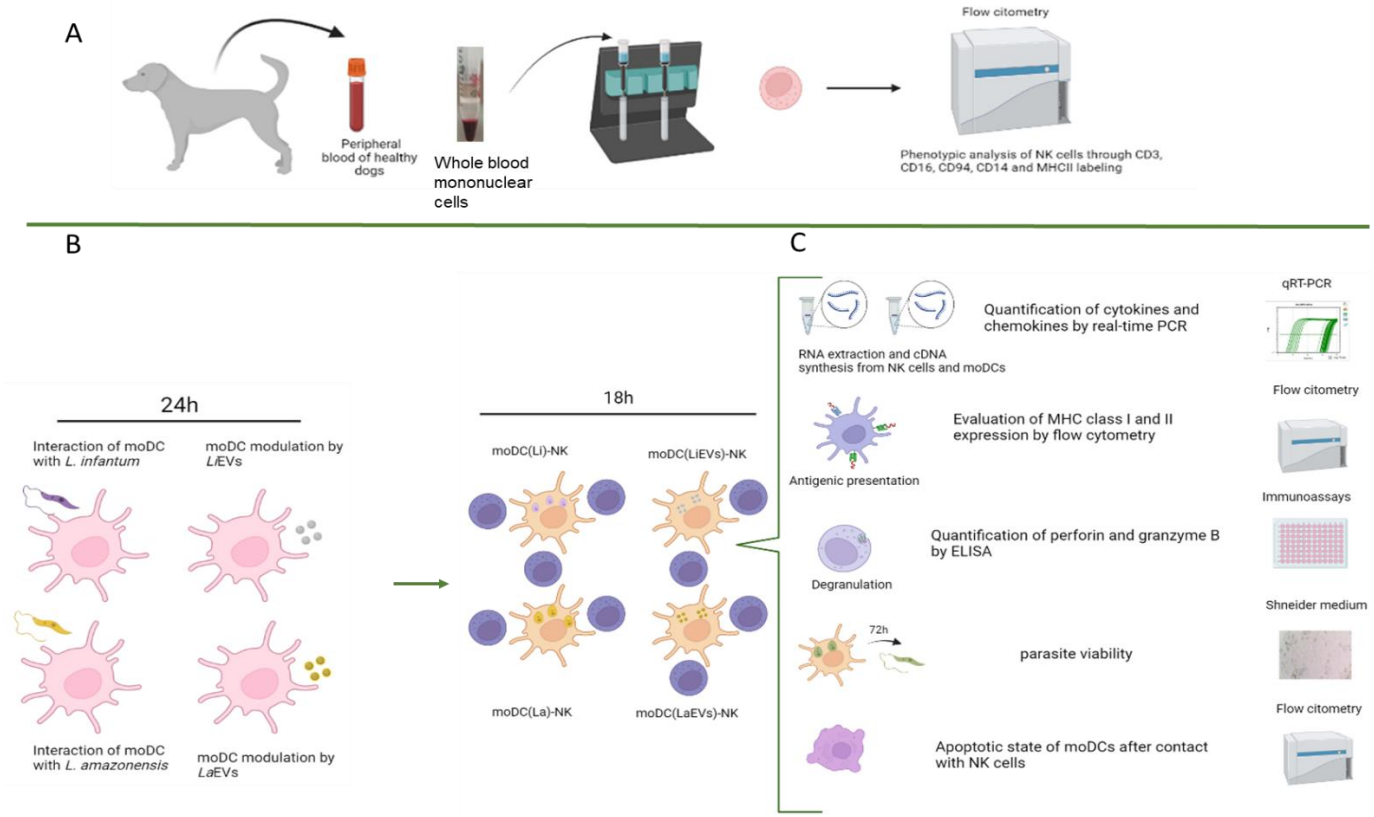


Figure 28. Schematic representation of the experimental design followed in the present study. NK cells were isolated from PBMCs of healthy dogs (A) using magnetic separation. moDC were exposed to virulent promastigotes of *L. infantum* and *L. amazonensis* as well as promastigotes EVs (*LiEVs* and *LaEVs*) of both parasite species, for 24 h (B). Then, moDCs were placed in contact with NK cells for 18 h. The immune response was evaluated through the quantification of pro- and anti-inflammatory cytokines and chemokines gene expression by qRT-PCR, the expression of MHC molecules by multiparametric flow cytometry and quantification of the perforin and granzyme B by ELISA. After 42 h, infected moDCs were transferred to Shneider's medium to examine the viability of intracellular parasites moDC apoptosis was assessed by flow cytometry (C).

3.4.2. Isolation of NK cells

A MultiStand System constituted by MACS® Separators (Mini MACS), MS columns, NK Cell Isolation Kit coated magnetic microbeads and a MultiStand (Miltenyi Biotec, Germany) was used to sort NK cells. All the procedures and reagents used in this assay were performed according to the protocols described in the datasheet of NK Cell Isolation Kit microbeads provided by the manufacturer. The principle of this magnetic cell system is the use of microbeads, which are very small super magnetic particles, to sort the cell subset of interest. The mix of beads with PBMC suspension was passed through a column magnetically attached to MACS® Separator bound to the MultiStand. The columns and separator enable the formation of a high-gradient magnetic field that retains the beads and cells in the column.

Mononuclear cells were resuspended in 40 µL of PBS pH 7.2 supplemented with 0.5% FBS and 2 mM EDTA. Magnetic separation (MS) buffer was added to NK Cell Biotin-Antibody Cocktail (Miltenyi Biotec) and incubated for 5 min at 4°C, protected from light. Buffer (30 µL) was added to 20 µL of NK Cell MicroBead Cocktail and incubated for 10 min. at 2−8 °C. Then, the mononuclear cell suspension was passed through MS columns attached to a Mini MACS separator according to the manufacturer's instructions. Unlabeled cells, representing lymphocytes enriched in NK cells were eluted (which flow through) and the column was detached from the magnetic field.

After the collection of cell suspension (enriched in NK cells), it underwent a new magnetic separation to remove CD3 cells. Cells were centrifuged (10min., 500 ×g, 4°C) and the supernatant was discarded. The pellet was resuspended in 100 µL PBS supplemented with 3% FBS and 10 mM EDTA (cell sorting buffer). MagniSort™ Positive Selection Antibody (20 µL) was added to the cell suspension and mix well by 5 times pulse vortexing. After 10 minutes of incubation at room temperature, the cell suspension was washed with cell separation buffer and then centrifuged (5min, 300 ×g, 4°C). The supernatant was discarded, and the cells were resuspended in 100 µL of buffer and 20 µL of MagniSort™ Positive Selection Beads. The suspension was well mixed by five times pulse vortexing and incubated at room temperature for 10 minutes.

The cell suspension was passed through a MS column attached to a Mini MACS separator according to the manufacturermin.s instructions and eluted unlabeled cells were collected.

3.4.3. Immunophenotyping of NK cells

NK cells were immunophenotyped by multiparametric flow cytometry analysis. NK cells were washed once with cold 1× PBS (Lonza, Belgium) (300 ×g, 10min. 4°C), fixed with 2% paraformaldehyde (Sigma-Aldrich) (w/v) for 20 min. at 4°C and resuspended in PBS. Then NK cells were incubated with the monoclonal antibodies directly conjugated with fluorescent dyes listed in Table 8.

Table 8. Surface marker antibody panel for NK cells

Laser	488 nm	640 nm	
Emission filters	525/40	665/20	660/20
Flurochrome	FITC	APC	Alexa Fluor® 647
Biomarker	MHC II CD3 CD16	CD94	CD21
Monoclonal antibody	Rat anti Dog MHC Class II Monomorphic MOUSE ANTI DOG CD3 Human CD16 Monoclonal Antibody	CD94 Monoclonal Antibody	Mouse anti Canine CD21
Clone	YKIX334.2 CA17.2A12 eBioCB16	HP-3D9	CA2.1D6
Company	Bio-Rad Bio-Rad Invitrogen	Invitrogen	Bio-Rad

Cell acquisition was performed on a CytoFLEX system cell analyzer (Beckman Coulter), and Flowjo_V10 analyzed data. The FSC-H vs SSC-H gate was used to remove debris and pyknotic cells as well as the very large debris. Singlet gate was used to define the non-clumping cells based on pulse geometry FSC-H *vs* FSC-A.

3.4.4. moDC-NK cell co-cultures

Dog moDCs (1×10^5 cells/well) were seeded in 96-well plates with *Leishmania* promastigotes, at a ratio parasite-DC of 3:1 in 300 µL of RPMI supplemented with 10% heat-inactivated FBS (v/v). moDCs exposed to *Leishmania* EVs (10 $\mu g.mL^{-1}$) were also evaluated. Plates were incubated at 37ºC in a humidified atmosphere containing 5% of CO_2 for 24 h. In parallel, resting-moDCs were used as negative control.

After 24 h of DC exposure to *Leishmania* promastigotes and EVs, NK cells were added to the plate at 1:1 moDC-to-NK cell ratio for an additional 18 h. At the same time, resting-NK cells was used as negative controls.

After the incubation period, the tubes were centrifuged at 500 ×g for 10 min. at room temperature (RT). Supernatants were transferred to new tubes and kept at -20ºC for later use in ELISAs. To each tube (pellet of NK cells) was added RLT, which is a lysis buffer used to lyse tissues and cells, allowing the release of RNA / DNA / protein (350 µL RNeasy Mini Kit, Qiagen, Germany) and for each mL of RTL 0.1 µL of 2-mercaptoethanol (RLT-ME, Sigma-Aldrich) was added. The samples were kept at -80 ºC for further extraction of ribonucleic acid (RNA) as described in 2.3.12.1.

3.4.5. Immunoassays

Once activated, NK cells use cytotoxic mechanisms to kill target cells. This mechanism consists of the production of two proteins, perforin and granzyme B. Perforin is a pore-forming protein in cell membranes and granzymes are serine proteases that enter the target cell through the pores formed by perforin and activate de caspase cascade leading to cell apoptosis. Thus, we were able to quantify the production of perforin and granzyme in co-cultures supernatants by ELISA.

Release of perforin and granzyme B was estimated in co-cultured supernatants of unprimed moDC-NK, PMA stimulated moDC-NK, *Leishmania* infected moDC-NK, and *L*EVs stimulated moDC-NK, after 18 hours of incubation, using the Dog/Canine Perforin 1 PRF1 ELISA Kit (Quimigen Unipessoal LDA, Portugal) and Canine Granzyme B

ELISA Kit (Quimigen Unipessoal LDA) respectively, according to the manufacturer's instruction.

Anti-Canine PRF1 specific antibody and monoclonal antibody specific for Granzyme B were used to pre-coated the 96-well plates respectively for perforin and granzyme detection. The canine PRF1 present in the standards or in our samples binds to the capture antibody. Subsequently, the detection of biotinylated canine anti-PRF1 antibody is added to form an Ab-Ag-Ab sandwich. After a wash step, streptavidin-HRP is added and unbound conjugate is removed with wash buffer. In turn, for the detection of Granzyme B, the standards and test samples were pipetted into the plate wells and a biotinylated goat polyclonal detection antibody specific for granzyme B was added to the wells. In both ELISAs, the wells were washed with TBS buffer. After the unbound biotinylated antibody was removed, Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed with TBS buffer.

Then the addition of substrate TMB, results in the production of a blue color product that changes to yellow after the addition of an acid stop solution. The density of the yellow color is directly proportional to the amount of granzyma B or Canine PRF1 captured on the plate. Optical density (OD) was read at 450 nm in a microplate reader (TRIADTM 1065, DYNEX Technologies, USA).

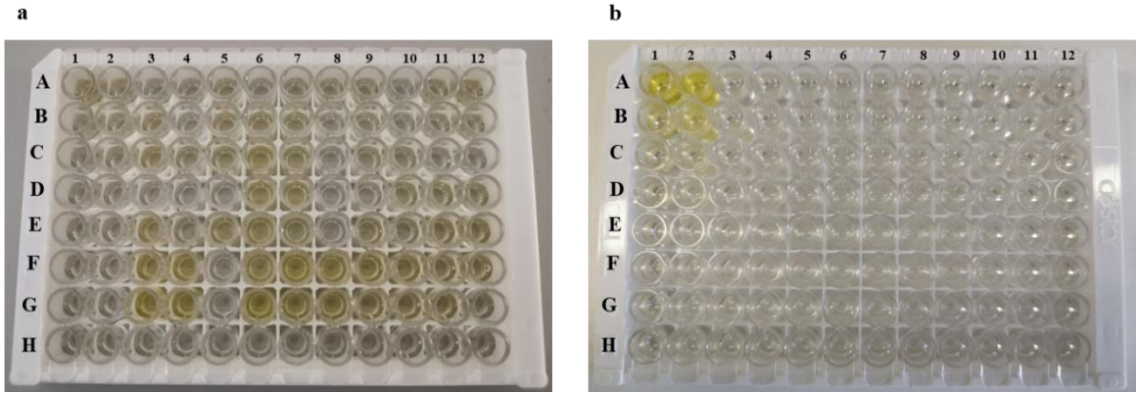


Figure 29. Representative image of the perforin and granzyme B commercial ELISA. Plate of moDC-NK cell co-culture samples after the addition of stop solution. Plate to evaluate the release of perforin (a) and granzyma B. Standards - Column 1 and 2 (A-F), blank - Column 1 and 2 (G and H), Samples - Column 3 to 12.

3.4.6. Real-time PCR

RNA isolated from NK cells that were in co-culture with infected moDCs stimulated EVs stimulated moDCs was used to examine the gene expression of the following molecules through real-time PCR (as described in 2.3.13):

(i) Chemokines - CCL3/MIP-1α and CCL4/MIP-1β;

(ii) Cytokines - IL-3, IFN-γ, TNF-α, IL-8 and IL-13.

RNA obtained from moDCs exposed to *L. infantum* and *L. amazonensis* promastigotes and stimulated by EVs of both *Leishmania* strains and then co-cultured with NK cells were used to examine:

(i) Costimulatory molecules - CD80 /CD88;
(ii) Cytokines IL-12p40 and IL-10 by real-time PCR.

In parallel, costimulatory molecules, chemokine and cytokine gene expression of resting NK and NK cells cocultured with unprimed moDC (unstimulated and unexposed to parasites) were also analysed.

3.4.7. moDC apoptosis

Apoptotic cells were assessed by multiparametric flow cytometry analysis. Unprimed moDC (negative control), moDC exposed to *L. infantum* and *L. amazonensis* promastigotes and stimulated by EVs shed by *L. infantum* and *L. amazonensis* promastigotes, were incubated for 24 h. Then NK cells were added to moDCs and the co-cultures were incubated for 18 h. The viability status of co-cultured NK cells was also examined (as described in 2.3.8co).

After incubation, cultures were washed one time with 200 mL of cold 1× PBS (300 ×*g*, 10 min., 4ºC) and incubated with the commercial kit TACS^{TM} Annexin V FITC (R&D Systems, USA), according to the manufacturer's instructions. Before flow cytometry acquisition, cells were treated with 1μL of propidium iodide (PI) (R&D Systems). FL1-H (Annexin V FITC) *vs* FL5-H (PI) gate on unprimed-moDC was used to delimit annexin

V FITC$^-$/PI$^-$ population (viable cells), annexin V FITC$^+$/PI$^-$ (pre-apoptotic cells) and annexin V FITC$^{+ \text{ or } -}$ /PI$^+$ cells (apoptotic cells).

3.4.8. Viability of moDC-intracellular parasites

The viability of *L. infantum* and *L.amazonensis* uptaken by moDC parasites were evaluated after being in contact with NK cells.

After 24 h of infection, *L.infantum* and *L.amazonensis* infected moDC were placed in contact with NK cells for 18 h (42 h total). Then the medium was removed and the wells were washed two times with 1× PBS to clean possible free parasites, and 300 µL of complete SCHN medium was added. The plate was incubated for 72 h at 24ºC to eventually promote the parasite release from the infected moDC and the differentiation of intracellular viable amastigotes into extracellular promastigotes. Viable parasites were estimated under an optical microscope using a Neubauer counting chamber.

3.4.9. moDC surface expression of MHC molecules

moDCs incubated for 24 h with *L. infantum* and *L. amazonenis* promastigotes in the proportion of 1 moDC:3 parasites and EVs of both *Leishmania* species in co culture with NK cells were used to examine the MHC expression by multiparametric flow cytometry. Cells were then harvested and washed with 100 µL of 1× PSB at 500 ×g for 10 min. to remove free parasites and traces of proteins present in the culture medium. Cell suspension was fixed with 2% paraformaldehyde (Sigma-Aldrich) (w/v) for 20 min. at 4ºC and incubated with a panel of monoclonal antibodies (Table 9).

Cells were incubated on ice for 30 min. and washed with 1× PBS at 500 ×g for 10 min. to eliminate Ab that did not bind specifically to cells. Sediments were then resuspended in 100 µL of PBS 2% FBS. In parallel, unprimed moDCs in co-culture with NK cells were used as negative control.

Tabela 9 – Panel of monoclonal antibodies for labeling MHC molecules

Laser	488 nm	
Emission filters	525/40	585/42
Flurochrome	FITC	PE
Biomarker	MHC II	HLA ABC
Monoclonal antibody	Rat anti Dog MHC Class II	Mouse anti Human HLA ABC
Clone	YKIX334.2	W6/32
Company	Bio-Rad	Bio-Rad

The controls for cytometer calibration and fluorescence adjustment, ensuring the correct reading of the samples, were cells individually labelled with each of the antibodies used and cells stained with both antibodies. Cell acquisition was performed on a CytoFLEX system cell analyzer (Beckman Coulter) and Flowjo_V10 analyzed data (Tree Star Inc., USA) was used to import and evaluateflow cytometry data. The FSC-H vs SSC-H gate was used to remove debris and pyknotic cells as well as the very large debris. Singlet gate was used to define the non-clumping cells based on FSC-H vs FSC-A pulse geometry.

3.4.10. Data analysis

Assays were realized in, at least, five different dogs. Each sample was analyzed in duplicate or triplicate. The Wilcoxon test for paired samples was used to perform the statistical analysis, as is the non-parametric equivalent of the paired samples t-test. It should be used when the sample data are not normally distributed, as is the case of the present study.

Differences were considered significant with a 5% significance level ($p<0.05$). GraphPad Prism version 9 for Windows (GraphPad Software, USA) was used for statistical analysis and graphical representation of data.

Since moDCs are stimulated by EVs or infected, it is difficult to draw a conclusion about NK effect (the parasite or EVs or the interaction with NK cells). whether it is the fact that the cells are stimulated or infected, or Then a relation was established with infected or primed moDCs (no NK cells present) obtained in the first part of this work (Chapter 1). In this case, the mean and SEM of the data are represented by column bars graph.

3.5. Results

3.5.1. The population of cells magnetically purified were enriched in NK cells

The cell population isolated from PBMCs and magnetically purified using the MicroBead Cocktail and MagniSort™ Positive Selection Antibody were evaluated by multiparametric flow cytometry, using the surface markers CD3, CD21, CD16, CD94, and MHCII. After analyzing the size (FSC) and the granular content (SSC), the singlet gate was used to define non-agglutinating cells and eliminate doublets (Fig. 28)

Within the cell population, there were $CD3^+$ and $CD21^+$ cells as well as $CD3^-$ and $CD21^-$ cells in addition to $CD16^+$, $CD94^+$, and $MHCII^+$ cells (markers chosen to identify NK cells). Therefore, dog NK cells should present the following phenotype: $CD3^-CD21^-CD16^+CD94^+MHCII^+$. However, the commercial kit used to purify NK cells did not seem to be able to completely separate the NK cells from the other lymphocytes, as is the case of $CD3^+$ (T cells) and $CD21^+$ cells (B cells), which were present in a considerable proportion, ≈ 27% and ≈ 19%, respectively.

Therefore, the magnetically purified cell population was enriched in NK cells, and for convenience, designated as NK cells through this chapter. Moreover, this cell population were morphological homogenous when observed by optical microscopy and cells were round, showing small to medium-sized cells without contamination with mononuclear cells or granulocytes, which are bigger cells (Fig. 29).

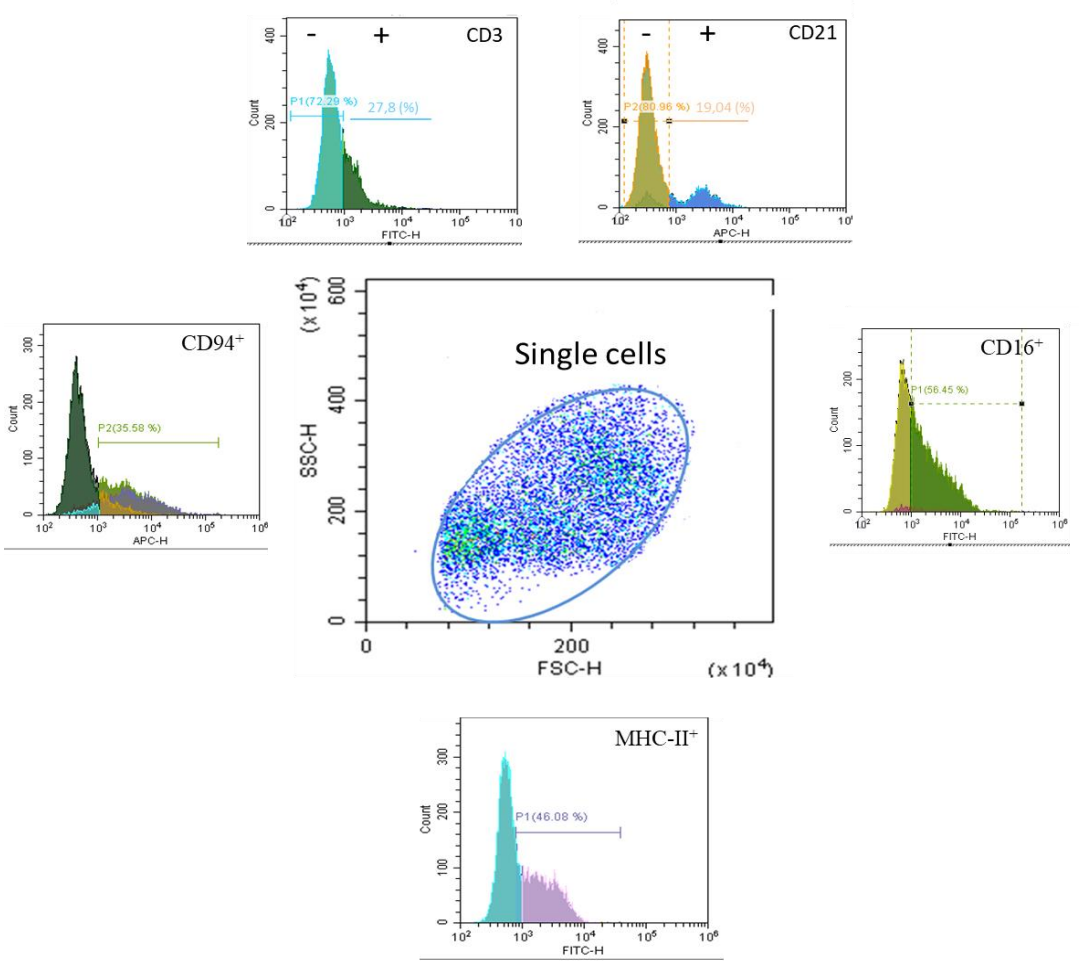


Figure 30: Imagem representative of NK cells immunophenotyping. Cells magnetically separated from PBMCs were evaluated by multiparametric flow cytometry. Total cells were gated on FSC-H vs SSC-H pseudocolour graph using the auto gate tool. Gate strategy using the surface markers CD3 (blue), CD21 (orange), CD16 (green), CD94 (dark blue) and MHC-I (light blue).

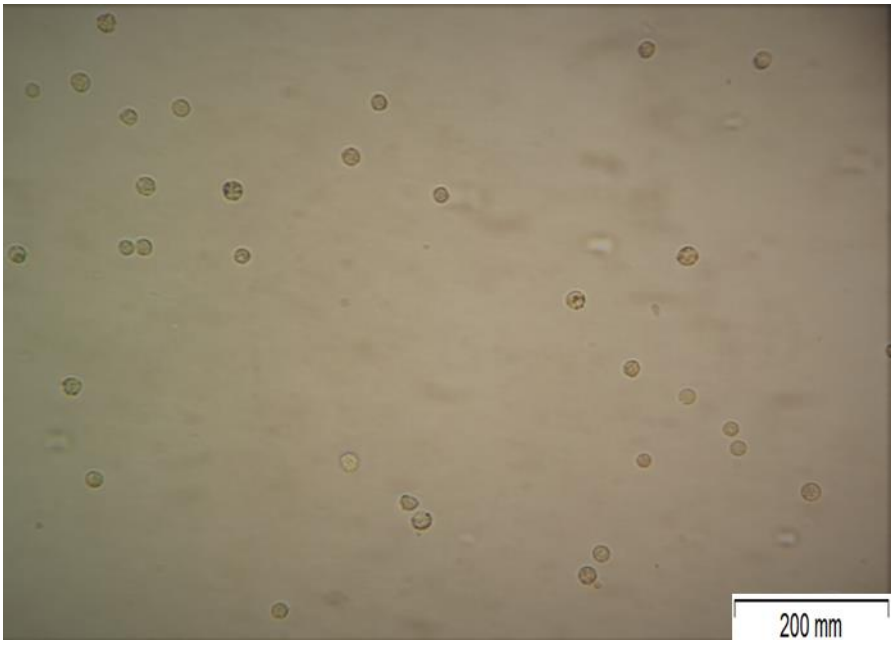


Figure 31. Image representative of purified cell population enriched in NK cells. After the isolation of PBMCs, NK cells were purified by magnetic separation, observed by optical microscopy and images were acquired.

3.5.2. NK cells keep viability when in co-culture with infected moDCs

To confirm that NK cells in co-culture with unprimed moDCs, *L. infantum* and *L. amzonensis* infected moDCs, and *LiEVs* and *LaEVs* stimulated moDCs were viable, NK cells were evaluated by multiparametric flow cytometry. In parallel, NK cells were also analyzed. When compared to resting NK cells, NK cells that were in co-culture with *L. amazonensis* -infected moDCs showed a marked increase (P = 0.0234) in the proportion of viable cells and a slight decrease in pre-apoptotic cells as well. The frequency of viable NK cells also significantly increased when in co-culture with *LaEVs* stimulated moDCs (P = 0.0234). After 18 hours of incubation, the number of apoptotic cells was reduced in all cases and the pre-apoptotic cells only showed a slight increase in NK cells treated with DMSO (Fig. 30)

Taken together these results indicate that NK cells in co-culture for 18 h with unprimed-moDCs, infected moDCs and EV-stimulated moDCs keep their viability. However, it is interesting to highlight that *L. amazonensis* infected moDCs and *La*EV stimulated moDCs seem to enhance NK cells viability.

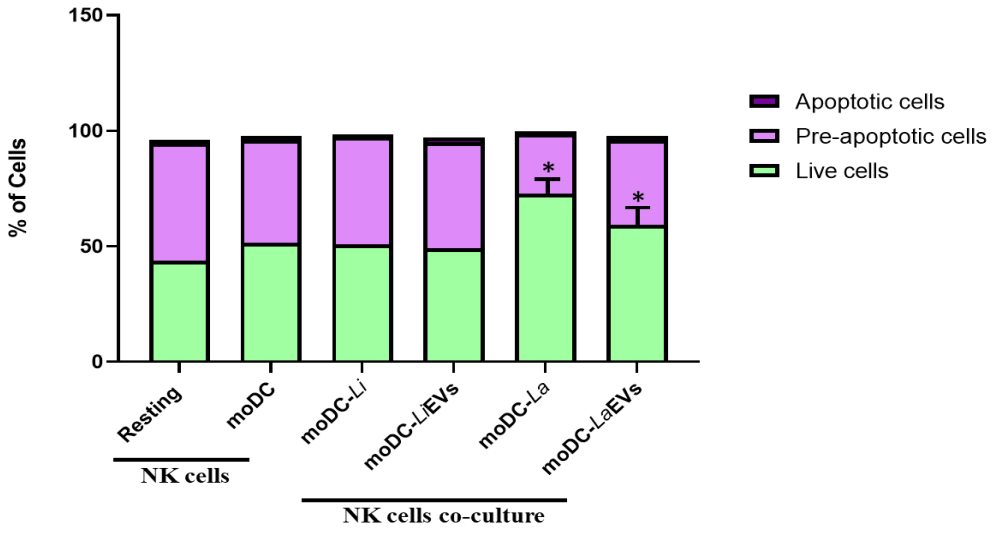


Figure 32. Viability of NK cells when in co-culture with *L. infantum* and *L. amazonensis* infected moDC. The viability of NK cells in co-culture with *L. infantum* (Li) and *L. amazonensis* (La) infected moDCs and moDCs stimulated by *L. infantum* (*LiEVs*) and *L. amazonensis* (*LaEVs*) EVs was assessed by multiparametric flow cytometry, using annexin-V and propidium iodide (A). In parallel, the viability of resting NK cells was also evaluated. The median of five dogs and three replicates per sample are represented by stacked bars. Nonparametric Wilcoxon's test was used for statistical comparisons and *(P<0.05) indicates a significant difference when compared with resting NK cells.

3.5.3. NK cells in co-culture with L. *amazonensis* infected moDCs generate IFN-γ and IL-13

The gene expression of pro-inflammatory cytokines by NK cells in co-culture with *Leishmania* infected moDCs and moDCs exposed to EVs (*LiEVs* and *LaEVs)* were quantified by real-time PCR.

When compared with resting NK cells, gene expression of IFN-γ was significantly increased in NK cells that were in co-culture with L. *amazonensis* infected moDCs (P = 0.0313) (Fig. 31a). In turn, co-culture with *L. infantum* infected moDCs led to a significant decrease of IFN-γ (P = 0.0156) and TNF-α gene expression (P = 0.0273) in NK cells (Fig. 31b).

On the other hand, NK cells only presented a significant accumulation of IL-13 mRNA when in co-culture with moDC infected by *L. amazonensis* (P= 0.0156) or stimulated by *LaEVs* (P= 0.0313) (Fig. 31c)

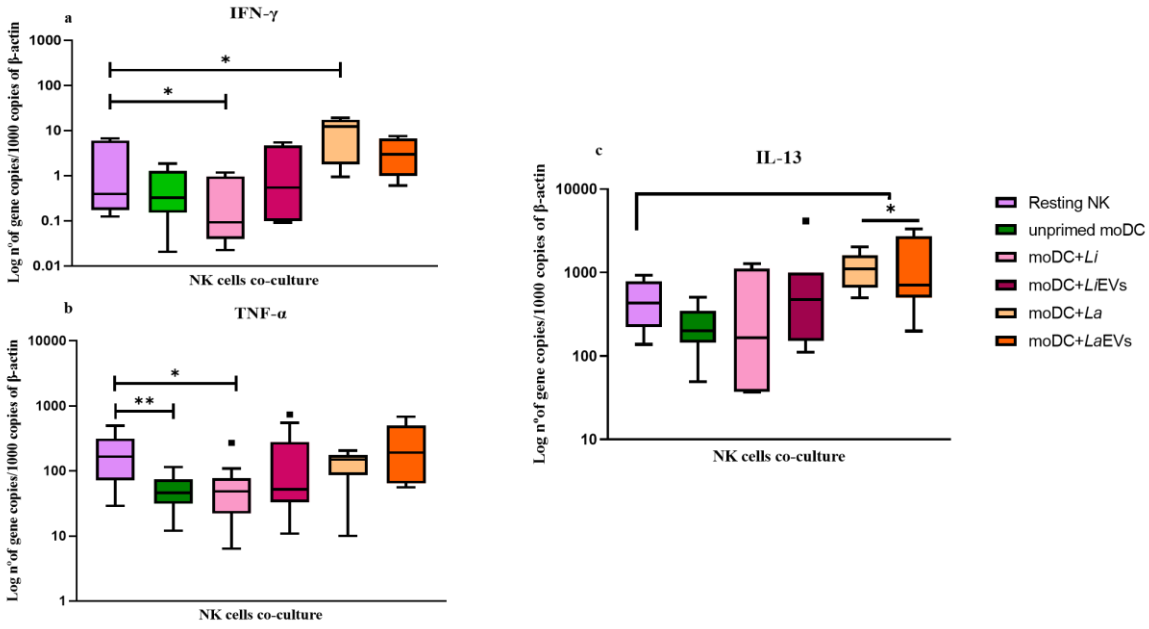


Figure 33. Gene expression of pro-inflammatory cytokines by NK cells in co-culture with *Leishmania* infected moDCs. mRNA of KC cells co-cultured with infected moDCs (Li and La) and moDCs stimulated by parasite EVs (*LiEVs* and *LaEVs*) were used to evaluate IFN-γ (a), TNF-α (b), and IL-13 (c) gene expression by real-time PCR. In parallel, cytokine gene expression of NK cells cocultured with unprimed moDCs and NK cells alone was also assessed. The results of five dogs performed in duplicate are represented by Tukey box plots, including the median, interquartile range and minimum and maximum values. Black squares represent outliers. Nonparametric Wilcoxon's test was used for statistical comparisons. * (P<0.05) and ** (P<0.01) indicate significant differences.

Therefore, these results indicate that moDCs infected by *L. infantum* parasites do not induce NK cells to generate pro-inflammatory cytokines. Indeed, these infected cells seem to inhibit NK cells to generate proinflammatory cytokines. In contrast, *L. amazonensis* infected moDCs induce NK cells to generate IFN-γ and IL-13, but not TNF-α, and *LaEVs* stimulated moDCs also promoted NK cells to generate IL-13.

3.5.4. *L. infantum* and *L. amazonensis* infected moDCs promote the generation of chemokines by NK cells

The gene expression of CCL3, CCL4 and CXCL8 (IL-8) was evaluated by real-time PCR in NK cells in co-culture with *L. infantum* and L.amazonensis infected moDCs or moDCs stimulated by parasite EVs (*LiEVs* and *LaEVs).*

moDCs stimulated by EVs from both *Leishmania* species induced NK cells to accumulate a significantly higher amount of CCL3 ($P_{LaEVs\text{ -}moDCs \text{ and } LiEVs\text{ -}moDCs}$ = 0.0313) and CCL4 mRNA ($P_{LaEVs\text{ -moDCs, }LiEVs\text{ -moDCs}}$ = 0.0156). moDCs infected with *L. amazonensis* or *L. infantum* (P_{CCL3}= 0.0313, P_{CCL4}=0.0156) also promoted NK cells to significantly increase the gene expression of CCL3 (Fig. 32a) and CCL4 (Fig. 32 b).

Moreover, with the exception of NK cells in co-culture with *LaEVs* -moDCs, NK cells of all the other co-cultures (unprimed moDCs, *L. infantum* and *L. amazonensis* infected moDCS, and *LiEVs* stimulated moDCs) significantly upregulated IL-8 gene expression ($P_{\text{Li-moDCs}}$ = 0.0391; $P_{\text{La-moDCs, }LiEVs\text{ -moDCs}}$ = 0.0078; $P_{LaEVs\text{ -moDCs}}$ = 0.0313) (Fig. 32c).

Furthermore, the contact with unprimed moDCs also induced NK cells to increase CCL4 (P= 0.0156) and CCL8 gene expression (P= 0.0391).

Taken together these results indicate that the co-culture with *L. infantum* and *L.amazonensis* infected moDCs and EVs stimulate moDCs lead NK cells to generate CCL3, CCL4 and CXCL8 which are chemotactic for $CD8^+$ T cells, $CD4^+$ T cells and neutrophils, respectively.

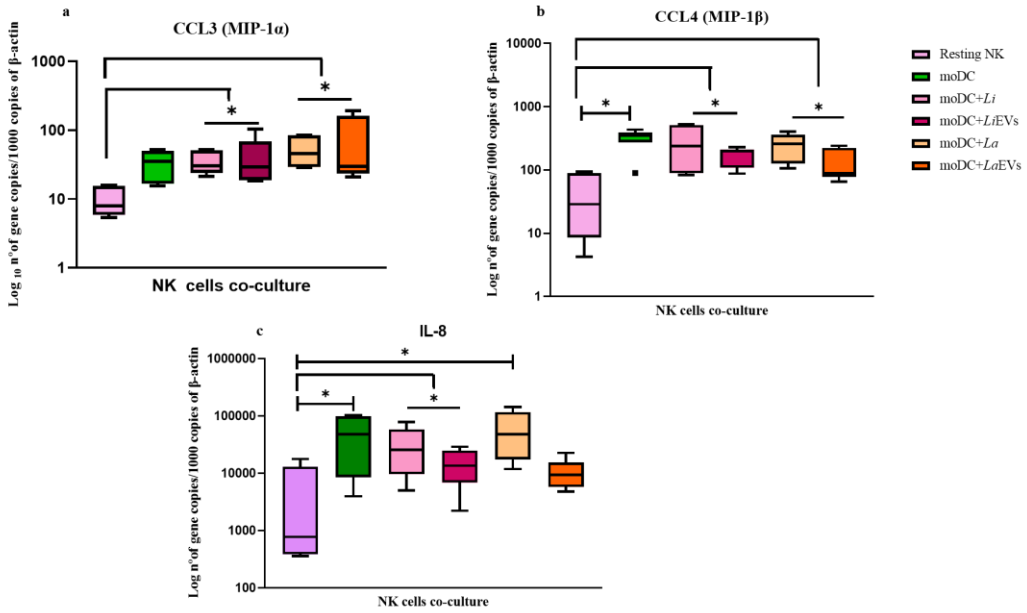


Figure 34. Gene expression of chemokines in NK cells that were in co-culture with *Leishmania* infected moDCs. Infected moDCs (Li and La) and moDCs exposed to parasite EVs (*LiEVs* and *LaEVs)* were used to evaluate CCL3 (a), CCL4 (b) and IL-8 (c) gene expression by real-time PCR. In parallel, chemokines of unprimed moDCs cocultures and NK cells alone were also assessed. The results of five dogs performed in duplicate are represented by Tukey box plots, including the median, interquartile ranges, and minimum and maximum values. Black squares are indicative of outliers. Nonparametric Wilcoxon's test was used for statistical comparisons. *(P<0.05) indicate significant differences.

3.5.5. *L. amazonensis* infected moDCs trigger the release of perforin by NK cells

Perforin concentration was quantified by ELISA in supernatants of NK cells co-cultured with infected (*L. infantum* or *L. amazonensis)* moDCs and EVs (*LiEVs* or *LaEVs*) stimulated moDCs.

Overall, there was an increase in the concentration of perforin in supernatants of co-cultures. However, when compared with the level of perforin found in the supernatant of NK cell culture, the amount of perforin in supernatants of NK cells-*L. amazonensis* infected moDCs co-cultures (P = 0.0078) and NK cells-EVs stimulated moDCs co-cultures (P_{LiEVs} = 0.0156 and P_{LaEVs} = 0.0078) were significant high (Fig. 33a)

In contrast, the granzyme B concentration detected by ELISA in the supernatants of co-cultures was similar to NK cell culture (Fig. 33b).

Thus, *L. amazonensis* infected moDCs and *LiEVs* or *LaEVs* stimulated moDCs induce NK cell degranulation, releasing perforin. However, the process of NK degranulation induced by *L. infantum* infected moDCs seems to be more restricted being released lower levels of perforin. Although there was a release of perforin into the extracellular medium, this was only accompanied by a minimal release of granzyme B.

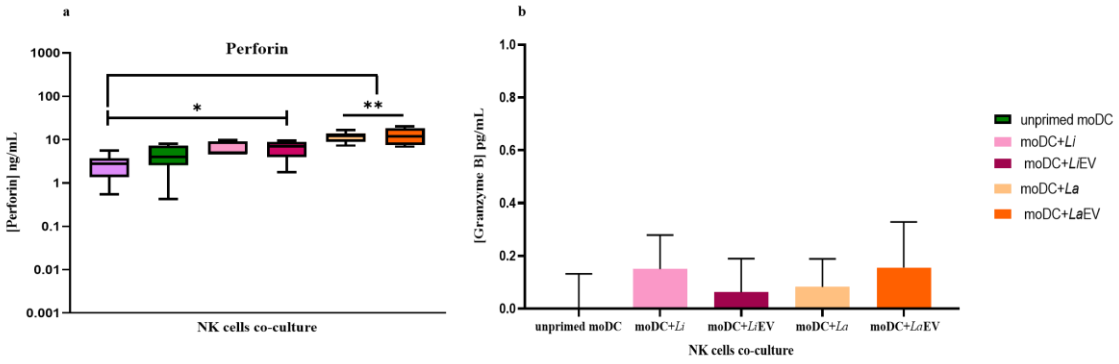


Figure 35. Degranulation of NK cells in contact with *Leishmania* infected moDCs. Supernatants of NK cells co-cultured with infected moDCs (*Li* and *La*) and *LiEVs* and *LaEVs* stimulated moDCs were used to analyse by perforin (a) and granzyme B (b) release by ELISAs. In parallel, supernatants of NK cells-unprimed moDCs co-cultures and NK cell cultures were also evaluated. The perforin results of five dogs performed in duplicate are represented by Tukey box plots (a) and by columns bar chart (b) the concentration of granzyma B concentration after withdrawing granzyme produced by NK cells,. Nonparametric Wilcoxon's test was used for statistical comparisons. * ($P<0.05$) and ** ($P<0.01$) indicate statistically significant differences.

3.5.6. NK cells induce infected moDCs to upregulate IL-10 and IL-12p40 gene expression

To evaluate the effect of NK cells on the generation of pro- and anti-inflammatory cytokines by *Leishmania*-infected, EV-stimulated (*LiEVs* and *LaEVs)* and unprimed moDCs, cytokine gene expression of co-cultured moDCs was quantified by real-time PCR. The results were compared with the cytokine gene expression of moDCs (infected, stimulated and unprimed) that had no contact with NK cells.

NK cells induced unprimed moDCs (uninfected and unstimulated) to upregulate IL-10 and IL-12p40 gene expression, showing atleast a 10-fold increase.

NK cells cocultured with *L infantum*-infected moDCs highly upregulate IL-10 gene expression but not of IL-12p40, which was similar to uninfected or unstimulated moDCs

In contrast, *Li*EV stimulated moDCs that were in coculture with NK cells evidenced increased gene expression of both IL-10 (Fig. 34b) and IL-12p40 (Fig. 34a) that were more than 10-fold when compared with unstimulated moDCs that also were in contact with NK cells. Moreover, the upregulation of IL-12p40 was highly increased, reaching more than 100 fold increase.

L. amazonensis infected moDCs and *La*EV stimulated moDCs that were in co-culture with NK cells showed increased gene expression of IL-10 and a marked increase of IL-12p40.

These results indicate that NK cells have a non-negligible effect on the generation of cytokines by moDCs, especially by infected and EV-stimulated moDCs. Curiously, *L.infantum* infected moDCs generate mainly the regulatory cytokine IL-10 while in *L. amazonensis* infected moDCs predominate the upregulation of IL-12p40 which is a subunit of IL-12 and IL-23. Both these pro-inflammatory cytokines direct the proliferation and polarisation of T cells towards an inflammatory phenotype.

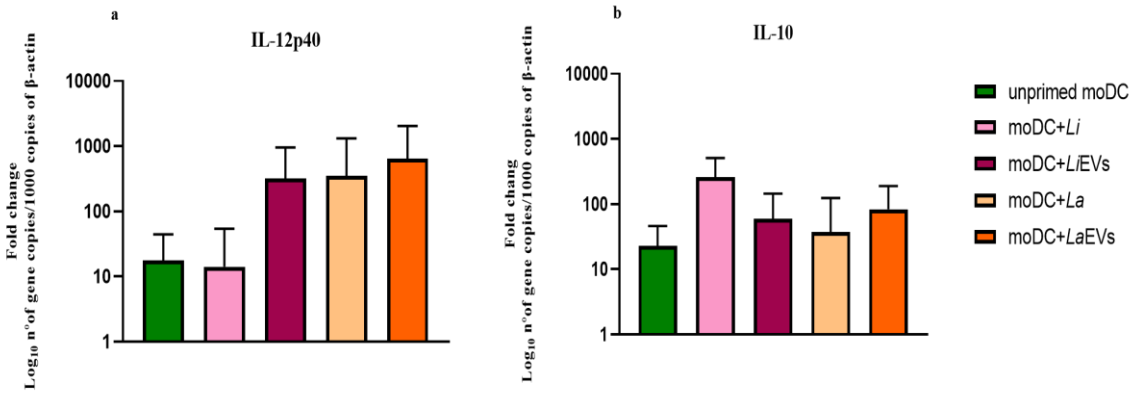


Figure 36. Gene expression of anti- and pro-inflammatory cytokines by infected moDCs that have been in co-culture with NK cells. Infected moDCs (Li and La), EVs primed moDCs (*LiEVs* and *LaEVs)* and unprimed moDCs that werein contact with NK cells were used to evaluate gene expression of IL-10 and IL-12p40 by real-time PCR. The fold change of five dogs by-comparing with moDCs that have not contacted with NK cells is represented by column bar chart showing the mean and SEM.

3.5.7. NK cells favor the expansion of MHCII$^+$ moDCs subset among infected moDCs

To assess the influence of NK cells in the expression of MHCI and MHCII by *L. infantum* and *L. amazonensis* infected moDC and moDC exposed to EVs (*LiEVs* and *LaEVs)* the surface expression of MHCI and MHCII molecules were evaluated by multiparametric flow cytometry. In parallel, the surface expression of MHC was also evaluated in unprimed (uninfected and unstimulated) moDC that were in co-culture with NK cells. The results were compared with the frequency of MHCI$^+$ and MHCII$^+$ moDCs (infected and EVs stimulated) that have not contacted NK cells.

The contact with NK cells leads to an increase of MHCII$^+$ moDCs, MHCI$^+$MHCII$^+$ moDCs and an accentuated augment of MHCI$^+$moDCs among the unprimed moDCs population.

However, a marked decrease in the frequency of MHCI$^+$ moDCs infected with *L. infantum* was observed concerning *L.infantum* infected moDCs that were not in co-culture with NK cells. On the contrary, the frequency of MHCI$^+$MHCII$^+$ moDCs increased (≈18-fold

increase) (Fig. 35c) when in co-culture with NK cells, as well as MHCII$^+$moDC that showed a high frequency (≈ 2-fold increase) (Fig. 35b). The frequency of MHCII$^+$ and MHCI$^+$MHCII$^+$ moDCs infected with *L. amazonensis* showed two and five-fold increases respectively, and *LiEVs* -stimulated moDCs revealed the expansion of MHCI$^+$, MHCII$^+$ and MHCI$^+$MHCII$^+$ moDCs (≈ 2 to 4 fold increase). Curiously, *LaEVs* -stimulated moDCs that were co-cultured with NK cells showed a higher fold increase of MHCI$^+$MHCII$^+$ moDCs (≈14), a moderate fold increase (≈ 4 fold increase) of MHCI$^+$ moDCs and a slight increase (≈ 1.16 fold increase) of MHCII$^+$ moDCs (Fig. 35a).

Therefore, these results suggest that among the population of *L. infantum* -infected moDCs that had contact with NK cells there was a loss of MHCI$^+$moDC subset and expansion of MHCII$^+$moDC subset, pointing towards the possibility of antigen presentation to T lymphocytes. Among the populations of *L.amazonensis*, infected moDCs and *LiEVs* stimulated moDCs the frequency of MHCI$^+$ moDCs, MHCII$^+$ moDCs and MHCI$^+$MHCII$^+$ moDCs have a moderate increase. But the subset of MHCII$^+$ moDCs of LaEVs -stimulated moDCs population did not seem to contribute to the increase of antigen presentation when compared with *LaEVs* -stimulated moDCs that had no contact with NK cells.

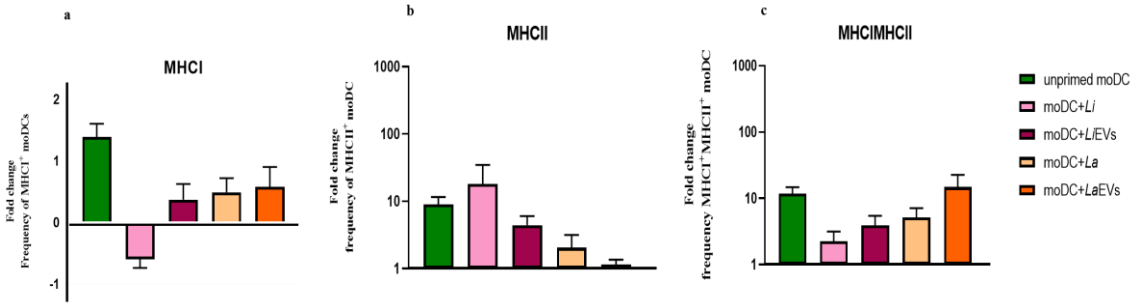


Figure 37. Frequency of MHCI, MHCII$^+$ and MHCI$^+$MHCII$^+$ moDCs subsets among Leishmania infected moDCs that were in co-culture with NK cells. The proportion of MHCI$^+$(a), MHCII$^+$ (b) and MHCI$^+$MHCII$^+$ (c) moDCs was evaluated in infected moDCs (*Li* and *La*), EVs stimulated moDCs (*LiEVs* and *LaEVs*) and unprimed moDCs that were exposed to NK cells. The fold change of five dogs by comparing with moDCs that did not contact NK cells is represented by column bar charts showing the mean and SEM.

3.5.8. NK cells promote the gene expression of the co-stimulatory molecules CD86 in *L.infantum* infected moDCs

The gene expression of CD80 and CD86 was evaluated by real-time PCR in *L. infantum and L. amazonensis* infected moDCs, EVs stimulated moDCs and in unprimed moDC that were in co-culture with NK cells. To examine the possible effect of NK cells on moDCs, the gene expression levels of CD80 and CD86 were compared with moDCs (unprimed, infected and stimulated) that have not contacted with NK cells.

Unprimed moDCs that were co-cultured with NK cells showed an increased gene expression of CD80 (Fig. 36a) and CD86 (Fig. 36b). However, the contact with NK cells led to a reduction of CD80 in infected and EVs-stimulated moDCs. In contrast, the accumulation of CD86 mRNA showed a considerable fold increase in *L. infantum* -infected moDCs and EVs-stimulated moDCs. In *L. amazonensis* -infected moDCs that were in co-culture with NK cells, the level of CD86 gene expression did not evidence important changes, being similar to infected moDCs that had no contact with NK cells.

Although the difference between the roles played by CD80 and CD86 remains elusive it has been considered that CD80 have a higher affinity for T cell CD28 whereas CD86 has weaker interactions. Engagement with CD28 is required to enhance T cell differentiation and cytokine production whereas lower affinity between DC and T cell costimulatory molecules can lead to suboptimal T cell responses (Linterman *et al.*, 2014, Wang *et al.*, 2015). Therefore, NK cells may drive *L. infantum* infected and EVs stimulated moDCs to mainly express CD86, which is described as the more abundant and earlier expressed (Sansom *et al.*, 2003).

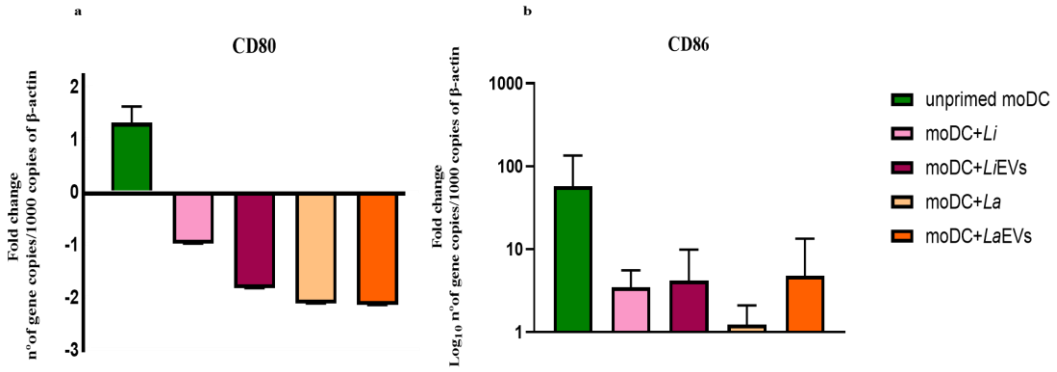


Figure 38. Gene expression of co-stimulatory molecules in *Leishmania* infected moDCs that were in co-culture with NK cells. RNA extracted from infected moDCs (*Li* and *La*), stimulated moDCs (*LiEVs* and *LaEVs*) and unprimed moDCs were used to evaluate the gene expression of CD80 (A) and CD86 (B). The fold change of five dogs by comparing with moDCs that have not contacted NK cells is represented by column bar charts showing the mean and SEM

3.5.9. NK cells do not cause the apoptosis of infected moDCs

To assess the possible effect of NK cells in the apoptosis of *L. infantum* and *L. amazonensis* infected moDCs and EVs stimulated moDCs, as well as in unprimed moDCs, the levels of apoptotic cells was assessed by multiparametric flow cytometry. The obtained data were compared with the frequency of apoptotic moDCs (unprimed, infected and stimulated) that have not contacted NK cells.

Regarding unprimed moDCs, NK cells seem to induce a slight fold increase in cell apoptosis. In contrast, the levels of apoptotic cells among infected (*L.infantum* or *L.amazonensis*) or EVs stimulated (*LiEVs* or *LaEVs)* moDCs exposed to NK cells were inferior to moDCs that did not contact NK cells (Fig. 37).

Thus, it appears that NK cells do not induce cell death of infected or stimulated moDCs.

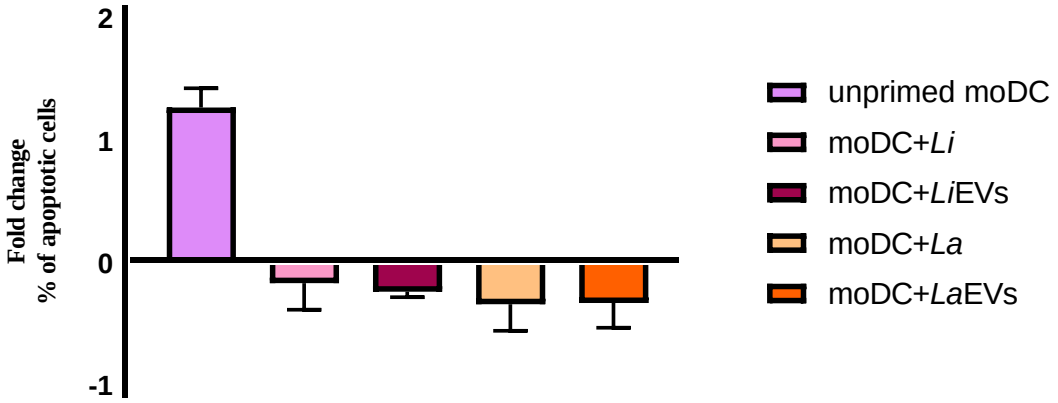


Figure 39. Apoptosis levels among infected moDCs that were in co-culture with NK cells. The percentage of apoptotic, infected (Li or La) or stimulated by EVs shed by *L. infantum* (*LiEVs)* or *L. amazonensis* (*LaEVs)* moDCs in co-culture with NK cells was assessed by multiparametric flow cytometry using annexin-V and propidium iodide. The apoptosis fold change of five dogs by comparing with unprimed, infected or stimulated moDCs that had no contact with NK cells is represented by a column bar chart indicating the mean and SEM.

3.5.10. Parasites internalized by moDCs that were exposed to NK cells maintain viability and replicate

The viability of *L. infantum* or *L. amazonensis* parasites internalized by moDCs that were in co-culture with NK cells were assessed following the transfer of infected moDCs to complete SCH medium and incubation at 24°C for 72 h.

The intracellular amastigotes differentiated into motile promastigotes, suggesting that the internalized parasites maintained viability. When compared to the concentration of *Leishmania* promastigotes used to infect moDCs, a significantly higher number of viable parasites were observed after 114 h (24 h of moDC infection plus 18 h of co-culture with NK cells plus 72 h of parasite culture) that appear to maintain their ability to replicate (P_{Li} = 0.0020; P_{La}= 0.0010) (Fig. 38a and b).In addition, a higher density of *Leishmania* rosettes observed in the culture medium indicates that the parasites were replicating (Fig. 38).

Thus, after 18 h in co-culture with NK cells, *L. infantum* and *L. amazonensis* (Fig. 38a and 38b) internalized parasites of lived moDCs have survived and retained the ability to

replicate, indicating that NK cells did not cause extensive lysis of moDCs and that internalized parasites were viable.

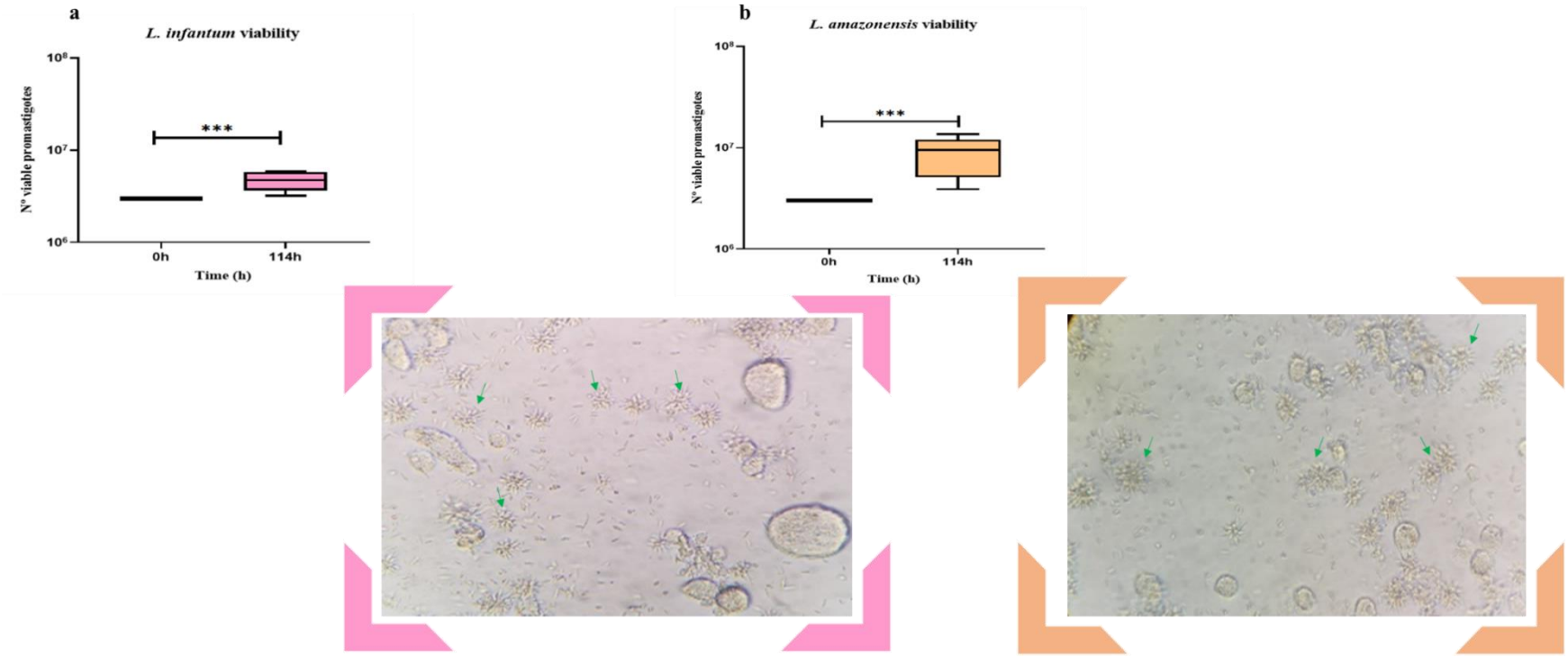


Figure 40. Viability of *L. infantum* and *L. amazonensis* parasites phagocytosed by moDCs that were in co-culture with NK cells. moDCs of five dogs infected with *L. infantum* or *L. amazonensis* promastigotes for 24 h and incubated with NK cells for another 18 h, were transferred to Schneider medium and incubated at 24°C for 72 h. The estimated viable promastigotes are represented by Tukey box plots, including the median, interquartile ranges and minimum and maximum values. The non-parametric Wilcoxon test was used for statistical comparisons. *** ($p<0.001$) represents statistically significant differences when comparing 0 h (initial concentration of *Leishmania* promastigotes) *vs* 114 h (24 h contact with moDCs +18 h in co-culture with NK cells + 72 h in parasite culture medium allowing intracellular amastigotes to differentiate promastigotes).

3.6. Discussion

NK cells are well characterized both in man and in mice. In men they are characterized by the expression of CD56 and CD16 while in mice it is by CD11b. In contrast, canine NK cells to date are not well characterized.There are several studies in this sense but they end up being controversial, for example in some studies the authors consider that cells that present $CD3^{+}$ are considered NK cells and other authors discard this possibility. Thus, further studies are needed achieving a better characterization of canine NK.

In the current study, CD16, CD94 and MHCII were used as NK molecular markers, which are the most consensual in the literature for the identification of canine NK.

Since canine NK cells express fewer CD5 molecules on the cell surface (Grøndahl-Rosado *et al.,* 2016), dog NK cells were characterized by the isolation of $CD5^{lo}$ cells from PBMCs derived from canine whole blood by immunomagnetic separation. Cells can be maintained or expanded in culture for days or months. In this way, the cells were cultured for 21 days in a medium supplemented with a cocktail of cytokines (such as IL-2 and/or IL-15) (Somanchi *et al.,* 2011). Flow cytometry analysis of activated $CD5^{lo}$ cells indicated that they were positive for CD45 and MHCII, but negative for CD3, CD4, CD5, and CD21. In turn, through qRT-PCR, activated canine $CD5^{lo}$ cells were found to express high levels of NK cell-related receptors (NKp30, NKp44, NKp46, NKG2D and CD244), as well as perforin 1 and granzyme B. Another putative marker for canine NK cells is CD94 (KLRD-1). Experiments with dog-specific anti-CD94 Ab have identified a population of NK cells, representing about 7.7% of PBMCs, included within the $CD5^{dim}$ population (Van Helden *et al.,* 2015). A gene paralogous to the mice Ly49 receptor, which binds to MHCI distinguishing between healthy and infected or altered cells, has been identified in the canine genome (Bushnell *et al.,* 2017, Schneider *et al.,* 2017).

$CD5^{dim}$ is not specific for identifying circulating NK cells (based on differential gene expression analysis of canonical NK markers), while NKp46 showed high agreement for canonical NK markers identified in human and mouse NK cells. NKp46 expression in dogs may not identify all NK subtypes at a steady state. Identification of cells by the low density of the CD5 receptor does not indicate a specific population of canine NK cells.

For this reason, $CD3^{-}NKp46^{+}$ cells may not identify all canine NK cells, although transcriptional evidence points to these surface markers as a more reliable phenotype to identify a homogeneous NK cell population expressing genes that are translated to cell surface proteins not previously described in dogs, including KRLB1 (NK1.1), CD96, KLRF1 and KLRK1 (NKG2D), among others.

In the present study, the percentage of gate cells, which was determined to characterize the phenotype of dog cells isolated from canine PBMCs by magnetic separation, showed a predominant frequency of $CD3^{-}CD21^{-}CD16^{+}CD94^{+}MHCII^{+}$, which is compatible with NK cells. However, the isolated cells also included approximately between 15 to 25% $CD3^{+}$ and $CD21^{+}$, so a pure NK cell population was not obtained. However, there are various studies where the characterized dog NK cell population also expresses CD3 molecules (Yasuda et al., 2009, Lee et al., 2018, Gingrich et al., 2021). Currently, it is difficult to correctly identify dog NK cells due to the lack of one or more specific markers.

Resting NK cells are not fully activated but may kill or secrete cytokines upon additional stimulation provided by pathogens, cytokines, and/or accessory cells. Some pathogens directly activate NK cells after NK receptor binding or TLR activation (Borrego *et al.,* 1998, Canossi *et al.,* 2016). However, it is believed that DCs are the most potent stimulators of NK cells (Carretero *et al.,* 1997, Braud *et al.,* 1998, Lanier, 2008) being necessary for the *in vivo* activation of NK cells (Braud *et al.,* 1998). The interaction of NK cells with DC results in maturation, activation, and production of cytokines by both cells. In turn, *in vivo* studies performed in murine models of cutaneous or visceral leishmaniasis showed that during the initial phase of infection NK cells are activated by conventional DC in a TLR9, IL-12 and IL-18 dependent manner, helping to restrict the tissue parasite load. In the present study, we found that dog NK cells stay viable when in co-culture with moDCs. Curiously, *L. amazonensis* infected moDCs and *LaEVs* stimulated moDCs, but not *L.infantum* infected moDCs or *LiEVs* stimulated moDCs, seem to prolong NK cell lifespan. In a previous study (Ross and Caligiuri, 1997), it was observed that IL-15 avoids apoptosis of NK cells and induces the proliferation of NK cells. Therefore, dog moDCs infected by *L. amazonensis* or stimulated by *LaEVs* may secrete IL-15 favouring the survival of dog NK cells, improving the cytotoxic potential of the host and leading to the destruction of target cells and eventually to parasite inactivation.

DCs and NK cells have a crucial role in modulating innate and adaptive immune responses through complex cell-to-cell crosstalk and the synthesis of soluble immune mediators. Upon stimulation, NK cells secrete large amounts of cytokines, as is the case of IFN-γ, TNF-*α* and GM-CSF, and chemokines, such as CCL3, CCL4, and CCL5 (Walzer *et al.,* 2005). Thus, NK-cell–induced DC activation is dependent on both TNF-α and IFN-γ secretion and cell-cell contact. Thus, the help of NK cells might be critical for optimal DC activation and subsequent induction of T cell immune response in conditions of poor inflammation, but when NK cell activation occurs through direct recognition of target cells (Shortman and Liu, 2008).

Studies have shown that IL-12 produced by DC is also crucial for the activation of NK cells, namely for IFN-γ synthesis (Ferlazzo *et al.*, 2004). IFN-γ and TNF produced by NK cells contribute to the development of a Th1-type response in addition to their cytotoxic potential. Control of all forms of leishmaniasis requires differentiation by IL-12 and the recruitment of IFN-γ-producing helper T cells (Mougneau *et al.,* 2011), which are crucial for inducing IFN-γ activated MΦs capable of killing intracellular amastigotes (Lykens *et al.,* 2010), in addition to the activation of mechanisms that prevent hyperinflammation and mediate the resolution of tissue damage (Peters and Sacks, 2006). In the current study, the moDCs stimulated by EVs shed by *L. infantum* and *L. amazonensis or* infected by *L. amazonensis* evidence upregulation of IL-12p40 and only NK cells that were in contact with *L.amazonensis* infected moDCs increase IFN-γ gene expression. A previous study by Xin and collaborators (2007) reported that the upregulation of the IL-12p40 gene in moDCs infected with *L. amazonensis* appears to be relatively weak and transient. Although it was not possible to establish a direct relationship between IL-12 release by dog moDCs and IFN-γ induction by canine NK cells, *L.amazonensis* infected moDCs induce NK cells to generate IFN-γ, which can contribute to the efficient control of *L.amazonensis* infection in the dog.

DCs are not the only cell type involved in developing an effective T cell immune response. Some studies have demonstrated that NK cells have a protective role in leishmaniasis, being the primary source of IFN-γ for the development of a Th1 immune response (Scharton and Scott 1993, Bogdan *et al.,* 2012). Indeed, reduced numbers of NK cells and low pro-inflammatory immune mediators, such as IFN-γ and TNF-α were observed in

patients with diffuse CL when compared to localized CL (Bogdan *et al.,* 2012). Recently, a contribution of NK cells to the immunopathology of CL was also demonstrated mainly through the release of granzyme B. It was suggested that NK cells can contribute to cytotoxicity activity in CL patients (Messlinger *et al.,* 2018).

TNF-α seems to be the key to initiating granuloma formation, which is associated with host resistance to *L. infantum* infection in VL mouse model as well as in canine and human hosts (Pearson and Sousa, 1996, Murray, 2001, Sanchez *et al.,* 2004). However, in the present study, the interplay of infected or EVs stimulated moDCs with dog NK cells did not induce the generation of TNF-α. On the contrary, *L.infantum* infected moDCs seem to inhibit the synthesis of TNF-α, which can favor parasite survival.

There seems to be a divergent role for NK cells according to the leishmaniasis clinical form and disease severity. It has been seen that patients with the diffuse form of CL exhibited a lower frequency of NK cells, lower levels of cytokines, such as IFN-γ, and downregulation of TLR expression, as is the case of TLR1, TLR2, and TLR6 (Salaiza-Suazo *et al.,* 1999, 2014) unlike patients who present the localized form of CL, who exhibit high levels of cytokines and higher frequency of NK cells (Salaiza-Suazo *et al.,* 1999). Furthermore, in mice lacking $CD4^+$ T cells, IFN-γ production by NK cells was not sufficient to control *L. major* (Wakil *et al.,* 1998). On the other hand, *Leishmania* parasites also use a variety of strategies to manipulate MΦ and DC functions to favor their survival and replication. One of the major mechanisms used by *Leishmania* is to inhibit the production of Th1 polarizing cytokine (IL-12) as well as prevent DCs from successfully presenting parasite antigens to T cells, resulting in impaired cell-mediated immunity.

The Th2 cytokine IL-13 is involved in infection susceptibility to several *Leishmania* species and the use of IL-13 deficient mice and IL-13 transgenic mice demonstrates that IL-13 is important for the generation of Th2 cells (Matthews *et al.,* 2000; Alexander *et al.,* 2002, Murray *et al.,* 2006). Since IL-13 is a negative regulatory factor of MΦ function, the production of IL-13 after infection by *L. amazonensis* may be, in part, a mechanism used by the parasite to evade the immune system. Interestingly, despite this initial Th2 environment, IFN-γ was produced. These data correlate with murine and human *L.*

braziliensis infection that after a short period of a negative modulated immune response, a strong Th1 immune response predominates (Rocha *et al.,* 1999, de Oliveira *et al.,* 2004). An independent role for IL-13 in *L.mexicana* infection was demonstrated by comparing disease progression in wild-type B6/129 and IL-13$^{-/-}$mice. IL-13$^{-/-}$ mouse developed lesions similar in size to wild-type animals after 8 weeks of infection. However, in contrast to wild-type mice in which the disease continued to progress, IL-13$^{-/-}$ mice showed a cure due to the development of a Th1 response. Thus, it is posssible that IL-4 play a critical role in the early development of the lesion and that IL-13 promotes a chronic non-healing infection (Alexander *et al.,* 2002). Recently, it was demonstrated that IL-13 is the predominant Th2 cytokine in human *L. guyanensis* lesions inhibiting the expression of the β2 chain of IL-12R in lymphocytes (Bourreau *et al.,* 2001). Another study by Zaatar (2022) showed that *L. major* infected mice injected with IL-13 resulted in parasite load increase. Twelve days after IL-13 injection, IFN-γ levels decrease significantly, suggesting that exogenous IL-13 hinders the Th1/Th2 shift by inhibiting the production of IFN-γ, which contributes to disease exacerbation. Moreover, Coelho and colleagues (2010) showed that a few hours after exposure to *L. amazonensis* , human PBMCs release high levels of IL-13 and IL-10 followed by low IL-12 production of, leading to a predominantly Th2 immune response.

In the present study, NK cells that were in contact with *L. amazonensis* infected moDCs or with *LaEVs* stimulated moDCs seem to increase the generation of the anti-inflammatory cytokine IL-13, pointing towards the activation of Th2 immune response. However, NK cells also seem to be able to generate IFN-γ, which promotes a Th1 immune response. It is probably, that in the case of canine *L.amazonensis* infection, modulation of NK cells by DC lead to a balance between Th1 and Th2 immune responses, avoiding the predomination of a pro-inflammatory immune response and favoring the establishment of a chronic infection.

Another candidate for limiting the NK cell response after *Leishmania* infections is the cytokine IL-10, which is generated by MΦ, Th1 cells, and regulatory T cells (Belkaid *et al.,* 2002, Anderson *et al.,* 2007, Nagase *et al.,* 2007), but also by human and mouse activated NK cells (including those from LCL patients) (Akuffo *et al.,* 1999, Maasho *et*

al., 2003, Maroof *et al.,* 2008). It should be emphasized that both inhibitory and stimulatory effects on NK cells have been attributed to IL-10 in various settings (Shibata *et al.,* 1998; Tu *et al.,* 2008). The role of IL-10 in the activation or suppression of NK cells in leishmaniasis has not yet been defined. Indeed, in response to *Leishmania* infection, MΦ can release IL-10 (Bortell *et al.*, 2021), which is one of the cytokines capable of antagonizing NK cell functions by suppressing the release of activating cytokines (Clark *et al.,* 2018).

When evaluating the production of IL-10 in infected or stimulated moDCs in contact with NK cells, we obtained an increase in its generation, but a more abrupt increase was observed in moDCs infected with *L. infantum,* suggesting the impairment of NK activity, mainly in what concerns the release of pro-inflammatory cytokines. And in fact, NK cells in contact with *L. infantum infected* moDCs show a very low generation of IFN-γ and TNF-α.

Macrophage inflammatory protein (MIP)-1α and MIP-1β are two CC chemokines that induce lymphocyte migration. MIP-1α preferentially mediates the chemotaxis of $CD8^+$ lymphocytes and MIP-1β the $CD4^+$ T cells (Menten *et al.,* 2002, Lukacs *et al.,* 1996, Schall *et al.,* 1990, Le *et al.,* 2004). Both chemokines are recognized by the T lymphocyte cell receptor, CCR5. NK cells can secrete MIP-1α in a cytokine-dependent manner (Salazar-Mather *et al.,* 1998, Bernardini *et al.,* 2006). CCL3/MIP-1α, CCL4/MIP-1β and CCL5/RANTES were found in the supernatant of stimulated NK cells (Fauriat *et al.,* 2010)

According to a previous study by Mayra and Sanabria (2008), co-cultures of *L. amazonensis* amastigote-infected DCs and activated NK cells, but not resting NK cells, result in a significant increase in the production of IL-12p40/p70, TNF-α, CCL3, CCL5, and CXCL10, whereas *L. amazonensis* promastigote-infected DCs lead NK cells to produce IL-12, IFN-γ, IL-1α, TNF-α, IL-10, CCL3, and CXCL10 (Sanabria *et al.,* 2008. These results show that the state in which NK cells are found (resting or activated) modulates the response of DCs to *Leishmania* promastigotes and amastigotes. Previous studies in *L. donovani* murine model showed high levels of CCL2, CCL3 and CXCL10 during the first week of infection which can be associated with the recruitment of MΦ at the site of

infection for parasite clearance (Cotterell et al., 1999, Steigerwald *et al.,* 2005). Furthermore, high levels of CCL2, CCL3, CCL4, CCL8, and CXCL10 were reported in human CL (Ritter *et al.,* 1996, Campanelli *et al.,* 2010, Diaznet *et al.,* 2013). In turn, the findings of the current study indicate that *L. infantum* or *L. amazonensis* infected moDCs and *LiEVs* or *LaEVs* stimulated moDCs direct the generation of both CCL3 and CCL4 by NK cells, which are responsible for the recruitment of lymphocytes to the sites of infection, favoring a cellular immune response (helper and cytotoxic) in the parasite target organs, which can promote the control of *L.infantum* and *L. amazonensis* infection in dogs.

Interleukin-8 (IL-8) is a member of the CXC chemokine subfamily and is produced by leukocytes (monocytes, T cells, neutrophils, and NK cells) and non-leukocyte somatic cells (endothelial cells, fibroblasts, and epithelial cells). Its production is induced by inflammatory stimuli, such as IL-1. IL-8, also known as CXCL8, activates neutrophils inducing chemotaxis, granule exocytosis, and respiratory burst (Baggiolini and Clark-Lewis, 1992, Mukaida *et al.,* 2003). The production of IL-8 by NK cells in the context of PBMC demonstrates that this response also occurs in the presence of other immune cells, when additional signals are present, including other cytokines and cell-cell interactions. This cytokine is secreted by tissue-resident MΦ in response to *Leishmania* infections and plays a key role in the early stages of infection (Alexander *et al.,* 1999). Monocytes isolated from *L. major* infected individuals exhibited high levels of IL-8. Both IL-8 and neutrophils are involved in the early defense against *Leishmania* at the site of infection (Hammond *et al.,* 1996, Müller *et al.*, 2001). Furthermore, *L. major* infected neutrophils also secrete high levels of IL-8 that lead to increased neutrophil infiltration and parasite phagocytosis (Laufs *et al.*, 2002, Scapini *et al.,* 2001).

In the present work, IL-8 was produced by NK cells that were in contact with *L. infantum* or *L. amazonensis* infected moDCs and with *LiEVs* stimulated moDCs, suggesting the recruitment of neutrophils to parasite target organs, which can elicit a parasite load reduction. A study by Pereira and collaborators (2017) highlights the effector functions of canine PMN exposed to *L. infantum* promastigotes, *in vitro*, indicating that in the early phase of infection PMNs promote parasite load reduction through the extracellular death mechanisms (NET).

Besides the generation of immune mediators after activation by moDCs, in the current-study the cytotoxic activity of canine NK cells was also analyzed through the study of perforin and granzyme B.

A likely explanation of how cytotoxic cells mediate inflammation and tissue injury in the CL is that, after cytotoxic cells degranulate, granzyme B and perforin are released into the extracellular space, inducing apoptosis of infected MΦ. In addition, extracellular granzyme B can indirectly induce inflammation through the activation of pro-inflammatory cytokines and the degradation of extracellular matrix substrates, contributing to tissue injury. Thus, granzymes can play a role in amplifying inflammation in *Leishmania* infections, contributing to tissue damage (Campos *et al.*, 2017).

In the current study, it was found that *L. amazonensis* infected moDCs, but not *L. infantum* infected moDCs, and *LiEVs* or *LaEVs* stimulated moDCs induce NK cells to release perforin. Since there was degranulation and release of perforin one would expect the release of granzyme, but the levels of granzyme B were very low. These low granzyme B release may be related to the ELISA kit used since it was for humans, which could not have good efficacy when applied to dogs. Even so, the low amount of apoptotic moDCs that were found in co-culture with NK cells can be a consequence of low amounts of granzyme B.

NK cells activate DCs to induce T cell proliferation and differentiation (Zitvogel *et al.*, 2002, Cooper *et al.*, 2004). DC maturation is demonstrated by the upregulation of surface markers, such as MHCII and costimulatory molecules B7-1 (CD80) and B7-2 (CD86) known to induce T cell receptor signaling and promote T cell activation (de Saint-Vis B et al., 1998; Tseng SY *et al.*, 2001). The CD86 costimulatory factor is synthesized and expressed on the surface of DCs and other APCs and mediates Th cell recognition through antigen presentation by MHCII molecules. Activation of DC by PAMPs induces cytotoxic $CD8^+$ T cells that recognize peptides complex with MHCI molecules.

Injection of cells after *in vivo* activation of NK CD244 (NK cell receptor 2B4) promoted IL-12 release and increased expression of CD40, CD83 and CD80. On the other hand, infection of the bone marrow CD with *L. mexicana* amastigotes did not increase the expression of CD-display indicators (CD80, CD54, MHCII) (Hernandez *et al.*, 2001).

When NK cells are cultured with immature DCs in the presence of maturation stimuli, such as lipopolysaccharide (LPS), strongly enhance DC maturation, specifically up-regulating the production of the co-stimulatory molecule CD86 and IL-12. Infection of mouse MΦ with *L. donovani* or *Mycobacterium tuberculosis* failed to up-regulate or decrease the expression of CD80 and CD86 (Kaye *et al.,* 1994, Saha *et al.,* 1994). These effects on costimulatory molecule expression may represent strategies used by pathogens to induce anergy or otherwise cause advantageous immune suppression (Brodskyn *et al.,* 2000).

NK cells recognize target cells through the different expressions of MHCI molecules and through surface receptors that interact with MHCI epitopes. When recognizing these epitopes, some of these target cell send negative signals to the cell, preventing the phenomenon of cytotoxicity, they are the so-called inhibitory receptors. Thus, if a cell does not express or express MHCI molecules different from the other cells of the organism, the binding with the inhibitory receptors will not trigger the cytotoxic mechanism. MHCI upregulation during moDCs maturation protects these cells from NK lysis (Lanier 1998, Long 1999, Ravetch and Lanier 2000, Moretta *et al.*, 2001)

In the current study, after contact with NK cells, *L. infantum* -infected moDCs exhibit an expansion of $MHCII^{+}$moDCs. In turn, both EVs and the two *Leishmania* species negatively modulate the co-stimulatory molecules CD80. The downregulation of CD80 could influence T cell activation. Even so, NK cells seem to be unable to downregulate CD86. Although both molecules bind to the same T cell co-stimulatory molecules performing the same function, which is mediate T cell activation together with the recognition of the pathogen antigen complexed with MHC molecules, the reduction of CD80 could lead to suboptimal T cell activation

$CD8^{+}$ T cells recognize peptides that are presented in MHCI molecules via the T cell receptor (TCR). Although peptides presented via MHCI mainly derive from endogenous antigens, various exogenous cell-associated antigens have also been shown to be uploaded onto the MHCI pathway, by a process referred to as cross-presentation. *Leishmania* antigens were also shown to be cross-presented (Bertholet *et al.*, 2006).

Our findings evidence e that after contact with NK cells, decreased frequency of $MHCI^+$ moDCs among *L. infantum suggests* that parasite antigens will not be presented to $CD8^+$ T lymphocytes. Therefore, NK cells can modulate moDC activity promoting a mix of pro-inflammatory/regulatory environments, influencing the process of cross-presentation and antigen presentation, impairing immune response mediate by cytotoxic T cells and helper T cells in addition to the very low release of granzyme B that mediates apoptosis and reduced apoptotic levels of primed moDCs, supporting parasite survival

Taking into account that the major activity of NK cells is to lyse infected cells (and cells altered by malignancy), we investigated the viability of parasites internalized by moDCs. At least some moDC internalized parasites were viable and maintained their replicative capacity, being in conditions of establishing infection. The survivors are probably the most virulent parasites that managed to resist to DC degradation processes and NK cells. Some authors consider the possibility of these parasites may be better adapted transitional forms able to survive in the intracellular environment (Ribeiro-Gomes and Sacks, 2012). Corroborating these findings we show that even in co-culture with NK cells, infected or stimulated moDCs tend to have lower levels of apoptotic cells when compared to moDCs that have no contact with NK cells. Thus it appears that activation or the performance of dog NK cytotoxic activity were impaired by *L. infantum* and *L. amazonensis* infected moDCs and also by EVs (*LiEVs* or *LaEVs*) stimulated moDCs.

3.7. Conclusions

NK cells play a key role in the immune response against infection and can establish multiple interactions with innate leukocytes, including DCs, driving the destruction of infected cells. Taking into account the limited information available on the interplay of NK cells with DCs in CanL, this study aimed to investigate the influence of *L. infantum* and *L. amazonensis* infected DCs on NK cells activity and the effect of NK cells in the immune activity of infected DCs. Thus, DCs were *in vitro* differentiated from peripheral blood mononuclear cells of healthy dogs (moDCs) and infected with *L. infantum*, which is the species of *Leishmania* that mainly causes CanL worldwide. In this study, moDCs were also infected with *L. amazonensis*, which had been also found in infected dogs of Latin America and primed by EVs shed by both parasite species for comparative studies. These cells were then co-cultured with a cell população enriched in conventional NK cells. Both differentiated moDCs and isolated NK cells were from the same dogs. The generation of cytokines was evaluated in both cells while the gene expression of chemokines and degranulation were assessed in NK cells in addition to the analyse of MHC and co-stimulatory molecules in moDCs. To complement these data, it was verified the viability of intracellular parasites and moDC apoptosis.

In summary, the findings of this study indicate that *in vitro*:

- NK cells seem to favor the predomination of $MHCII^+$ moDCs among *L. infantum* infected moDCs, indicating that moDCs can present parasite antigens to $CD4^+$ T lymphocytes, which in association with the generation of chemokines by NK cells, can direct the recruitment of other leukocytes, including lymphocytes, to the infection sites that can lead to the establishment of a Th-specific immune response. However, the reduced expression of the co-stimulatory molecule CD80 can drive a less efficient T cell activation. Moreover, moDCs can generate high levels of the regulatory cytokine IL-10, which can impair the effector T lymphocytes, pointing to an inefficient cellular immune response.

- The accentuated reduction of $MHCI^+$ moDCs among *L. infantum* infected moDCs should lead to the activation of NK cells and moDC destruction through the release of granules with perforin and granzyme B. However, the low degranulation

of NK cells does not seem to be enough to cause the apoptosis of moDCs in addition to the low generation of cytokines that play a crucial role in the activation of MΦ microbicide pathways (IFN-γ and TNF-α).

- Therefore, it can be raised the hypothesis that *L. infantum* parasites modulate the interaction of NK cells and moDCs ensuring their survival, which was confirmed in the current study. Indeed, intracellular parasites were viable and able to replicate, demonstrating that could establish infection in the dog.

- On the other hand, *L.amazonensis* infected moDCs promote NK cells to generate chemokines, attracting other leukocytes to the site of infection, and IFN-γ and IL-13 in addition to the generation of IL-12p40 and IL-10 by infected moDCs. IL-12, IL-13 and IFN-γ are pro-inflammatory cytokines involved in the establishment of a Th1 immune response and IL-10 is an immunosuppress cytokine that can downregulate MHCII expression. Moreover, it is recognized that IL-13 can impact skin biology. *L. amazonensis* despite being found in the dog is one of the species that causes human cutaneous leishmaniasis. Previous studies reported that IL-13 regulates cell-mediated immunity improving resistance to other cutaneous species of *Leishmania*, as is the case of *L. major* and *L. mexicana*. Therefore, the interaction of DCs with NK cells can lead to the establishment of a balance between pro-inflammatory and regulatory cytokines in addition to the parasite resistance conferred by IL-13.

- Although contact with NK cells improves the expression of MHC molecules by *L. amazonensis* infected moDCs also decreases the expression of costimulatory CD80 not compensated by CD86 increase, which can difficult antigen presentation and T cell activation. Even so, degranulation of NK cells releasing high perforin levels and low amounts of granzyme B did not seem to be enough to cause apoptosis of infected cells, since it was verified a decrease in the proportion of apoptotic moDCs in addition to the viability of intracellular parasites.

- Despite *L. amazonensis* infection presenting several differences when compared to *L. infantum* infection, it also seems that *L. amzonensis* parasites can modulate

the activity of dog moDCs and the interaction with NK cells, avoiding the establishment of a fully activate cellular immune response.

- moDCs primed by EVs shed by *L. infantum* or *L. amazonensis* promastigotes induce NK cells to generate chemokines, attracting other leukocytes, and also promote degranulation that was not sufficient to induce moDCs apoptosis.

- The interplay of *L.amazonensis* EVs-primed moDCs and NK cells leads to the generation of IL-12p40, IL-10 and IL-13 and the upregulation of MHCI and CD86 expression indicating that EVs can modulate dog immune response similar to *L. amazonensis* intracellular parasites.

- The interplay of *L. infantum* EVs-primed moDCs with NK cells enhances $MHCI^+$ and $MHCII^+$ moDCs subsets associated with the upregulation of CD86, indicating that moDCs can present antigens to $CD4^+$ and $CD8^+$ T cells. However, since the generation of cytokines by moDCs and NK cells was residual, the activation of specific cellular immune response may be at least partial impaired.

4|Final Remarks

4. Final remarks

Canine leishmaniasis is a serious disease for which there is no parasitological cure. However, it has been known that the development of an effective cellular immune response is decisive to control the infection and prevent the development of the disease. Thus, the crosstalk between innate and acquired immunity is essential, as the innate immune response is crucial to prevent the establishment of infection or decrease the proportion of viable parasites that can reach the final host cell, while the adaptive immune response is important to control replication and promote parasite death.

DCs and NK cells are effector cells of innate immunity. DCs are specialized in the capture and presentation of antigens and are considered a bridge between innate and adaptive immunity, by being attracted and activated by elements of the innate response and enabling the sensitization of T lymphocytes of the adaptive immune response, while NK cells represent a nonspecific line of defense, recognizing and lysing infected cells, recruit neutrophils and MΦ, activate DCs, T and B lymphocytes.

The crosstalk between NK cells and DC results in maturation, activation, and cytokine production by both cells. This interaction either in the periphery or in the secondary lymphoid organs acts as a key player connecting innate and adaptive immune responses against pathogens.

In some infectious diseases, such as parasitic diseases, EVs have been gaining impact as mediators in the parasite-host interaction. These nanovesicles allow the transfer of virulence factors and effector molecules from the parasites to the host, thus regulating host gene expression and immune responses and consequently mediating the pathogenesis process.

It is therefore important to advance the immunological research to determine the mechanisms of resistance and susceptibility to *Leishmania* parasites.

This study aimed to investigate the interaction between the innate and adaptive immune response of the canine host against *L. infantum*, which is the main etiological agent of CanL, by examining DCs and NK cell immune activation that are involved in this crosstalk. In parallel, was analysed the immune activation of moDCs and the crosstalk with

NK cells in *L. amazonensis* infection since both these species of *Leishmania* can naturally infect the dog, as well as the role played by EVs shed by *L infantum* and *L. amazonensis* promastigotes.

To the best of our knowledge, this is the first study investigating *in vitro* the immune response displayed against *L. infantum* and *L. amazonensis* as well as their parasitic fractions (antigens and EVs) by canine moDCs and the crosstalk with NK cells, indirectly foreseeing the role played by these cells in the activation of the acquired immune response.

Dog moDCs can sense *L. infantum* parasites, become activated, present a pro-inflammatory profile (IL-1β and IL-18) and promote the migration (CXCL16) of cytotoxic T ($CD8^+$) lymphocytes in addition with the predominance of $MHCI^+$ moDCs and positively regulated the co-stimulatory molecule CD86. Therefore, dog moDCs can promote the crosstalk with NK cells and establish a bridge with acquired immune response. Contrarily, *L. amazonensis* appeared to drive moDCs into an anergic state. In turn, *LaEVs* also signaled moDCs through TLR2, induced positive regulation of CD80/CD86 and can attract leukocytes. *LiEVs* promote the expansion of $MHCI^+$ moDCs and positively regulate CD86 in addition to the release of chemokine that attracting leukocytes (CXCL16) can promote the crosstalk with NK cells and induce the activation of cytotoxic T cells. (Fig. 39).

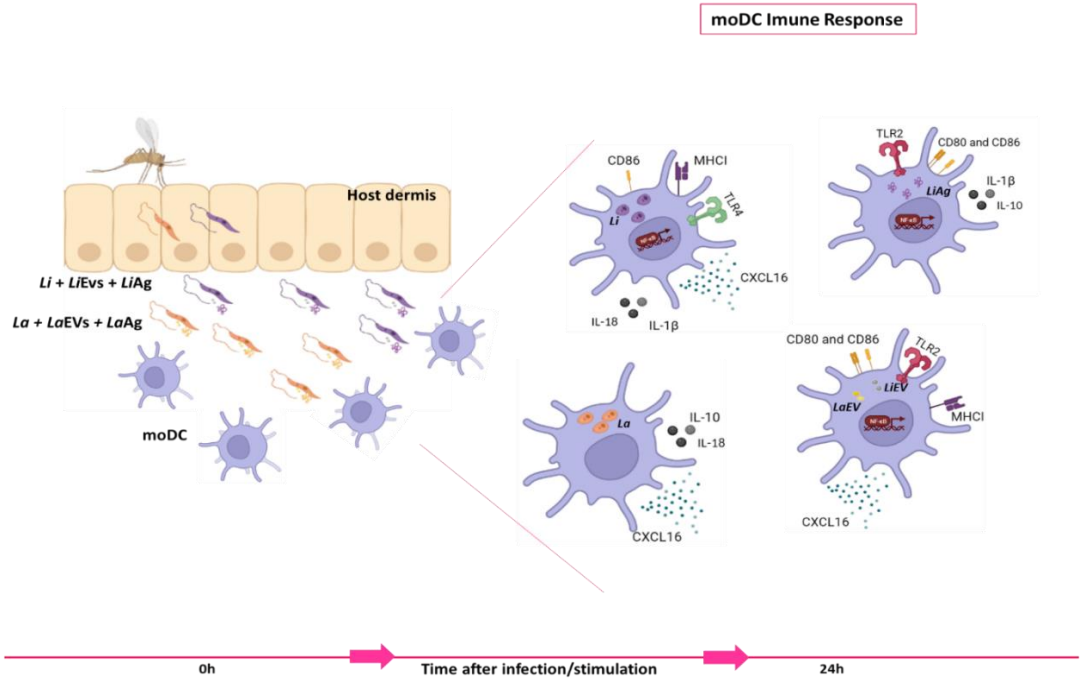


Figure 41. Interaction between dog DC and *L.infantum* and *L. amazonensis* parasites or EVs shed by *L. infantum* and *L. amazonensis* or parasite antigen (Ag) at the early phase of infection. After deposition of promastigotes by the vector into the host dermis, DC are recruited to the inoculation site and *Leishmania* promastigotes are internalized. Depending on the species of *Leishmania* parasite EVs or antigens, DCs elicit different types of response. *L. infantum* (Li), *LiAg* and both EVs (*LiEVs* and LaEVs*)* upregulate innate sensors (TLR4, TLR2), and immune mediators (cytokines and chemokines or cytokines or chemokines only), as well as co-stimulatory molecules and induce the predomination of $MHCI^{+}$ moDCs. Li, *LiAg* and *LaEVs* promote the activation and translocation of NF-κB to DC nucleus. In turn, *L. amazonenesis* (La) induced the release of immune mediators (anti- and pro-inflammatory cytokines and chemokine CXCL16). DCs –dendritic cells; EVs – extracellular vesicles. Image of my own authorship, created with BioRender.com (accessed August 2022).

The contact of infected moDCs with NK cells seems to promote the predominance of $MHCII^+$ moDCs, suggesting the possibility of antigen presentation to Th lymphocytes. When in contact with NK cells the *Li*EV-stimulated moDCs led to the expansion of $MHCI^+$ and $MHCII^+$ moDCs which can lead to activation of Th and cytotoxic T cells while moDCs exposed to *LaEVs* can promote the increase of $MHCI^+$moDCs, which may activate cytotoxic T cells (Fig. 40).

Thus, *L. infantum* and *L. amazonensis* establish different immune relationships with canine DCs, which may be associated with parasite-host co-evolution. Moreover, EVs show great potential for establishing a bridge with acquired immune response, being good candidates for future therapeutic or prophylactic studies.

Personalized medicine is a new approach that aims to adapt medical treatment to the individual characteristics of each patient. It is based on scientific information about the genetic and protein characteristics of cells that determine how well or poorly a given treatment will work. In veterinary medicine, these new perspectives have been considered to a small extend and explored, so this work can open new avenues to better understanding the responses of DC and NK cells when there is contact with *Leishmania* or with parasite EVs, which can lead to the development of newprophylatic and therapeutic strategies, such as DC based vaccines and also EVs as vehicles of immune mediators (eventually associated to antileishmanial drugs) for future personalized immune treatments.

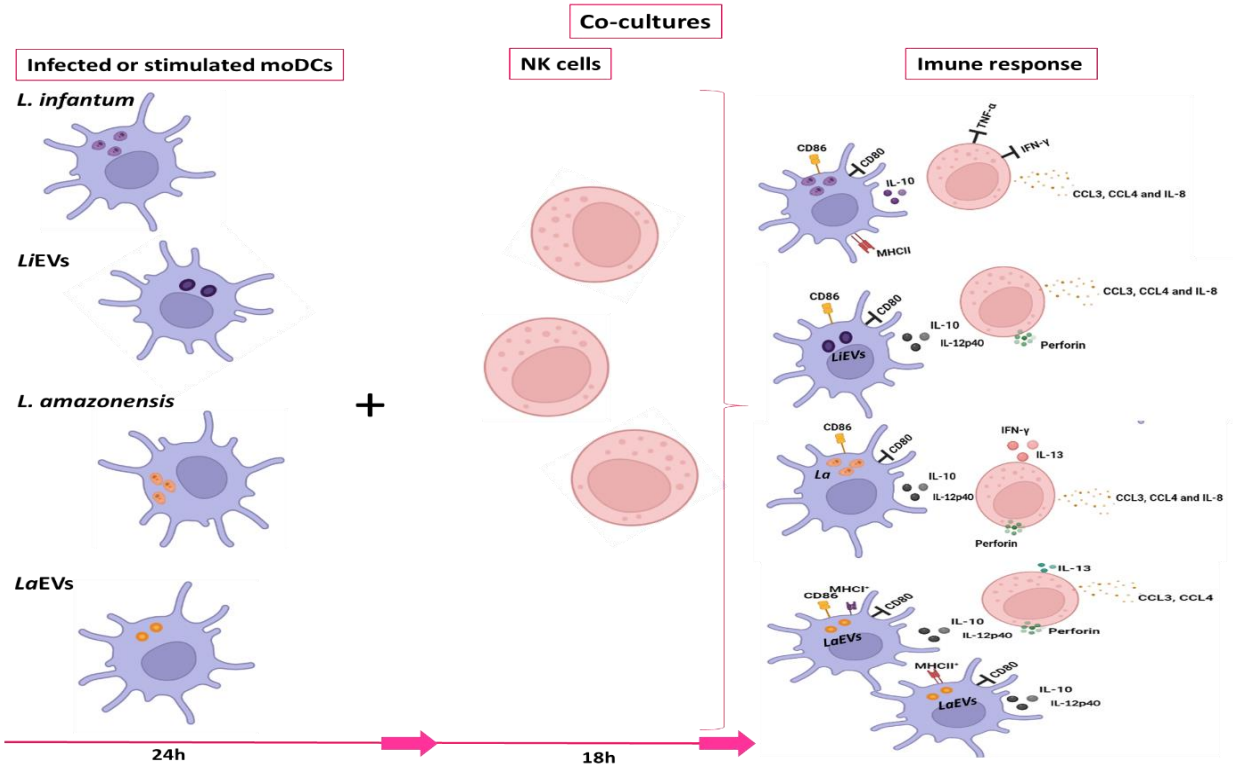


Figure 42. Crosstalk between DC and NK cells. Together with phagocytic cells, NK cells represent the first line of defense against pathogens using two different mechanisms of action: cytolytic destruction of infected cells and secretion of pro-inflammatory cytokines. The crosstalk between *Li*-moDCNK cells and *LiEVs* -moDC- NK cells generates immune mediators (cytokines and chemokines). *Li*-moDC-NK cells still directs the predomination of MHCII$^{+\Phi}$DCs cand *LiEVs* -moDC- NK cells led to degranulation of NK cell. In turn, *Li*-moDCs-NK cells inhibit the generation of the cytokines (IFN-γ and TNF-α. *La*-moDC--NK cells and *LaEVs* -moDC-NK cells generate immune mediators (anti- and pro-inflammatory cytokines and chemokines) and *LaEVs* -moDC-NK cells furtherexpand MHCI^{+} moDCs, promoting granule exocytosis of NK cells. Image of my own authorship, created with BioRender.com (accessed August 2022)

5|Reference List

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