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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF CELL-MEDIATED IMMUNITY TO *LEISHMANIA INFANTUM*

ANÁLISE FUNCIONAL DA IMUNIDADE CELULAR NA INFECÇÃO POR *LEISHMANIA INFANTUM*

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SUMMARY

Leishmania infantum is the causative agent of zoonotic visceral leishmaniasis (ZVL), a disease frequently characterized by specific impairment of cell-mediated immune responses and uncontrolled parasitization. Regulatory T cells (Treg) have been shown to be involved in the direct induction of immunosuppresion of effector immune response during chronic *Leishmania* infections. The present study aims firstly to investigate the possible involvement of Treg cells during *L. infantum* infection in a susceptible animal model. Results indicated that $CD4^+CD25^+$ regulatory T cells are present in *L. infantum*-infected BALB/c mice and exhibit phenotypic and functional characteristics of Treg. The presence of high levels of *foxp3* gene expression and surface expression of αEβ7 integrin (CD103) suggest a predisposition for Treg retention within sites of *L. infantum* infection, as is the case of the spleen and draining lymph nodes, consequently influencing local immune response and increased susceptibility. However, no evident Th polarization despite chronic parasitism in both spleen and liver was observed during *L. infantum* infection in this model. Th1 and Th2 effector immune responses seemed inadequate, perhaps due to Treg expansion. Foxp3-expressing-$CD4^+CD25^+$ T cells are indeed capable of producing TGF-β and may contribute to immunosuppression and better control of parasite-mediated-immunopathology during infection. Surprisingly, IL-10 producing-$CD4^+CD25^-Foxp3^-$ T cells were also identified as an additional source of IL-10 and represent a type 1 regulatory T (Tr1) cell subset that is being induced *in vivo* by *L. infantum* parasites. These findings suggest that distinct regulatory T cells develop in response to *L. infantum* and may play a possible role in promoting parasite persistence and the establishment of chronic infection in this particular experimental model of infection. Having demonstrated that Treg-mediated immunosuppression is evident in *L. infantum* susceptible infection model, the next step would be to verify if in a resistant experimental model of infection, immunosuppresive Treg can or cannot be modulated by the parasite. This would represent the development of a parasite strategy able to upregulate immunosuppressive cells, dampen effector immune response in their favour, and promote expansion, ultimately regulating the regulator. So to elucidate on

immunosuppressive Treg function induced by *L. infantum* parasites and the underlying mechanisms involved in the direct host-parasite interactions and immune regulation, the second part of this study focuses on the role of TLR-2 on Treg function during *L. infantum* experimental murine infection by investigating the influence of TLR-2 on Treg kinetics, immune response, parasite-associated pathology and the outcome of *L. infantum* infection. To achieve this, TLR-2 deficient mice (TLR-$2^{-/-}$) and their wild-type C57BL/6 mice (WT) were infected with *L. infantum* parasites and comparative analysis was done to see whether or not the presence or absence of TLR-2 produces any differential effect on the host parameters related to Treg dynamics and protective immunity. Defective TLR-2 signalling had a visible effect on outcome of *L. infantum* infection. Higher rates of parasite multiplication were observed in both spleen and liver of TLR-$2^{-/-}$ knock-out mice, despite the ability in forming well-defined and structured liver granulomas. These granulomas were apparently ineffective in parasite clearance, when compared to wild-type mice. Defective TLR-2 signalling did induce during late infection high retention of memory Treg which seemed to be associated to high parasite load and low IFN-γ levels. TLR-2 signalling pathway may play a role in Treg modulation and consequently in *L. infantum* pathogenesis. Functional TLR-2 signalling in WT may be important in providing tight control over $FOXP3^{+}$ committed Treg populations, negative Treg regulation and more protective immunity, giving rise to enhanced immunity and more effective response against infection. The presence or absence of TLR-2 did not seem to influence IL-10 or TGF-β expression, and it did not seem to correlate with $CD103^{+}$ $FOXP3^{+}$ Treg detected late during infection in TLR-$2^{-/-}$ mice. Detection of high levels of suppressive Treg in *L. infantum*-infected TLR-2 deficient mice was not accompanied by associated inductions of immunosuppressive cytokines. The presence of high levels of immunosuppressive Treg in infected spleen, in the absence of TLR-2, suggests that this receptor in particular plays an important role in regulating the regulators, thus orchestrating effective innate and acquired immunity against *L. infantum* infection.

SUMÁRIO

Leishmania infantum é o agente responsável pela leishmaniose visceral zoonótica, uma parasitose frequentemente caracterizada por alterações específicas da imunidade celular e parasitismo progressivo. Sabe-se actualmente que as células T reguladoras (Treg) são na verdade linfócitos T que se encontram directamente envolvidos na indução de mecanismos de imunossupressão da resposta imunológica durante infecções crónicas, como por exemplo *Leishmania*. Deste modo, este estudo tem como objectivo analisar o papel das Treg durante a infecção com *L. infantum* em modelo animal susceptível. Os resultados obtidos indicam que os linfócitos T $CD4^+CD25^+$ estão presentes em murganhos BALB/c infectados com *L. infantum* e exibem características fenotípicas e funcionais de Treg. A detecção de níveis elevados de expressão do gene *foxp3* e do marcador de superfície αEβ7 integrina (CD103) sugere a predisposição para a retenção de Treg nos locais preferenciais de infecção por *L. infantum*, como é o caso do baço e dos gânglios linfáticos, influenciando a resposta local e induzindo susceptibilidade. No entanto, apesar de neste modelo de infecção se ter observado parasitismo crónico no baço e no fígado não parece ter havido uma resposta Th polarizada o que pode estar relacionado com a expansão de Treg. Linfócitos T $CD4^+CD25^+$ que expressam Foxp3 demonstraram ter capacidade de produzir TGF-β, contribuindo para a imunossupressão e controlo da imunopatologia induzida pelo parasita. Supreendentemente, linfócitos T $CD4^+CD25^-Foxp3^-$ produtores de IL-10 também foram identificados como fonte adicional de IL-10, representando uma sub-população de linfócitos T reguladores do tipo 1 (Tr1) cuja diferenciação foi induzida pelo parasita. Estes resultados sugerem que diferentes tipos de células T reguladoras podem ser estimuladas durante a resposta imunológica à infecção por *L. infantum,* contribuindo para a persistência do parasita e o estabelecimento da infecção crónica neste modelo experimental. Tendo demonstrado que a imunossupressão mediada por Treg é evidente no modelo susceptível de *L. infantum*, o próximo passo seria verificar se num modelo experimental de resistência, a supressão evidenciada pelas Treg seria regulada ou não pelo parasita, representando deste modo o desenvolvimento pelo parasita de uma estratégia para promover a expansão de células imunossupressivas e a inibição da resposta efectora do hospedeiro, isto é regulando os

reguladores. Assim para estudar a função imunossupressora das Treg induzida por *L. infantum* e os mecanismos envolvidos na interacção hospedeiro-parasita e na regulação imunológica, a segunda parte deste estudo avalia o papel do receptor TLR-2 na função das Treg durante a infecção experimental por *L. infantum* e analisa a influência deste receptor na cinética das Treg, na resposta imunológica e na patologia. Para tal, murganhos mutantes C57BL/6 para o gene TLR-2 (TLR-$2^{-/-}$) e os murganhos "wild-type" C57BL/6 foram infectados com *L. infantum*. A análise comparativa foi efectuada de modo a verificar se a presença ou ausência de TLR-2 produz um efeito diferencial nos parâmetros do hospedeiro associados à dinâmica das Treg e à imunidade protectora. A ausência de sinalização TLR-2 teve um efeito visível no desenrolar da infecção. Elevadas taxas de multiplicação do parasita foram observados no baço e fígado dos murganhos TLR-$2^{-/-}$ apesar de se ter evidenciado a presença de granulomas hepáticos aparentemente bem estruturados e definidos. Estas formas granulatomosas são, aparentemente, ineficazes na eliminação do parasita comparativamente aos murganhos "wild-type". A ausência de sinalização TLR-2 induziu a retenção tardia de Treg de memória, associada à elevada carga parasitária e níveis reduzidos de IFN-γ. O TLR-2 poderá desempenhar um papel na regulação das Treg e consequentemente na patogénese por *L. infantum*. Nos murganhos "wild-type", a sinalização via TLR-2 pode ser importante no controlo das populações de Treg $FOXP3^{+}$, na regulação negativa das Tregs e no desenvolvimento de imunidade protectora mais eficaz contra o parasita. A presença ou ausência de TLR-2 não influenciou a expressão de IL-10 ou de TGF-β e não está relacionado com a detecção de Treg $CD103^{+}$ $FOXP3^{+}$ durante a fase tardia da infecção nos murganhos TLR-$2^{-/-}$. Elevados níveis de Treg nestes murganhos não foram acompanhados pela indução de citocinas imunossupressoras. A presença de níveis elevados de Treg no baço de animais infectados, na ausência de TLR-2, sugere que este receptor poderá desempenhar um papel importante na regulação dos reguladores, mediando desta forma a imunidade inata e adquirida desenvolvida pelo hospedeiro durante a infecção por *L. infantum*.

OBJECTIVES

The main objective of this work is to investigate on whether regulatory T cells (Treg) control immune responses to *Leishmania infantum* and contribute to susceptibility to infection. To further study a possible mechanism of host-parasite interaction, toll-like receptor (TLR) - 2 was selected and evaluated as the candidate receptor in parasite-induced regulation of Treg and protective immunity to infection. In order to achieve this, the following specific objectives were delineated:

1 – Phenotypic characterization of regulatory T cell populations during *L. infantum in vivo* infection:

- Identification of Treg populations using specific markers;
- Kinetics of Treg during *in vivo L. infantum* infection;
- Evaluation of Treg and effector immune responses by assessing pro- and anti-inflammatory cytokines.

2- Evaluation of the effect of TLR-2 modulation on Treg population during *L. infantum in vivo* infection:

- Comparison of Treg *in vivo* cell populations of gene disrupted TLR-$2^{-/-}$ knock-out and wild-type mice;
- Comparison of the evolution of parasite burden;
- Evaluation of the effect of TLR-2 on Treg kinetics;
- Evaluation of the effect TLR-2 on effector immune responses.

ABBREVIATIONS

Ab - antibody

Ag - antigen

AIDS - acquired immunodeficiency syndrome

AMP - adenosine monophosphate

AP – acid phosphatase

APC - allophycocyanin

APCs - antigen presenting cells

ATP - adenosine triphosphate

BMDM - bone marrow derived macrophages

Bp – base pair

BSA - bovine serum albumin

C; G; A; T - cytosine; guanine; adenine; thymine

Ca^{2+} - calcium ion

CCR – CC-chemokine receptor

CD - cluster of differentiation

cDNA – complementary deoxyribonucleic acid

CIE – counterimmunoelectrophoresis

CL – cutaneous leishmaniasis

cm - centimetre

CSF - colony stimulating factor

Ct - cycle threshold

CTLA – cytotoxic T- lymphocyte antigen

DAT - direct agglutination test

DC – dendritic cells

DCL – diffuse cutaneous leishmaniasis

DDT - dichloro-diphenyl-trichloroethane

DLA - dog leukocyte antigen

DNA - deoxyribonucleic acid

dsDNA- double-stranded deoxyribonucleic acid

DNase – deoxyribonuclease

dNTP - deoxyribonucleotide triphosphate

E – efficiency of amplification

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme-linked immunosorbant assay

FAST – fast agglutination screening test

FCS - fetal calf serum

FGT – formol gel test

FGV- first generation vaccines

FITC - fluorescein isothiocyanate

FL - Fluorescence

FML – fucose mannose ligand

Foxp3 – forkhead box P3

FR4- folate receptor 4

FRET – fluorescence resonance energy transfer

FSC - forward scatter light

g - gram

g - relative centrifugal force

G – gauge

GITR – glucocorticoid-induced tumor-necrosis factor receptor family related gene

GPI – glycosylphosphatidylinositol

GIPLs – glycoinositol phospholipids

H_3PO_4 – phosphoric acid

HAART – highly active antiretroviral therapy

HBSS – Hanks' balanced salt solution

HCl – hydrochloric acid

HIV – human immunodeficiency virus

HLA - human leucocyte antigens

HPRT - hypoxanthine guanine phosphoribosyl transferase

LeIF – *Leishmania* elongation initiation factor

ICT – immunochromatographic test

IDO – indoleamine 2,3-dioxygenase

IFA – indirect immunofluorescence antibody

IFN-γ - interferon-gamma

IgG - immunoglobulin G

IL - interleukin

iNOS - inducible nitric oxide synthase

ip - intraperitoneal

IPTG - isopropyl-beta-D-thiogalactopyranoside

KATEX – latex agglutination test

kb – kilo base

kDa – kiloDalton

kDNA – kinetoplast DNA

KO – knock-out mice

LACK - *Leishmania* homolog of receptors for Activated C Kinase

LDA – limiting dilution assay

LN – lymph nodes

LPG – lipophosphoglycan

LV - liver

LZ - leishmanization

M; mM; mM - molar; milimolar; micromolar

mAb – monoclonal antibody

MAP – mitogen-activated protein

MCL – mucocutaneous leishmaniasis

mg; mg - miligrama; microgramа

Mg^{2+} - magnesium ion

MHC - major histocompatibility complex

MHC I - major histocompatibility complex class I

MHC II - major histocompatibility complex class II

ml; ml - millilitre; microlitre

MLEE – multi-locus enzyme electrophoresis

MPL-SE – monophosphoryl lipid A stable emulsion

mRNA - messenger ribonucleic acid

MST – Montenegro skin test

Mya – millions years ago

N – Avogadro constant

NaN_3 – sodium azide

ND – not detectable

NF-AT – nuclear factor activated T cells

NF-κβ – nuclear factor kappa-light-chain-enhancer of activated B cells

NK - natural killer cells

nm; mm - nanometer; millimeter

NNN - Novy, Nicolle, MacNeal

NO - nitric oxide

Nramp – Natural resistance-associated macrophage protein

ºC - degree centigrade

PAGE - polyacrylamide gel electrophoresis

PAMPs – pathogen-associated molecular patterns

PBMC – peripheral blood mononuclear cells

PBS - phosphate buffer saline

PCD – programmed cell death

PCR - polymerase chain reaction

PE – phycoerythrin

PerCP - peridinin chlorophyll-a protein

pg – picograms

PG – phosphoglycans

pH - power of hydrogen

pi – post-infection

PKC – proteinase kinase C

PKDL – post-kala-azar dermal leishmaniasis

PNA - lectin peanut agglutinin

PPG – proteophosphoglycans

RFLP – restriction fragment length polymorphism

Rn- fluorescence emission with subtracted background fluorescence signal

RNAi – ribonucleic acid interference

RPMI - Roswell Park Memorial Institute

RT - reverse transcription

SD – standard deviation

SDS - sodium dodecyl sulfate

SEM – standard error

sf – scurfy

SGV- second generation vaccines

S/N – signal versus noise ratio

SOCS – suppressor of cytokine signalling

SP - spleen

SPF – specific pathogen-free

spp - species

SSC - side scatter light

ssrRNA - small subunit ribosomal RNA

TCR – T-cell receptor

TGF-β - transforming growth factor - beta

Th - T helper cell

TLR-2 – Toll-like receptor-2

TLR2-/- - TLR-2 deficient mice

TMB - 3, 3', 5, 5'-tetramethylbenzidine

TNF-α - tumor-necrosis factor alpha

Tr1 – T regulatory 1 cells

Treg; iTreg; nTreg – regulatory T cells, induced regulatory T cells, naturally occurring regulatory T

cells

Tris – hydroxymethylaminomethane

TSA – thiol-specific antioxidant

U – units

μm – micrometer

VL – visceral leishmaniasis

VLP – viable parasite load

v/v; w/v - volume/volume; weight/volume

WHO - World Health Organization

WT – wild-type mice

X-Gal - 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

ZVL – zoonotic visceral leishmaniasis

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CHAPTER II. MATERIALS AND METHODS

CHAPTER I. INTRODUCTION

1. The genus *Leishmania*

Leishmaniasis is a vector-borne disease caused by a pathogenic protozoan parasite belonging to the family *Trypanosomatidae*, genus *Leishmania* Ross, 1903. These parasites infect the mononuclear phagocytic system of the mammalian host and are transmitted by the bite of a female sand fly of the family *Psychodidae*, genus *Phlebotomus* Rondari, 1843 or *Lutzomyia* França, 1924. In man, *Leishmania spp*. can cause a variety of symptoms that range from disfiguring cutaneous and mucocutaneous lesions, widespread destruction of mucous membranes, to visceral disease affecting amongst others, haematopoietic organs.

2. Biology of *Leishmania*

2.1. Morphology and life cycle

Leishmania is a unicellular digenetic endoparasite that morphologically alternates between intracellular aflagellated amastigotes within the vertebrate host and extracellular flagellated promastigotes within the intestinal tract of the phlebotomine sand fly. The amastigote stage is a round or ovoid-like organism, 2-6 μm in diameter, containing a nucleus, a kinetoplast and an internal flagellum. The amastigotes multiply by binary fission within the acidic and hydrolase-rich environment of the parasitophorous vacuoles of macrophages. The promastigotes have an elongated cell body (about 10-20 μm by 2.5-5 μm) with a central nucleus, a kinetoplast and a long external flagellum that emerges from the anterior part of the cell and confers motility (Figure 1).

Most *Leishmania* species (subgenus *Leishmania*) are suprapylarian parasites: that is, their development is restricted to the midgut of the digestive tract of sand flies. Members of the subgenus *Viannia* are peripylarian parasites: they enter the hindgut before migrating forward into

the midgut. Infection initiates when the sand flies feed from an infected host and ingests blood containing macrophages infected with amastigotes. The infected blood meal passes to the posterior

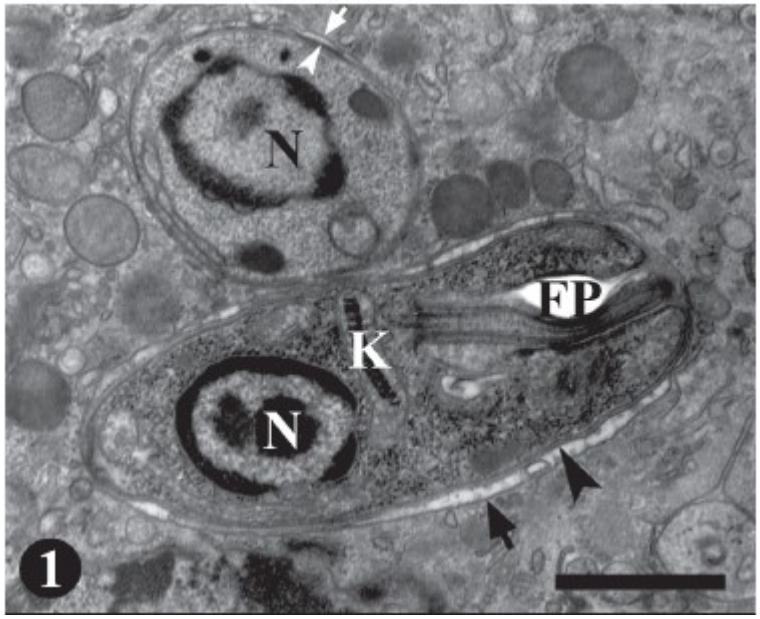


Figure 1. Transmission electron microscopy of *Leishmania*-infected host cells from hamster skin lesion showing amastigote and promastigote forms inside host cell vacuoles. Amastigote with the characteristic round shape, a centrally located nucleus, a bar-shaped kinetoplast, and large lipid inclusions. Promastigote form with the typical elongated body shape and a free flagellum. Arrowheads: parasite membrane; arrows: host parasitophorous vacuole membrane. N nucleus; K kinetoplast; FP: flagellar pocket (adapted from Corrêa and Soares, 2006).

abdominal gut where the infected macrophages are lysed and *Leishmania* amastigotes released. These begin to differentiate into several distinct developmental stages as they migrate anteriorly from the posterior hindgut or midgut to the stomodeal valve, which forms a junction with the foregut. Within 6–9 days, amastigotes transform into dividing procyclic promastigotes, relatively resistant to digestive enzyme activity, which in turn eventually attach to the lining of the midgut epithelium, rapidly multiply and differentiate into highly infectious metacyclic promastigotes. Each of the intermediate developmental promastigote stages is characterised by morphological and

functional changes aimed at ensuring its' survival in the sand fly vector. Depending on the parasite and vector species, there may be additional blood meals during maturation period, but most parasites can complete development within the timeframe of a single digestive cycle. The metacyclic promastigotes are freely motile and accumulate just behind the stomodeal valve, well positioned for egestion from the mouthparts of the sand fly upon uptake of a second blood meal and infection of a new host. Some of these promastigotes will survive, invade new cells and differentiate once again into amastigotes, hence completing its' life cycle. Once inside the skin, the promastigotes are phagocytosed by resident tissue macrophages, dendritic cells and neutrophils, transformed into amastigotes within the phagolysosome of macrophages. Replication of amastigotes can cause host cells to rupture and release parasites capable of invading other macrophages (Figure 2).

2.2. Pathogenecity

Disease progression is dependent on both the species of *Leishmania* involved and the genetics and immune status of the host. *Leishmania* pathogenesis involves key issues such as promastigote differentiation to the mammalian-infective amastigote stage, parasite responses and adaptations to life in host macrophages, genetic differences between major pathogenic species, and the intimate interface between the parasite and its' host cell (McConville et al., 2007). Parasite virulence factors and host mechanisms are inextricably linked to pathogenesis.

2.2.1. Parasite virulence factors

A successful completion of the life cycle of *Leishmania* parasites in the sand fly requires that they survive the digestive enzymes; avoid expulsion from the gut; and at a later stage, migrate anteriorly and break free from the midgut epithelium for transmission to the mammalian host. Several

molecules are central to vector-parasite interactions during critical stages of the *Leishmania* life cycle.

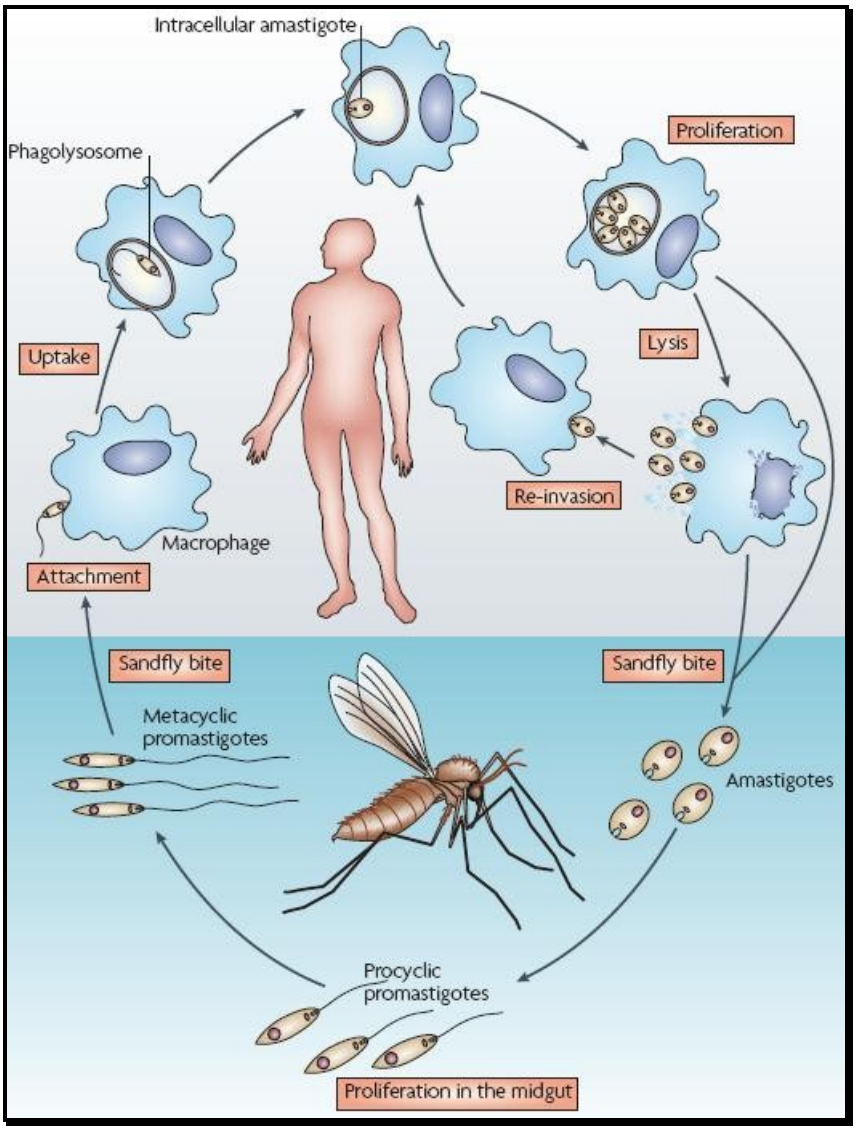


Figure 2. The *Leishmania* lifecycle. The promastigote form of *Leishmania* is transmitted into the skin by female phlebotomine sandflies. Once transmitted, the parasites are internalized by macrophages and dendritic cells in the dermis where they loose their flagella, transforming into the amastigote form. The amastigotes multiply, destroy the host cell and infect other phagocytic cells. The amastigotes disseminate through the lymphatic and vascular systems, eventually infiltrating the bone marrow, liver and spleen (reproduced from Handman, 2001).

Leishmania phosphoglycans share a common phosphorylated galactose-mannose disaccharide structure **[**-6Gal(β1-4)Man(α1)-PO_4-**]** and include glycosylphosphatidylinositol (GPI)-anchored molecules such as lipophosphoglycans (LPG), secreted extracellular phosphoglycans (PG) and secreted glycoproteins as acid phosphatase (AP) and proteophosphoglycans (PPG). Other GPI-anchored glycoconjugates include the small surface GPI lipids (GIPLs) and the surface protease or metalloproteinase gp63 (Yao et al., 2003).

Proteolytic enzymes secreted by the sand fly to digest the blood meal create a hostile environment for the parasites (Sacks et al., 2001; Volf et al., 2001; Ramalho-Ortigão et al 2003). Transitional *L. major* forms transforming from amastigotes to promastigotes in midgut of *Phlebotomus papatasi* are highly sensitive to proteolysis, and up to 50% of parasites can be killed in the first two days after blood feeding (Pimenta et al., 1997). To overcome this, *Leishmania* parasites have evolved specific abilities to modulate the activity of midgut digestive enzymes in their natural or competent vector species by inhibiting or delaying the peak of enzymatic activity. Glycoconjugates of *Leishmania* appear to play a part in protecting *Leishmania* promastigotes from these enzymes. Analysis of parasite mutants that are deficient in the synthesis of all PG were shown to be highly sensitive to the conditions in the early blood fed midgut (Sacks et al., 2000) although the addition of glycoconjugates from a *L. major* strain enhanced survival in *P. papatasi* (Schlein et al., 1990). These studies give emphasis on the importance of secreted phosphoglycans in protection of *Leishmania* parasites from digestive enzymes.

Glycoconjugates also seem to be important virulence factors when *Leishmania* is in the vertebrate host. LPG is the largest and most abundant surface glycolipid of promastigotes and forms a dense glycocalyx on their surface. It has been implicated as an adhesion molecule that mediates the interaction with the midgut epithelium of the sand fly in the subgenus *Leishmania*, thus preventing their loss with the excreted blood meal (Pimenta et al., 1992). LPG variations have also been implicated in the specificity of various *Leishmania* to different *Phlebotomus* species and thus promote vectorial competence to the invertebrate hosts (Pimenta et al., 1994; Sacks et al., 1995;

Kamhawi et al., 2000). LPG undergoes several important modifications during the life cycle that are characteristic for each *Leishmania* species. During the acquisition of virulence features in the sand fly midgut, LPG elongates, the number of repeating units doubles from about 15 in procyclic promastigotes to about 30 in metacyclic forms (Descoteaux et al., 1999). These structural changes are required for the detachment of infectious metacyclic promastigotes from the sand fly midgut (Sacks et al., 2001).

LPG has also been implicated in many steps required for establishment of macrophage infections apart from its' survival in the insect vector (Sacks et al., 2000; Ilg et al., 2001). Studies demonstrated that *Leishmania* phosphoglycans$^{-/-}$ were unable to survive in activated macrophages but retained the ability to persist indefinitely in the mammalian host without inducing disease in non-activated macrophages (Spath et al., 2003). Later, the same outcome was reported in a model of *L. major* mutant specifically lacking LPG, revealing that the previous findings may be related to LPG (Spath et al., 2003).

By acting as an activator of the complement cascade and binding to serum proteins, LPG facilitates promastigote attachment to macrophages and contributes to the ability of metacyclic promastigote to resist complement-mediated lysis in the mammalian host (Puentes et al., 1988). This is accompanied by an absence of proinflammatory response and no oxidative burst thus enabling promastigotes silent entry and easy access to a safe haven, the inside of the macrophage. LPG serves as an excellent cover masking direct recognition of parasite molecules by macrophage receptors and therefore bypassing macrophage activation. In fact, attachment and internalization of *L. donovani* promastigotes to bone marrow derived - macrophages failed to induce phosphorylation of several kinases involved in innate macrophage function such as production of proinflammatory cytokines and nitric oxide (NO) (Privé et al., 2000). Failure to activate macrophages during the invasion process may contribute to the successful establishment of *Leishmania* within the mammalian host.

Another known function for LPG is related to its' ability in manipulating signalling pathways and subverting or attenuating normal macrophage function. To turn off host microbicidal functions, *Leishmania* activates macrophage phosphotyrosine phosphatases and inhibits protein kinase C (PKC) activity (Descoteaux et al., 1993; Nandan et al., 2000). Once inside the phagosome of the macrophage, *Leishmania* is now faced with the dangers of subsequent phagosome maturation involving interactions with endosomes and lysosomes, vacuoles containing antimicrobial proteins. In the case of phagosomes harbouring *L. donovani* promastigotes, interactions with late endosomes and lysosomes are inhibited (Desjardins et al., 1997). Inhibition of phagosome maturation is dependent on LPG repeating unit domain since phagosomes harbouring phosphoglycan-defective mutants quickly mature (Desjardins et al., 1997). The exact mechanism by which LPG exerts such an effect on the phagosome remains unclear; however, the insertion of LPG in cellular membranes (Tolson et al., 1990) may alter their fusogenic properties (Miao et al., 1995).

It is noteworthy to state that the diverse mechanisms used by promastigotes to invade macrophages, manipulate innate function and evade and survive host defense mechanisms may not be common to all *Leishmania* species. Different *Leishmania* species place different emphasis on the importance of canonical virulence determinants, including LPG (Turco et al., 2001). Overall, previous studies establish a role for LPG in many but not all of the steps previously identified in macrophage invasion and survival. In fact new evidence now suggests that LPG is detrimental to long-term survival in the vertebrate host, as it is associated with activation of dendritic cells (DC), natural killer (NK) cells and NKT cells (Becker et al., 2003; Amprey et al., 2004; Aebischer et al., 2005); this is perhaps the reason why it is rapidly downregulated (at least three orders of magnitude) (Turco et al., 1991) in amastigotes. Due to its structural characteristics and GPI-anchor, LPG has also been identified as a *Leishmania* ligand capable of activating some "pathogen-associated molecular patterns" (PAMPs) recognising receptors, otherwise known as toll-like receptors (TLRs). This growing family of receptors are now known to be key players in the detection of pathogens

and the induction of anti-microbial immune response. The importance of *Leishmania* – TLR interactions will be discussed in further detail in section 7.3.

The process of differentiation of metacyclic promastigotes to amastigotes is one of the major developmental transitions in the *Leishmania* life cycle and a key event in establishing infection in the mammalian host. *In vitro* studies suggest that two environmental factors are sufficient to induce differentiation of promastigotes to amastigote-like forms (axenic amastigotes). A mild rise in temperature (33-37ºC) and a decrease in pH to 5.5 are the conditions that mimic the environment of macrophage phagolysosome. *In vivo* studies show that other factors, such as host serum components, may be required for differentiation (Bee et al., 2001). Several works indicate that *Leishmania in vitro* differentiation can be triggered by pharmacological agents that induce protein misfolding and promastigote heat shock response (Wiesgigl & Clos, 2001; Barak et al., 2005), by mitogen-activated protein (MAP) kinase signalling that are involved in modulating flagellum length (Wiese, 2007) and proteolytic systems involving protein turnover and degradation as key processes in autophagy and consequent promastigote-amastigote differentiation (Belesterio et al., 2007).

To be able to survive in host macrophages, amastigotes have thought to have acquired (1) amino acid permeases that allow them to scavenge host amino acids from the lumen of phagolysosome vacuole in competition with the host transporters (McConville et al., 2007), (2) the capacity of endocytosing and degrading host proteins using lysosomal proteinases (Besterio et al., 2007) and (3) lack of stage specific changes in mRNA levels and constitutive expression of enzymes involved in central carbon metabolism confer selective advantage by allowing these parasites to exploit variable nutrient conditions in the mammalian host.

Leishmania parasites are highly capable of manipulating host response in its favour. Depending on parasite stage and species, *Leishmania* can evade humoral innate defenses, remodel intracellular compartments and pathways, and impair macrophage and DC mechanisms (Sacks et al., 2002a;

Engwerda et al., 2004; McMahon-Pratt et al., 2004). Parasites interfere with intracellular kinases and phosphatases activity, downregulate activating-type signalling pathways, upregulate suppressive-type signalling pathways, and affect transcription factors and gene expression (Buates et al., 2001; Bertholet et al., 2003; Rodriguez et al., 2004). In turn, macrophage responsiveness and secretion of cytokines, surface molecule expression, and generation of leishmanicidal mechanisms (reactive oxygen and nitrogen intermediates) are compromised (Bertholet et al., 2003; Engwerda et al., 2004; Ray et al., 2000). Parasite effects extend to dendritic cells, which are critical to antigen presentation, T-cell co-stimulation, and efficient development of acquired Th1 responses (Brandonísio et al., 2004). Effects on dendritic cells include inhibition of migration, maturation and activation, and of interleukin (IL-) 12 production (Brandonísio et al., 2004). More detailed immunological aspects of host-parasite interactions will be further discussed in section 7.

Different species of *Leishmania* often exhibit strong preference for specific sand fly vectors and can cause different types of diseases in the human host. Comparative genome analysis may help identify the molecular basis for these differences. To date three completed *Leishmania* genomes (*L. major, L. infantum, L. braziliensis*) are in the public domain (Ivens et al., 2005; Peacock et al., 2007) with sequencing of a fourth (*L. mexicana*) in progress (http://www.genedb.org/). These first four species were chosen to represent the main species complexes of the *Leishmania* subgenus together with the best-characterised species of the *Viannia* subgenus. Importantly, the three completed genomes represent species that usually give rise to distinct disease types. Smith (2007), details on the remarkable similarity of the three genomes (only ~200 of the ~8300 genes are differently distributed) as well as some striking differences. The genes that differ between the three species are good candidates for functional analysis to determine their roles in establishment of infection. The most divergent, *L. braziliensis*, possesses 47 genes that are absent from the other two species. In comparison, *L. infantum* has 27 species-specific genes while *L. major* has only 5. A number of the other differentially distributed genes are found in 2 out of the 3 species. Some of these species-specific sequences have already been analysed at the molecular level. Examples

include the *L. infantum* A2 gene that encodes an amastigote-specific repeat-containing protein previously characterised in *L. donovani*, the only *Leishmania* sequence to date that confers a change in virulence phenotype when introduced into *L. major* by genetic transfection (Zhang et al., 2003); and the HASP and SHERP genes, expressed from a single locus (absent in *L. braziliensis*) in infective stages of *L. major* and *L. infantum*, with their protein products localizing in the plasma membrane and intracellular membranes, respectively, in these species (Denny et al., 2000; Knuepfer et al., 2001). Despite the well-conserved synteny between the three model species, *L. braziliensis* may have the capacity for more extensive genomic reorganisation, given the putative retrotransposons and RNA interference (RNAi – post-transcriptional mechanism for gene silencing) machinery very recently found in this species (Peacock et al., 2007). The availability of RNAi in *L. braziliensis* may play a role in the biological differences and different disease outcomes observed between the two subgenera. This finding has major practical applications and provides an alternative tool to targeted gene replacement for silencing gene expression in these parasites and reducing the degree of host pathogenecity.

3. Origin, evolution and taxonomy

It has been differently proposed that the genus *Leishmania* first appeared either in the 'Old World' (Africa, Asia and Europe) (Kerr et al., 2000; Momen et al., 2000) or in the 'New World' (the Americas) (Noyes et al., 1997; Stevens et al., 2001). The 'New World' origin is supported by the high genetic diversity of neotropical *Leishmania* species and by combined amino acid, DNA, and RNA polymerase-based trees, which root in America. This claim has received support by the description of a monoxenic insect flagellate from Costa Rica that branches at the root of the *Leishmania* clade (Yurchenko et al., 2006). In fact, a recent revision of current taxonomy (Lukes et al., 2007) proposes that the ancestor of the 'New World' leishmaniasis evolved in South America in the Paleocene or Eocene, ≈46–36 Mya and then migrated via the Bering land bridge to Asia. The

Leishmania lineage would have, then, dispersed through Central and/or Southeast Asia during the Miocene, 24–14 Mya (Fernandes et al., 1993), where a major diversification gave rise to *L. aethiopica*, *L. major*, *L. gerbilli*, *L. turanica*, *L. tropica*, and the *L. donovani* complex (Croan et al., 1997; Noyes et al., 1997). *L. infantum* would have then split from the early *L. donovani* lineage ≈1 Mya, and *L. donovani* soon thereafter invaded India and Africa. Closing the circle, after 500 years ago, *L. infantum* MON-1 European strains were transferred to South America, represented by the species formerly designated *L. chagasi*, considered synonymous with *L. infantum* (Maurício et al., 2000). The two main reservoir hosts of the *L. donovani* complex are humans and canids whose historical movements likely would have influenced the distribution of *L. donovani* and *L. infantum*.

Leishmania species must adapt to varied and heterogeneous environments during their life cycle. Efficient biological mechanisms are essential for the long-term and short-term survival of the parasite. *Leishmania* parasites basically present a clonal population structure. The great diversity of *Leishmania* species arises from different genetic processes such as asexual multiplication, gene amplification, occasional genetic exchanges through interindividual recombination, intrachromosomic and intragenic recombination (Victoir et al., 2005). Concerning interindividual recombinations, some studies suggest genetic exchange in *Leishmania* despite the lack of evidence of a sexual stage. For example, in the 'New World', hybrids between *L. braziliensis* and *L. peruviana*, and *L. guyanensis* and *L. braziliensis*, have been described (Da-Cruz et al., 1992; Belli et al., 1994; Dujardin et al., 1995; Bañuls et al., 1997) and in the 'Old World' hybrids have been reported between *L. major* and *L. arabica* (Evans et al., 1987; Kelly et al., 1991) and between *L. infantum* and *L. donovani* (Hide et al., 2006 and 2007) and between *L. infantum* and *L. major* (Ravel et al., 2006). However, the occurrence of pseudo-recombination events and cross-species genetic exchanges is not frequent enough to disturb the propagation of clones stable in space and time. It is postulated that the asexual model is enough to ensure parasite fitness in various environmental conditions and that sexual recombination is not necessary for the production of a large repertoire of genotypes (Ayala et al., 1998; Victoir and Dujardin, 2002).

The classification of *Leishmania* was initially based on ecobiological criteria such as vectors, geographical distribution, tropism, antigenic properties and clinical manifestation (Bray, 1974; Lumsden, 1974; Pratt and David, 1981; Lainson and Shaw, 1987) - for example, *L. guyanensis (*isolated in Guyana), *L. peruviana* (isolated in Peru), *L. infantum* (isolated from a child in Tunisia) and *L. gerbilli* (isolated from gerbils). All members of the genus *Leishmania* are parasites of mammals. The two subgenera, *Leishmania* and *Viannia*, are separated on the basis of their location in the vector's intestine (Lainson and Shaw, 1987).

However, biochemical and molecular analysis showed that these criteria were often inadequate and thus other criteria such as the patterns of polymorphism exhibited by kinetoplast DNA (kDNA) markers, proteins or antigens came to be used to classify *Leishmania* (Kreutzer and Christensen, 1980; Arnot and Barker, 1981; Pratt and David, 1981; Barker et al., 1986). For epidemiological purposes, the most useful taxonomic technique is isoenzyme analysis or multi-locus enzyme electrophoresis (MLEE) (Miles et al., 1980; Evans et al., 1984; Rioux et al., 1990). MLEE detects different alleles of housekeeping genes by scoring the electrophorectic mobility of enzymes they encode. It is still considered the 'gold standard' for *Leishmania* species identification and genetic diversity studies.

Use of molecular techniques led to the publication of a taxonomic scheme by the World Health Organization (WHO, 1990) (Figure 4). Today 30 *Leishmania* species are known and approximately 20 are pathogenic for humans. These species generally present different epidemiological and clinical characteristics related to different genetic and phenotypic profiles. The validity of the classification scheme, considered by some workers as too arbitrary, has been questioned several times. Debate has centred on *L. panamensis*, *L. peruviana*, *L. chagasi*, *L. infantum*, *L. archibaldi*, *L. garnhami*, *L. pifanoi*, *L. venezuelensis* and *L. forattinii* (Bañuls et al., 1999, 2000; Mauricio et al., 2000, 2001; Cupolillo et al., 2001). *L. chagasi* is accepted as a synonym of *L. infantum* (see Mauricio et al., 2000) and *L. peruviana* has been validated as an independent species (Bañuls et al., 2000). The other species listed above are still under discussion.

In addition, a recent study has shown to have identified a potential new species of *Leishmania* causing VL in Thailand (Sukmee et al., 2008). Through a combination of PCR-RFLP (restriction fragment length polymorphism) on the ssrRNA (small subunit ribosomal RNA) and mini-exon genes it was shown that the *Leishmania spp.* found is clearly distinguishable from other members of the genus *Leishmania*.

4. Epidemiology

Leishmaniasis is an important parasitosis, widespread on all the continents except Antartica. Human leishmaniasis constitutes a major public health problem and the burden is increasing (Desjeux, 2001, 2004). This disease can present different clinical features depending on the infecting species of *Leishmania* and the host's immunological status: cutaneous leishmaniasis (CL), a disease that can result in unaesthetic stigma if multiple lesions occur; mucocutaneous leishmaniasis (MCL; also known as *espundia*) a mutilating disease; diffuse cutaneous leishmaniasis (DCL) a long-lasting disabling disease due to a deficient cellular-mediated immune response; visceral leishmaniasis (VL; also known as *kala-azar*) a disease often fatal if untreated; and post-kala-azar dermal leishmaniasis (PKDL) a chronic cutaneous disease characterized by a macular, maculo-papular or nodular rash that develops after VL treatment. In 2002, the WHO estimated that the number of persons at risk to be around 350 million through 88 countries and the number of new cases to be 2 357 000 per year (WHO, 2002). In fact, 2 million new cases (1.5 million for CL and 500 000 for VL) are considered to occur annually, with an estimated 12 million people presently infected worldwide. It is noteworthy to mention that under-reporting is substantial - only around 600,000 infections are officially reported each year (WHO, 2002). Despite the scarcity of reliable data, there is little doubt that the case-load worldwide is considerably higher than official reported figures.

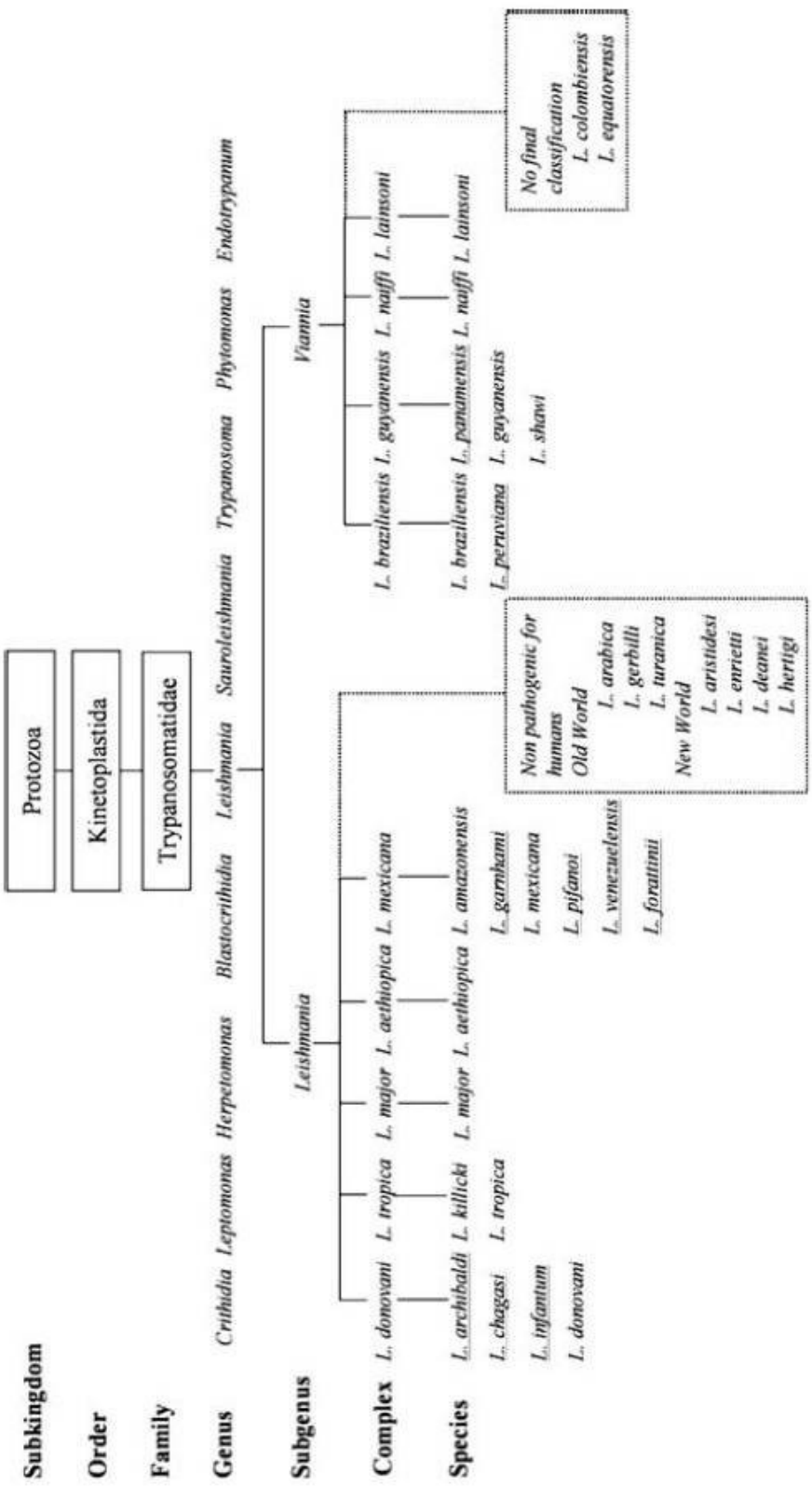


Figure 4. Taxonomy of *Leishmania*; underlined species are or have been questioned. (Based on the scheme published by the [WHO, 1990] with additions from the literature).

Of the 88 leishmaniasis-affected countries, 72 are classed as developing countries, including 13 of the least developed countries. Ninety percent of VL cases occur in just five countries — Bangladesh, India, Nepal, Sudan and Brazil. Ninety percent of CL cases occur in just seven countries — Afghanistan, Algeria, Brazil, Iran, Peru, Saudi Arabia and Syria. VL is of higher priority than CL as it is a fatal disease in the absence of treatment (Desjeux, 2004). Anthroponotic VL foci are of special concern as they are at the origin of frequent and large-scale tenacious epidemics with high case-fatality rates. Malnutrition is a well-known risk factor in the development of this form, and epidemics flourish under conditions of famine, complex emergencies and mass population movements. In Sudan, for example, a major decade-long epidemic of VL occurred from 1984 to 1994. Some studies estimate that the disease caused 100 000 deaths in a population of around 300 000 in the western upper Nile area of the country (Seaman et al., 1996). Despite the huge amount of research conducted on these pathogens in numerous scientific fields since the beginning of the last century, recent studies have also shown the reactivation of several other foci- in Italy, China, Brazil and Israel (Arias et al., 1996; Gradoni et al., 2003; Guan et al., 2003; Nasereddin et al., 2005) — and the emergence of new epidemic foci in Israel and Morocco (Jacobson et al., 2003; Al-Jawabreh et al., 2004; Guernaoui et al., 2005; Shani-Adir et al., 2005). Nevertheless, leishmaniasis is still considered as a neglected disease and is grossly underestimated, mainly due to lack of awareness of its serious impact on health.

5. Leishmaniasis

Leishmaniasis is a typical example of an anthropozoonosis although the majority of infections are originally zoonotic. The different epidemiological cycles are (i) a primitive or sylvatic cycle (human infection is accidental, transmission occurring in wild animal foci) e.g. *L. braziliensis*; (ii) a secondary or peridomestic cycle (the reservoir is a peridomestic or domestic animal, the parasite being transmitted to humans by anthropophilic sand flies), e.g. *L. infantum*; and (iii) a tertiary, strictly anthroponotic cycle, in which the animal reservoir has disappeared (or has not yet been

identified) and the sand fly vectors are totally anthroponotic, e.g. *L. donovani*. Nevertheless, many unknown factors remain. For example, the main animal reservoir of *L. braziliensis* is still unknown (Cupolillo et al., 2003). *L. tropica* was considered to be a strict anthroponosis, but several cases of canine infection have been described (Dereure et al., 1991; Banuls, 2007).

Cutaneous leishmaniasis, which accounts for more than 50% of new cases of leishmaniasis, is frequently self-healing in the 'Old World' but, when the lesions are multiple and disabling with disfiguring scars, it creates a lifelong aesthetic stigma. It results in formation of skin ulcers at the site of the sand fly bite, usually on exposed parts of the body, the face, neck, arms and legs. Its' most severe form, recidivans leishmaniasis, is very difficult to treat CL, is caused by several species of *Leishmania*: *L. major*, *L. tropica*, *L. aethiopica*, and *L. mexicana*, but also by *L. braziliensis*, *L. panamensis*, *L. peruviana, L. amazonensis*, *L. guyanensis, L. naiffi and L. lainson* (Murray et al., 2005). Dermatropic *L. infantum* has also proven to cause localized CL (Belazzoug et al., 1985). Time to lesion resolution varies between species and between individuals.

Diffuse cutaneous leishmaniasis occurs in individuals with defective cell-mediated immune response. Its' severity is due to disseminated lesions that resemble those of lepromatous leprosy, which never heal spontaneously and is subject to relapse after treatment with any of the currently available drugs. Because of the devastating consequences to the patient, it is recognized as a special public health problem. DCL is caused by *L. aethiopica*, *L. amazonensis* and *L. mexicana* occurs in anergic hosts with poor immune responses. This form of disease is restricted to a few foci in Ethiopia, Kenya, Venezuela and the Dominican Republic, suggesting an important role for the genetics of the parasite as well as the genetics of the host in determining the disease phenotype.

In mucocutaneous leishmaniasis ("espundia") the initial skin lesions cure, but the late development of metastatic lesions can lead to extensive destruction of oral-nasal and pharyngeal cavities with hideous disfiguring lesions, mutilation of the face and great suffering for life. MCL may also arise after inadequate treatment of some *Leishmania* species, and if untreated can lead to severe

deformities or even death. Although mostly related to *Leishmania* species of the 'New World' such as *L. braziliensis* but also more rarely by *L. panamensis* and *L. guyanensis* (Naiff et al., 1988; Saraiva et al., 1995; Osorio et al., 1998), MCL has been also reported in the 'Old World' due to *L. donovani*, *L. major* (Al-Gindan et al., 1983) and, in immmuno-suppressed patients, *L. infantum* (Alvar et al., 1990).

Visceral leishmaniasis, also known as *kala-azar*, is the most severe and often fatal syndrome. VL is caused by parasites belonging to the *L. donovani* complex – *L. donovani sensu stricto* in East Africa and the Indian subcontinent and *L. infantum* in south Europe, North Africa and Latin America (Boelaert et al., 2000). There are two types of VL, which differ in their transmission characteristics: zoonotic VL is transmitted from animal to vector to human and anthroponotic VL is transmitted from human to vector to human. In the former, humans are occasional hosts and animals, mainly dogs, are the reservoir of the parasite. Zoonotic VL is found in areas of *L. infantum* transmission whereas anthroponotic VL is found in areas of *L. donovani* transmission. Following an incubation period that generally lasts between 2 and 6 months, VL patients present symptoms and signs of persistent systemic infection (including fever, fatigue, weakness, loss of appetite and weight loss), parasitic invasion of the blood and reticulo-endothelial system (that is, the general phagocytic system), such as enlarged lymph nodes, spleen (splenomegaly) and liver (hepatomegaly) and hypergammaglobulinaemia (mainly IgGs from polyclonal B cell activation). Fever is usually associated with rigor and chills and can be intermittent. Fatigue and weakness are worsened by anaemia, which is caused by the persistent inflammatory state, hypersplenism (the peripheral destruction of erythrocytes in the enlarged spleen) and sometimes by bleeding. If left untreated, the disease has a high mortality rate mainly due to immunosuppression and secondary infections. Some individuals develop PKDL (Zijlstra et al., 2003), which appears within a few years of the complete cure of VL. PKDL patients are considered a major source of parasites for new infections because of the large number of organisms in the skin accessible to sand fly bites.

Human susceptibility to *L. infantum* is low therefore asymptomatic infections are common in healthy populations. Clinical disease is associated with age (infants below 2 years are most affected), malnutrition and immunosuppression (e.g. HIV co-infection).

Table 1. Human-infective species of the *Leishmania* genus. The main species complexes and subgenus are shown in bold; the species. * Species that can also be associated with cutaneous leishmaniasis (adapted from Smith et al., 2007).

Old World species	New World species	Disease type
***L. major* complex**	***L. mexicana* complex**	Cutaneous
L. (L.) major	*L. (L.) mexicana*	
L. (L.) tropica	*L. (L.) amazonensis*	
L. (L.) aethiopica	*L. (L.) pifanoi*	
	L. (L.) venezuelensis	
	L. (V.) (Viannia) subgenus	
	L. (V.) braziliensis	
	L. (V.) panamensis	
	L. (V.) guyanensis	
	L. (V.) perviana	
	L. (V.) lansoni	
	L. (V.) braziliensis	Mucocutaneous
L. (L.) aethipoica	*L. (L.) amazonensis*	Diffuse cutaneous
	L. (L.) pifanoi	
***L. donovani* complex**		
L. (L.) donovani		Visceral
*L. (L.) infantum**	*L. (L.) chagasi**	

VL has become a frequent infection in HIV-positive individuals in endemic areas and accelerates the onset of AIDS in these patients. Cases of HIV and visceral leishmaniasis co-infection have been reported in 35 countries worldwide (Chappuis et al., 2007). Both the cellular and humoral responses to *L. infantum* are diminished in co-infected patients (Moreno et al., 2000), leading to an increased parasite load in peripheral blood, lower sensitivity of serological tests, and a higher rate of treatment failure (Murray et al., 1999; Deniau et al., 2003). The impact of these co-infections was recognised as an alarming problem by international health authorities (Desjeux et al., 1995). Cases were reported showing a deadly overlapping between HIV pandemic and the zoonotic entity of VL (Gramiccia et al., 2005). In Europe, intravenous drug users have been identified as the main population at risk. The demonstration of unusual modes of anthroponotic transmission (i.e. by syringe exchange) (Cruz et al., 2002) and the high rate of relapses following antileishmanial treatments, were alarming features indicating a trend toward an even higher incidence. Highly active antiretroviral therapy (HAART) is used to modify the course of HIV infection. It has also been used to treat HIV-positive patients with VL and other infections. HAART appears efficient at preventing VL in individuals infected by *Leishmania* (de la Rosa et al., 2001), as reflected by the sharp decrease in the incidence of VL in Europe following the widespread use of HAART (Rosenthal et al., 2001; Lopez-Véléz et al., 2003). HAART is used to partially restore immune function but its efficacy in preventing VL relapses is disappointingly low (Mira et al., 2004).

6. Control strategies

The main control strategy includes: case finding and treatment, vector control, when feasible and, in zoonotic foci, animal reservoir control and vaccine development. It is impossible to devise a single control strategy for leishmaniasis control. A strategy combining these three approaches can help in eradication of the disease.

6.1. Diagnosis

One of the main challenges in the control of leishmaniasis is the clear need to reinforce screening and diagnosis of this disease in the peripheral health structures where patients are treated on the basis of clinical suspicion. Diagnostic tools for VL like, for example, invasive methods involving spleen, bone marrow and lymph node aspirations, are very difficult to technically implement and decentralise. Usually, there are no facilities to perform bone marrow or spleen aspiration in most of the district hospitals. Screening and diagnosis in such settings should rely on simple tests. The existing tools are based on serological, parasitological and molecular diagnosis.

In CL, serum antileishmanial antibody can be detected with standardised and sensitive assays (Romero et al., 2005) however, in practice (Vega-Lopez et al., 2003) diagnosis is made microscopically by identification of amastigotes in Giemsa or Leishman - stained lesion biopsies, scrapings, or impression smears. Combination of microscopy and culture increases diagnostic sensitivity to more than 85% (Ramirez et al., 2000) and culture (or DNA analysis) allows species identification. Detection of parasite DNA in lesion material is usually most sensitive in the diagnosis of both cutaneous and mucosal leishmaniasis, (de Oliveira et al., 2003; Faber et al., 2003; Vega-Lopez et al., 2003, Weina et al., 2004; Oliveira et al., 2005), but is seldom used as the only test producing positive results. Culture and DNA testing are technically difficult laboratory techniques that are not currently practical in some developing countries. Serological tests are rarely used in CL diagnosis because the number of circulating antibodies against CL-causing parasites tends to be low and the sensitivity can be variable. The specificity can also be variable, especially in areas where cross-reacting parasites (e.g., *Trypanosoma cruzi*) are prevalent (Reithinger & Dujardin, 2007). The Montenegro skin test (MST) is occasionally used in CL diagnosis (e.g., in epidemiological surveys and vaccine studies) because of its simple use and because of its high sensitivity and specificity (Wiegle et al., 1991). The main disadvantages of the MST are that it requires culture facilities to produce the MST antigen, that different antigen preparations impact test sensitivity, and that the test does not distinguish between past and present infections.

Diagnosis of VL is quite complex. Clinical manifestations lack specificity and differential diagnosis is important because diseases of other etiologies with a clinical spectrum similar to that of the leishmaniasis (e.g., leprosy, skin cancers, and tuberculosis for CL and malaria and schistosomiasis for VL) are often present in areas of endemicity. Non-leishmanial tests show that a reduction in the number of red and white blood cells and platelets (pancytopenia) was found to be highly specific (98%) for VL in suspected clinical patients in Nepal but the sensitivity was low (16%) (Boelaert et al., 2004). Marked polyclonal hypergammaglobulinemia (the production of high titres of non-specific antibody), a common finding in VL, can be detected by a formol gel test (FGT; also called the aldehyde test), which is still used in East Africa and Asia because of its simplicity and low cost. However, as the sensitivity of this test is poor (as low as 34%) (Boelaert et al., 2004), some experts have recommended its use to be discontinued (Sundar et al., 2003). Parasitological diagnosis remains the gold standard in VL diagnosis because of its high specificity (Herwaldt et al., 1999). Direct visualisation of amastigotes in clinical specimens in spleen aspirates is the classical confirmatory test used in regions where tissue aspiration is feasible and microscopy and technical skill are available. Diagnostic sensitivity for splenic, bone marrow, and lymph node aspirate smears varies, being higher for spleen (93–99%) than for bone marrow (53–86%) or lymph node (53–65%) aspirates (Young et al., 1939, Ho et al., 1948; Siddig et al., 1988; Zijlstra et al., 1991; Babiker et al., 2007). Serological methods are highly sensitive and being non-invasive they are comparatively more suited for diagnosing VL in endemic regions. These methods are either based on detection of antibodies (produced against parasite by polyclonal activation of B cells) or antigens. Several tests have been developed, but all have two major limitations. First, though serum antibody levels decrease after successful treatment (Kumar et al., 2001; Braz et al., 2002), they remain detectable up to several years after cure (Hailu et al.,1990; Bern et al., 2005; De Almeida et al., 2006). Second, a significant proportion of healthy individuals living in endemic areas with no history of VL are positive for anti-leishmanial antibodies owing to asymptomatic infections. Antibody-based tests must therefore always be used in combination with a standardized clinical case definition for VL diagnosis. Serological tests based on indirect fluorescence antibody (IFA)

(Harith et al., 1987), enzyme-linked immunosorbent assay (ELISA) (Kumar et al., 2001) or western blot (Santos-Gomes et al., 2000) have shown high diagnostic accuracy in most studies but are poorly adapted to field settings. Two serological tests have been specifically developed for field use and have been sufficiently validated — the direct agglutination test (DAT) and the rK39-based immunochromatographic test (ICT). The DAT is a semi-quantitative test that uses microtitre plates in which increasing dilutions of patient's serum or blood are mixed with stained killed *L. donovani* promastigotes (Harith et al., 1986; 1987). If specific antibodies are present, agglutination is visible after 18 hours with the naked eye. The fast agglutination screening test (FAST) is a simplified (single serum dilution at a cut off of 1:800 or 1:1600) and more rapid (2–3 hours) version of the DAT, and its diagnostic accuracy seems comparable (Schoone et al., 2001; Hailu et al., 2006), but further validation is needed. rK39 is recombinant surface antigen that is highly specific for VL. It is a 39-amino acid repeat that is part of a kinesin-related protein in *L. chagasi* and which is conserved within the *L. donovani* complex (Burns et al., 1993). The test was then developed into an ICT, or dipstick, format that was more suitable for field use and is currently the best available diagnostic tool for VL for use in remote areas. Antigen-based immunodiagnostic tests are found to be more specific than antibody based methods as they avoid cross-reactivity and can distinguish active from past infections and are also more useful in immunosuppressed patients. A latex agglutination test (KATEX) detecting a heat-stable, low-molecular-weight carbohydrate antigen in the urine of VL patients has also shown promising initial results (Attar et al., 2001; Sarkari et al., 2002). In the particular case of zoonotic canine leishmaniasis, infected dogs that apparently are asymptomatic are also infectious and constitute an important reservoir for transmission. Specific and more sensitive tools are needed to distinguish asymptomatic from symptomatic dogs. A very recent study by Rajasekariah (2008) has developed a canine ELISA assay based on a cocktail of exo-antigens prepared from three species (*L. infantum*, *L. donovani*, and *L. major*).

Although different molecular methods have successively been evaluated for leishmaniasis diagnosis (e.g., pulsed-field gel electrophoresis and multilocus enzyme electrophoresis) (Jaffe et

al., 1987), PCR-based assays currently constitute the main molecular diagnostic approach of researchers and health professionals. It is a definite breakthrough in the diagnosis and prognosis of VL as a non invasive method with excellent sensitivity and specificity. Polymerase chain reaction (PCR) identification is also currently used to identify different *Leishmania* species, diagnosis and control canine leishmaniasis (de Andrade et al., 2006; Gomes et al., 2007). A variety of nucleic acid detection methods targeting both DNA and RNA have been developed. Different DNA sequences in the genome of *Leishmania* like ITS region, gp63 locus, telomeric sequence, sequence targeting rRNA genes and 18SrRNA, ssUrRNA and both conserved and variable regions of kDNA minicircles have been documented in diagnosis and prognosis of VL (Salotra et al., 2001; Wortmann et al., 2001; Cortes et al., 2006). PCR formats range from simplified PCR methods for use in laboratory settings with minimal equipment, PCR-enzyme-linked immunosorbent assay (ELISA), a "reverse hybridization" method based on the capture of PCR amplicons by specific probes immobilized in ELISA microtiter wells and colorimetric visualization (Martin-Sanchez et al., 2002; De Donker et al., 2005) to more sophisticated, rapid and high-throughput methods in which *Leishmania* PCR products are analyzed during their amplification (real-time PCR) after staining with SYBR-green I dye or hybridization with fluorogenic probes (e.g., TaqMan or fluorescence resonance energy transfer [FRET]) (Rolão et al., 2004). In leishmaniasis diagnosis, PCR applications is particularly advantageous when parasite load is low (e.g. in blood and conjunctiva) (Cruz et al., 2002; Strauss-Ayali et al., 2004), in HIV-coinfection (Bossolasco et al., 2005; De Doncker et al., 2005), in the quantification of tissue parasite load and monitorization of disease progression and outcome (Mary et al., 2004), in evaluating the viability of parasites (van der Meide et al., 2005) when assessing the efficacy of drug therapies and predicting treatment outcomes and in the confirmation of clinical cure in VL (Maurya et al., 2005). In these cases, RNA should be preferred to DNA as an amplification target because the latter is still detected for a long time (estimated at 24 h) after parasite death (Quispe-Tintaya et al., 2005).

6.2. Reservoir and vector control

In the case of human reservoirs, serological diagnosis at the infection stage, if followed by a prompt treatment, will prevent the evolution to overt disease and reduce morbidity and mortality (Singh et al., 2006). In zoonotic VL, dogs are the main reservoir of *L. infantum*. Despite evidence from experimental studies showing a decreased incidence of VL in both dogs and children following serological screening of dogs and culling of sero-positive animals (Ashford et al., 1998; Palatnik-de-Sousa et al., 2001), the efficiency and acceptability of this control strategy is increasingly being debated (Tesh et al., 1995; Reithinger et al., 2002; Alvar et al., 2004). Culling of infected dogs is not considered an acceptable measure, both for ethical, socio-economical reasons and the low impact of this measure in situations of permanent transmission, such as Brazil (Moreira et al., 2004). Treating infected dogs is also not an effective control strategy as relapses are frequent and dogs can regain infectivity weeks after treatment, despite being clinically cured (Alvar et al., 1994; João et al., 2006). Moreover, the widespread veterinary use of VL drugs might lead to resistance in parasites. A new control approach is the use of deltamethrine-treated collars (Scalibor ProtectorBand, Intervet International BV) which reduced the risk of infection in dogs (by 54%) and children (by 43%) in a study conducted in Iran (Gavangi et al., 2002). Vaccination of dogs would nevertheless be the best strategy if an efficacious vaccine can be developed.

Sand flies are susceptible to the same insecticides as *Anopheles* mosquitoes, the malaria vector. Following the large scale antimalarial insecticide [dichloro-diphenyl-trichloroethane (DDT)] spraying campaigns that was implemented in the 1950s VL almost completely disappeared from the Indian subcontinent (Kaul et al., 1994). Unfortunately, the disease quickly re-emerged when these spraying campaigns were discontinued. The use of insecticide-impregnated bednets (ITNs), as an alternative to residual-insecticide house spraying, could concomitantly prevent VL and other vector-borne diseases, such as malaria. However there is limited evidence that bednets provide protection against VL (Chappuis, 2007). Case-control studies conducted in Bangladesh and Nepal showed that sleeping under a non-impregnated bednet during the warm months was a protective

factor against VL (Bern et al., 2005). Despite low usage, the mass distribution of ITNs in Sudan was accompanied by a 27% reduction in the incidence of VL in an observational study (Ritmeijer et al., 2007).

6.3. Treatment and vaccines

Several drugs are available for treating leishmaniasis. Pentavalent antimonial compounds, such as the lead molecule megalumine antimonate (glucantime®) and sodium stibogluconate, are the drugs used in first line chemotherapy. Pentavalent antimony is a prodrug that is reduced by glutathione to active trivalent species catalyzed by thiol-dependent-reductase (Franco et al., 1995). This trivalent is toxic to both amastigotes, and promastigotes. Potential targets that are inhibited by pentavalent antimonials are enzymes in glycolosis that are essential for adenosine triphosphate (ATP) levels in *Leishmania* and purine transporters and DNA topoisomerases, all essential for nucleoside metabolism (Berman et al., 1987; Lucumi et al., 1998; Demicheli et al., 2002). Antimonials are toxic drugs with frequent, sometimes life-threatening, adverse side effects, including cardiac arrhythmia and acute pancreatitis. The emergence of resistance against antimonials led to the use of other compounds such amphotericin B, pentamidine, paromomycin and allopurinol, considered to be second-line drugs. Pentamidine acts on the pathogen genome by hampering replication and transcription at the mitochondrial level, while Amphotericin B, an antifungal macrolide polyene, binds selectively to *Leishmania* cell membrane sterols, forming barrel shaped transmembrane pores and induces changes in membrane permeability through uncontrolled loss of ions. This leads to cellular dysfunction and eventually cell lysis (Brajtburg et al., 1985). However, it can also cause life-threatening adverse side effects such as hypokalemia (low potassium levels in the blood), nephrotoxicity and first-dose anaphylaxis. Its' lipid formulations that involve either phospholipids or detergents, referred to as "liposomes" and "mixed micelles" have been developed as drug delivery systems to minimize side effects. Other anti-fungals like ketoconazole, fluconazole and

terbinafine are found less effective. Amphotericin B is currently the most effective antileishmanial drug that induces the highest cure rates and has been recommended as first line drug in India, being more widely used now for treatment of VL. Paromomycin, an antibiotic, also exhibits good antileishmanial activity and acts primarily by impairing the macromolecular synthesis and altering the membrane properties of *Leishmania* (den Boer et al., 2006). Allopurinol inhibits the enzyme adenylosuccinate synthetase, which mediates the conversion of inosonic acid to adenosine monophosphate. Allopurinol hydrolyzes to allopurinol ribosides, an analogue of inosine, gets incorporated instead of ATP into leishmanial RNA, and there it interferes in the normal protein synthesis (Marr et al., 1977).

Current treatments against leishmaniasis are usually unsatisfactory due to some limitations including the route of administration of the drugs, their unaffordable cost and toxicity (Guerin et al., 2002; Croft et al., 2003; Singh et al., 2004). Efforts have been made to develop new leishmanicidal drugs and to find new strategies of drug design (Singh et al., 2004). Of interest for medicinal chemists is the pharmacological observation that certain molecules with anticancer activity and their derivatives might be also useful as antiprotozoal agents. It has been hypothesized that the cytotoxicity displayed by different antitumor drugs against protozoan parasites may be related to the fact that these parasites and cancer cells share diverse biochemical similarities which affect to DNA metabolism, protein kinases pathways, glucose catabolism enzymes, and polyamines metabolism (Klinkert et al., 2006). In addition, the growth of tripanosomatids parasites in their host and that of cancer cells show at least two common features that are (i) their mutual capacity for rapid cell division and (ii) some immune evasion and defense strategies. *Leishmania* parasites exhibit a molecular mechanism of programmed cell death (PCD) which shares some similarities with the apoptosis mechanism of cell suicide of multicellular organisms. Such mechanisms of PCD include proteins with caspase-like activity, cytochrome C release and DNA fragmentation. Therefore, the killing efficacy of some anticancer drugs against *Leishmania* may be due to the induction of programmed cell death in this protozoan parasite. Probably, the most promising drug

found is the antitumor compound miltefosine. Miltefosine is an alkylphosphocholine that is known to be toxic to both *Leishmania* promastigotes and amastigotes (Croft et al., 1987; Escobar et al. 2002). This drug has good gastrointestinal tract absorption and has the advantage of having more potent action when administered orally than when injected intravenously since it can cause erythrocyte lysis. Several proposed mechanisms for the mode of action of miltefosine include damage to the flagellar membrane (Santa-Rita et al., 2000), perturbation of alkyl-phospholipid metabolism and GPI-anchor biosynthesis (Lux et al., 1996), interference with ether-lipid remodelling through the inhibition of alkyl-lyso-phosphatidylcholine specific acyltransferase (Lux et al., 2000) and inhibition of the *de novo* synthesis of phosphatidylcholine (Lira et al., 2001). Miltefosine currently underwent Phase IV trial for the treatment of Indian VL and the final cure was 82% by intention to treat and 95% by per protocol analysis, with only 3 deaths out of 1,132 patients (Bhattacharya et al., 2007). However, Miltefosine is a teratogenic drug and its use is therefore strictly forbidden in pregnant women or in women who could become pregnant within two months of treatment. Non-adherence to the recommended regimen could lead to widespread parasite resistance and the increasing use of this drug in canine leishmaniasis in Europe might also increase the development of miltefosine resistance in *L. infantum*.

Leishmaniasis in general, but particularly CL, is probably one of the few parasitic diseases that are most likely to be controlled by vaccines (Khamesipour et al., 2006). The leishmanial life cycle is relatively uncomplicated and recovery from primary infection usually renders the host resistant to subsequent infections which only makes a successful vaccine even more feasible. Extensive evidence from animal models indicates solid protection can be achieved by immunisation using parasite-specific proteins, DNA or genetically attenuated parasites (Kedzierski et al., 2006; Khamesipour et al., 2006). In general, the leishmaniasis vaccines in development can be divided into three categories: (i) Live *Leishmania*; including new genetically modified constructs; (ii) First generation vaccines (FGV) consisting fractions of the parasite or whole killed *Leishmania* with or

without adjuvants; and (iii) Second generation vaccines (SGV) including all defined vaccines, i.e., recombinant proteins, DNA vaccines and combinations thereof.

'Leishmanization' (LZ), which simulates live infection by inoculation of live virulent *L. major* promastigotes, has been used as a vaccine for protection against CL (Senekji et al., 1941; Greenblatt et al., 1980). However, problems associated with LZ such as loss of parasite infectivity as a result of continuous sub-culturing, cases of immunosuppression in HIV-endemic areas and reduced responsiveness and chronic disease have lead to its discontinued use (Nadim et al., 1983). Until know this kind of vaccination is the only one that confers consistently high protection against LC. Therefore, a new interest has emerged in developing vaccines that use live parasites. New live vaccination strategies using mutant parasites, such as knock-out parasites lacking essential survival genes (Cruz et al., 1990; Alexander et al., 1998; Veras et al., 1999; Papadopoulou et al., 2002) or suicidal cassettes - *Leishmania* parasites genetically modified to commit suicide in response to external signals for self-destruction (Davoudi et al., 2005), are currently being tested. None of these constructs has reached clinical development, however this approach provides possibilities for induction of protection with a self-limiting infection, possibly without any associated pathology. At present there is only one prophylactic live vaccine in use. It is a mixture of live virulent *L. major* mixed with killed parasites that is being carried out in Uzbekistan (Sergiev et al., 1992; Gafurov et al., 1999).

Several SGV candidate DNA-derived proteins are currently under evaluation (Kedzierski et al., 2006) but the only one that has been engineered and assessed in human trials (Reed and Campos-Neto, 2003; Coler and Reed, 2005) is Leish-111f, a polyprotein antigen composed of three antigens expressed in both promastigotes and amastigotes across the *Leishmania* genus; *Leishmania* elongation initiation factor (LeIF), *L. major* stress-inducible protein (LmSTI1), and thiol-specific antioxidant (TSA), fused in tandem. This vaccine when combined with monophosphoryl lipid A-stable emulsion (MPL-SE) as adjuvant induces a potent Th1-type immune response and protects mice and non-human primates against CL (Campos-Neto et al., 2001, Coler et al., 2002, Skeiky et

al., 2002) and recently mice and hamsters against VL (Coler et al., 2007). However, Leish-111f failed to protect against canine leishmaniasis in a recent Phase III vaccine trial, where 95% showed evidence of *L. infantum* infection (Gradoni et al., 2005). The Leish-111f-MPL-SE product is the first defined vaccine for leishmaniasis in human clinical trials and has completed Phase I and II safety and immunogenicity testing in normal, healthy human subjects (Coler et al., 2007). Most of the recombinant derived proteins have also been tested as DNA vaccines (Kedzierski 2006) and are recommendable since they are usually highly immunogenic and elicit both humoral and cellular immune responses. Gp63 and LACK are the most extensively studied DNA vaccines against CL and VL but no development for use in humans have so far been reported.

Despite all efforts, no vaccine against any form of human leishmaniasis has yet been available. One major factor contributing against the development of a *Leishmania* vaccine is the lack of a conceived market for human leishmaniasis vaccines. In the absence of industrial interests, many candidate vaccines have remained as fine publications (Khamesipour, 2006). However, strong protective effects against canine leishmaniasis have been achieved with a vaccine developed in Brazil, a fucose manose ligand (FML)-Quillaja saponin formulation, capable of inducing in naturally exposed vaccinated dogs 92 and 95% protection to up to 3.5 years (Palatnik et al., 1994; Borja-Cabrera et al., 2002).

Other factors such as virulence, genetic differences between *Leishmania* species as well as host genetic factors controlling host protective immunity against different *Leishmania* also contribute to the problem and difficulties facing the design of a pan-*Leishmania* vaccine. An ideal anti-leishmanial vaccine would need to possess several attributes that include; (1) safety; (2) affordability to populations in need; (3) induction of $CD4^{+}$Th1 and $CD8^{+}$ T cell responses and long-term immunological memory; (4) effectiveness against both CL and VL; (5) stability and (6) effectiveness as a prophylactic.

7. Host – Parasite interactions

Cure in all forms of leishmaniasis is affected through cellular immune response capable of activating host macrophages to eliminate the parasite. Although leishmanial infections induce strong humoral responses, antibodies appear to play no role in protection and in fact are associated with non-healing forms of leishmaniasis. Not only do organisms of this genus have the ability to withstand, inhibit or circumvent the microbicidal activity of host macrophages, but under the appropriate circumstances, they can subvert the induction of both innate and adaptive immune responses.

Early classical experiments established that T-cell-deficient mice rapidly succumb to disease following inoculation with any one of several species of *Leishmania*, and that transfer of normal T cells confers resistance to the animals (Holaday et al., 1991). The $CD4^+$ subset of T cells is crucial for resistance, whereas $CD8^+$ T cells seem to participate more in the generation of immune memory than as effector cells involved in parasite elimination (Muller et al., 1991; Uzonna et al., 2004; Scott et al., 2005). It is universally accepted that the nature of the T-cell response is one of the crucial factors controlling experimental and human leishmaniasis (Tripathi et al., 2007). Engagement of pathogen recognition receptors, namely toll-like receptors, on the surface of host immune cells with *Leishmania* surface molecules has also gained significant importance in the development of innate immunity (Becker et al., 2003; de Veer et al., 2003; de Trez et al., 2004; Flandin et al., 2006; Whitacker et al., 2008). TLR signalling may also directly or indirectly regulate the immunosuppressive function of regulatory T cells (Liu et al., 2006; Sutmuller et al., 2006; Liu and Zhao, 2007) in immune responses and subsequently stimulate adaptive immunity thus influencing disease outcome.

Taking into consideration the main focus of the present dissertation, special attention will be given only on $CD4^+$ T cell-mediated response during acquired immunity to *Leishmania*.

7.1. Acquired immune responses against *Leishmania*

7.1.1. Th1 and Th2 responses

In human and experimental leishmaniasis, immunity is predominantly mediated by T lymphocytes. T cells play a major role in generating specific and memory T-cell responses to intracellular parasitic infections. T helper (Th) 1 and Th2 cells can be distinguished by the cytokines they secrete: Th1 cells secrete activators of cell-mediated immunity such as gamma-interferon (IFN-γ), while Th2 cells secrete cytokines such as interleukin (IL)-4, which promote antibody responses (Mosmann and Coffman, 1989). Cytokines can form a complex network of synergistic and antagonistic interactions, which not only induce but also control immune responses. The control of leishmanial infection is mediated by a Th1 type immune response, and experimental studies in murine models of CL (*L. major*) have established a clear-cut dichotomy between Th1-mediated protection and Th2-mediated disease susceptibility (Locksley et al., 1987; Heinzel et al., 1989; Scott et al., 1998; Sacks & Noben-Trauth, 2002). Resolution of infection is mediated by Th1 cells that produce IFN-γ. IFN-γ induces the production of nitric oxide (NO) in phagocytic cells that harbour *L. major* (principally macrophages), which leads to destruction of the parasite (Green et al., 1990; Liew et al., 1990). T-cell differentiation from naïve T cells (Th0) to either to Th1- or Th2-type effector cells depends chiefly on the priming during differentiation. IL-12 induces Th1 cells (regulated by transcription factor T-bet), to produce IFN-γ and tumor necrosis factor-alpha (TNF-α). IL-4 induces Th2 cells (regulated by GATA-3) to produce IL-4, IL-13 and IL-10. Infection with *L. major* in animal models resembles self-limiting CL in humans (Scott et al., 1998). Upon infection with *L. major*, mice of the resistant phenotype clearly develop a dominant Th1 phenotype of immune response to the parasite antigens. By contrast, susceptible BALB/c mice develop a typical Th2 response. Targeted disruption of the IFN-γ gene in resistant C57BL/6 mice causes these animals to become highly susceptible to *L. major* (Wang et al., 1994). However, the identification of the precise role of type 2 responses remains more elusive, and characterization of Th2 cytokine

functions is an area of continuing speculation and investigation. Studies on the role of IL-4, has provided some contradictory observations (Alexander et al., 1999). The apparent resolution of infection with *L. major* in BALB/c mice treated at the time of infection with an anti-IL-4 monoclonal antibody (Sadick et al., 1990; Chatelain et al., 1992) or in IL-4-deficient BALB/c mice (Kopf et al., 1996; Mohrs et al., 1999) helped to establish the view that early production of IL-4 drives the polarized Th2 response that is responsible for suppressing Th1-cell development and inhibiting the high-level secretion of IFN-γ. Early IL-4 response is shown to be confined largely to an oligoclonal population of $CD4^+$ T cells with a Vβ4Vα8 T-cell receptor (TCR) that recognize the *Leishmania* antigen LACK (*Leishmania* homologue of receptors for activated C kinase) (Launois et al., 1997). It has been proposed that LACK-specific Vβ4Vα8 $CD4^+$ T cells represent a unique lineage in BALB/c mice that are biased to produce IL-4 because their TCR has relatively low affinity for peptide/MHC (Malherbe et al., 2000). Despite the strong evidence for a disease-exacerbating role for IL-4, Scott, (1996) demonstrated that early IL-4 production does not necessarily predict susceptibility to *L. major* infection. In fact, (Noben-Trauth et al., 1996) found that IL-4-deficient BALB/c mice remained susceptible to *L. major* infection. These apparent differences may arise, in part, due to different parasite strains or species being examined or different tissue sites (footpad, ear or base of the tail) being infected. Also redundancy in IL-4 function via the compensatory activity of other cytokines such as IL-13, which shares many of the properties of IL-4, may obscure otherwise significant activity. Most importantly, early IL-4 production by LACK-reactive $CD4^+$ T cells has been observed in both susceptible and resistant *L. major*-infected mice (Reiner et al., 1993; Julia et al., 1999; Stetson et al., 2002), Resistant mice can also display an early, albeit transient, IL-4 response following *L. major* infection (Scott et al., 1996). So the critical defect in BALB/c mice seems to be in their inability to redirect the early IL-4 response along a normal Th1 differentiation pathway during infection. This indicates that the ability to redirect early Th2 response is the more probable determinant of resistance in the mouse model (Sacks and Noben-Trauth, 2002). A predominant Th2 response is not the underlying determinant of susceptibility in these animals.

The fact that IL-4$^{-/-}$ deficient mice developed progressive disease following infection with *L. major*, suggests that although IL-4 and IL-13 biological activity is lacking, these mice could be, under some circumstances, fully susceptible (Noben-Trauth et al., 1999). Consequently, it is possible that mechanisms or cytokines other than IL-4 and IL-13 can promote susceptibility to infection. The fact that the lesion size and number of parasites are reduced in these mice treated with anti-IL-10 antibodies (Ab) (Noben-Trauth et al., 2003) indicate that IL-10 could also play a role in susceptibility to infection with *L. major* (Sacks et al., 2004). IL-10$^{-/-}$ deficient BALB/c mice were more resistant to *L. major* than wild type mice (Kane and Mosser, 2001). Interestingly, it has been shown that IL-10 released by CD4^{+} T cells is also implicated in the persistence of *Leishmania* in healed lesions of resistant C57BL/6 mice since complete elimination of parasites occurred in these mice only when the IL-10 signalling pathway was abrogated with anti-IL-10R Ab (Belkaid et al., 2001). So the principal function of IL-10 is to limit the magnitude of an immune response, and it does so by inhibiting APC function, blocking cytokine and chemokine secretion, and inhibiting the induction of activities initiated by other cytokines, notably macrophage activation by IFN-γ (Moore et al., 2001). These clinical and experimental studies indicate that unfavourable infection outcomes are not related to Th2 dominance or a Th1 response defect per se but to concomitant expression of IL-10 that renders macrophages poorly responsive to activation signals.

The Th1/Th2 paradigm is important in determining disease outcome in murine CL. However, this dichotomy is not as influential during murine *L. donovani* and *L. chagasi* visceral disease, in which curative type 1 response is instead suppressed by IL-10 and transforming growth factor-β (TGF-β) without Th2 cytokine production (Wilson et al., 2005). *Leishmania chagasi* directly affects its local environment by activating latent TGF-β, and both *L. donovani* and *L. chagasi* suppress host macrophage responses to IFN-γ (McMahon-Pratt and Alexander, 2004). Protective immunity against *L. donovani* is dependent on IL-12-driven type 1 response and IFN-γ production. A disease-promoting role for IL-4 and the Th2 response in VL, however, is more difficult to identify. For example, the differential production of Th1 and Th2 cytokines does not control the rate of cure of

murine VL (Bogdan et al., 1993). No constant association between Th1 responses and resistance to disease with predominance of cells that produce IFN-γ has been identified in human VL (Khalil et al., 2005). The levels of IFN-γ and IL-4 are elevated during active disease and decline significantly after cure. The lack of IFN-γ production by peripheral blood mononuclear cells (PBMC) seems to predict progression of the infection into fulminant VL (Carvalho et al., 1985, 1989; Bacellar et al., 1991). However, it is likely that several mechanisms may participate in the inability of lymphocytes from VL patients to produce IFN-γ, one important point is related to the imbalance of cytokines produced in response to *Leishmania* antigens, i.e. high production of IL-4 and IL-10 and low IL-2 and IFN-γ production (Khalil et al., 2005). Although lymphocytes from patients with VL have a strong expression of mRNA for IL-4 and sera have high IL-4 levels, there is no evidence that IL-4 is involved in the down-regulation of the Th1 type of response in human leishmaniasis (Alexander et al., 2000; Wilson et al., 2005). It has been shown that *in vitro* addition of mAb against IL-4 did not restore the lymphocyte proliferative response or IFN-γ production in *L. chagasi*-stimulated PBMC from VL patients. IL-4 also did not suppress lymphocyte proliferative response or IFN-γ production in subjects cured of leishmaniasis (Ribeiro-de-Jesus et al., 1998). Thus, the prominent role of IL-4 as the leading Th2 cytokine in murine CL was not consistently seen in human VL.

Initially IL-10 was categorized as a Th2 cytokine (Hsieh et al., 1992) but with the newly discovered suppressor population of T cells, its Th2 candidature has been diluted out. Patients with VL have increased expression of mRNA for IL-10 in bone marrow and lymph node cells and high levels of IL-10 in *L. chagasi*-stimulated PBMC supernatants (D'Oliveira et al., 1997). The fact that IL-10 abrogates the effect of IL-12 in inducing IFN-γ production in *L. chagasi*-stimulated PBMCs of VL patients strongly suggests that IL-10 is a major immunosuppressive cytokine which is directly involved in the progression of *Leishmania* infection to visceral disease (Bacellar et al., 1996). Moreover, the addition of monoclonal antibodies to anti-IL-10 restores the lymphocyte proliferative response and IFN-γ production in PBMC from VL patients (Hailu et al., 2005).

Individuals with acute visceral leishmaniasis caused by *L. donovani* simultaneously express IL-10 and IFN-γ transcripts, and IL-10 decreases after resolution of disease (Ghalib et al., 1993; Karp et al., 1993).

In recent studies, (Anderson et al., 2007; Jankovic et al., 2007), it was demonstrated that infection-driven IFN-γ-producing Th1 cells are a crucial source of IL-10. IL-10 production by antigen-specific Th1 cells was necessary and sufficient to suppress immunity to a non-healing form of *L. major* in conventionally resistant mice, and, in *Toxoplasma gondii*-infected mice, the cells were required as a protective feedback mechanism to prevent lethal inflammation. The production of IL-10 by antigen-specific Th1 cells is a normal stage in their differentiation programme which is reached after full activation. Because splenic $CD25^-$ T cells from VL patients have elevated expression of both IFN-γ and IL-10, it is possible that at least some are IL-10–IFN-γ double producers, activated in a strong inflammatory setting as a mechanism of feedback control (O' Garra et al., 2007).

7.2. Immunosuppression

Many pathogens have evolved mechanisms to manipulate the immunoregulatory network of the host to their advantage, thereby generating conditions that ensure their survival for an extended period of time. A common strategy used by microorganisms to extend their survival involves the induction of regulatory responses that are normally associated with the termination of effector immune responses of the host. Although it has long been recognized that T cells with suppressive or anergic activity are generated *in vivo* during infection (Mahanty et al., 1996), it has only recently emerged that specialized subsets of regulatory T cells (Treg) also contribute to this regulatory network.

Several types of regulatory T cells have been described (Shevach et al., 2006) on the basis of their origin, generation and mechanism of action, with two main subsets identified: naturally occurring $CD4^+CD25^+$ regulatory T cells (referred to here as natural Treg or nTreg cells), which mainly develop in the thymus and regulate self-reactive T cells in the periphery, and inducible regulatory T cells (iTreg cells), which develop in the periphery from conventional $CD4^+$ T cells after exposure to signals such as regulatory cytokines, immunosuppressive drugs or antigen-presenting cells (APCs) conditioned by microbial products (O'Garra et al., 2004). According to Belkaid, (2007), nTreg are those cells that belong to the population of regulatory T cells that is present in the host before pathogen exposure, and iTreg as those that acquire regulatory function in the context of a given infection. Inducible regulatory T-cell populations include T regulatory 1 (Tr1) cells, which secrete IL-10 and T helper 3 (Th3) cells, which secrete TGF-β.

7.2.1. Naturally occurring Treg

Naturally occurring $CD4^+CD25^+$ regulatory T cells, that represent 5–10% of peripheral $CD4^+$ T cells in naïve mice, constitutively express surface CD25, the α-subunit of the IL-2 receptor, before activation **(**Sakaguchi et al., 1995) These cells also express cytotoxic T-lymphocyte antigen 4 (CTLA-4) (Read et al., 2000), glucocorticoid-induced tumor-necrosis factor receptor family-related gene (GITR) (Shimizu et al., 2002), OX40 (McHugh et al., 2002), CD39 and CD73 (Deaglio et al., 2007) and high levels of folate receptor 4 (FR4) (Yamaguchi et al., 2007). However, none of these markers are specific for nTreg cells, as they can also be expressed by activated T cells. The recent discovery of the expression of the X chromosome–encoded forkhead helix-winged transcription factor family member, forkhead box P3 (FOXP3) (Brunkow et al., 2001; Hori et al., 2002; Fontenot et al., 2003, Khattri et al., 2003), that is known to be crucial for the development and function of nTreg, is the most definitive signature of nTreg cells in mice (Shevach et al., 2006). However, its

expression in humans can also be transiently upregulated by activated non-Treg cells (Gavin et al., 2006).

Foxp3 expression can be induced *de novo* by conventional $CD4^+$ T cells which only renders the distinction between nTreg and iTreg cells less obvious. Very recent studies have given emphasis on epigenetic regulation of permanent expression of Foxp3 in truly committed Treg (Floess et al., 2007). Activated conventional $CD4^+$ T cells and TGF-β-treated cells displayed no Foxp3 DNA demethylation despite expression of Foxp3, whereas subsets of stable Treg, even upon extended *in vitro* expansion, remained demethylated (Baron et al., 2000). The unique Foxp3 promoter methylation profile in Treg suggests that a demethylated pattern is a prerequisite for stable Foxp3 expression and suppressive phenotype (Janson et al., 2008).

Treg cells require activation via T cell receptor (TCR) to exert regulatory function and their suppressive activity is related to their ability to inhibit IL-2 production via a mechanism requiring direct cell-contact (Thornton and Shevach, 2000). nTreg form long-lasting interactions with dendritic cells (DC) and impair their ability to subsequently activate effector T cells. Thus nTreg cells may inhibit T-cell responses indirectly by modulating the function of APC (Tang et al., 2006). CTLA-4-expressing nTreg cells induce the expression by APCs of the enzyme indoleamine 2,3-dioxygenase (IDO), which degrades tryptophan, and lack of this essential amino acid has been shown to inhibit T-cell activation and promote T-cell apoptosis (Fallarino et al., 2003). Controversy still exists regarding the molecular mechanisms underlying Treg *in vivo* and *in vitro* function, although several studies have suggested the involvement of TGF-β1 (Nakamura et al., 2004) and IL-10 (Asseman et al., 1999; Annacker et al., 2001) in Treg suppressive activity *in vivo*. More recently, adenosine and cyclic AMP have also been shown to contribute to Treg-cell suppressive activity (Bopp et al., 2007; Deaglio et al., 2007). However, the mechanisms of suppression by Treg cells are still largely unclear.

The main function of nTreg is to preserve immune homeostasis in the host by controlling excessive immune responses and the extent of immune-mediated pathology. This is of particular importance when maintaining self-tolerance and minimizing immunopathology normally associated to the onset of autoimmune diseases (Sakaguchi et al., 1995). One consequence of such control during infectious diseases is enhanced pathogen survival. Several persistent pathogens are known to establish chronic infections by engaging $CD4^+CD25^+$ regulatory T cells and taking advantage of their immunosuppressive function (Belkaid et al., 2002; McKee and Pearce, 2004; Campanelli et al., 2006; Vigário et al., 2007). In *L. major* infection of genetically resistant mouse strain, nTreg cells favour parasite survival and expansion. These cells accumulate at sites of infection and locally control the expression of Th1 effector mechanisms through IL-10-dependent and IL-10 independent mechanisms, and this prevents efficient elimination of the parasite (Belkaid et al., 2002; Yurchenko et al., 2006). In this model of infection, parasite persistence, as a result of immune suppression by nTreg cells, is necessary for the maintenance of protective immunity against the parasite. However, when the regulatory control is too excessive, the pathogen replicates without restraint and may compromise the survival of the host. For example, in humans infected with *Plasmodium falciparum*, a causative agent of human malaria, the removal of nTreg cells enhances *in vitro* proliferation of PBMCs and their production of IFN-γ in response to malaria antigens (Walther et al., 2005). In *L. major* infection of susceptible mice, nTreg were shown to suppress both Th1 and Th2 cells (Aseffa et al., 2002; Xu et al., 2003) by both IL-10/TGF-β – dependent and independent mechanisms (Liu et al., 2003). Also a role for Treg cells in restraining pathogenic responses during non-healing *L. amanzonensis* infection was observed (Ji et al., 2002).

Evidence from infection with different protozoan parasites suggests that nTreg cells recognize microbial antigens (Belkaid et al., 2002, Hesse et al., 2004, Hisaeda et al., 2005). In murine leishmaniasis, nTreg cells that accumulate at the site of infection can recognize parasite-derived antigens (Suffia et al., 2006). In addition, far from being anergic, as *in vitro* experiments had suggested (Thorthon et al., 2000), nTreg cells proliferate vigorously when they encounter their

cognate microbial antigens (Suffia et al., 2006). Notably, these cells are restricted to the site of infection and depend on antigen for their maintenance. Such compartmentalization provides a potential explanation to the concept of concomitant immunity, in which the host is immune to re-infection at a secondary site while maintaining a local chronic infection (Mendez et al., 2004).

One mechanism by which microorganisms might manipulate regulatory T-cell function is by creating an environment that favours the retention of regulatory cells at sites of infection. Integrin αEβ7 (also known as CD103 or ITGAE), the expression of which is positively modulated by TGF-β, has been shown to favour natural Treg-cell retention at sites infected by the parasite *L. major* (Suffia et al., 2005). In the same model of infection, CC-chemokine receptor 5 (CCR5) expression by nTreg cells was shown to be required for their migration to the infected sites (Yurchenko et al., 2006).

7.2.2. Induced Treg - Tr1/ IL-10 producing Treg

The mechanisms underlying the failure to control the growth and systemic spread of *Leishmania* parasites in human and murine visceral leishmaniasis are not well understood. Although the absence of antigen-specific Th1 response in the PBMC from VL patients is thought to be causally related to disease progression, the finding that these patients also express elevated IFN-γ mRNA in lesional tissue, as well as elevated serum levels of proinflammatory cytokines, suggests that their immunological defect cannot be explained simply by immune tolerance or Th2 polarization. As a possible homeostatic mechanism to control persistent infection-induced inflammation, elevated levels of the regulatory cytokine IL-10 have been reported repeatedly in clinical studies of VL. Elevated production of IL-10 is frequently found in human VL (*L. donovani*) (Caldas et al., 2005). Based on experimental models, IL-10-deficient or anti-IL10 receptor treated mice display enhanced resistance and leishmanicidal activity against *L. donovani* (Murphy et al., 2001; Murray et al., 2005). However, the extensive data generated fail to support a major role for nTreg in human VL.

It cannot be out ruled that $CD4^+CD25^+Foxp3^+$ Treg contribute to immune suppression during the earliest stages of infection (Nylén et al., 2007), however, extensive analyses of blood and splenic aspirates from Indian VL patients still do not support a major role for nTreg during overt disease. nTreg were not elevated in the blood and did not accumulate at the site of infection (spleen) in active VL cases; no changes in frequencies were observed with treatment. Moreover, antigen-specific IFN-γ responses were not rescued by depleting the PBMCs of $CD25^+$ cells (Nylén et al., 2007a). In contrast, a more recent study, demonstrated a probable immunosuppressive role for $CD4^+CD25^+$ regulatory T cells, during active VL, and post-treatment with amphotericin B, during reactivation in PKDL patients (Saha et al., 2007).

Recent studies now point out to the existence of an important source of IL-10-producing T cell subset, $CD4^+CD25^-Foxp3^-$ T cells, which seem to be the main cells involved in IL-10-mediated immune suppression in human (Nylén et al., 2007) and in murine VL (*L. donovani*) (Stäger et al., 2006). Treg cells that develop in peripheral lymphoid tissues, which are frequently $FOXP3^-$ have been termed adaptive or induced Treg (iTreg). Within this particular category of Treg, there are the so called antigen-specific $CD4^+$ T cells or Tr1 cells, known to produce large amounts of IL-10. Tr1 are involved in the down-regulation of immune responses via IL-10 and suppressing naïve and memory Th1 or 2 responses (Mills and McGuirck, 2004). High levels of splenic IL-10 mRNA was found primarily in the $CD25^-$FoxP3 $mRNA^{low}$ T cells, which accumulate in the spleen of VL patients. Thus, parasite-driven adaptive regulatory/Tr1 cells (Roncarolo et al., 2001) appear to be more important than nTreg cells in the suppression of anti-*Leishmania* immunity in VL.

7.2.3. Th3 immune response

Th3 cells are a regulatory T-cell subset, that was originally thought to be involved in oral tolerance, and that primarily secretes TGF-β (Chen et al., 1994; Fakaura et al., 1996). Because TGF-β1 knock-out (KO) mice exhibited severe multi-organ inflammations, TGF-β1 has been thought to be

an important immune regulatory cytokine. This cytokine provides help for IgA induction and has suppressive properties for both Th1 and Th2 cells. TGF-β1 was suggested to be involved in the regulatory function of $CD4^+CD25^+$ regulatory T cells (Annunziato et al., 2002; O'Garra et al., 2004), though the molecular mechanism of TGF-β1 induction in such regulatory-type T cells remains to be elucidated. As the expression of CD25 and *Foxp3* is induced in T cells under experimental conditions (in the presence of IL-4, IL-10 and TGF-β), Th3 cells are also referred as iTreg that are generated from naïve T cells and that expand upon encounter with antigen (McGuirck and Mills, 2002). Th3 cells can be distinguished from Th2 cells by cytokine profiles; Th3 cells produce much more TGF-β1 and IL-10 and less IL-4 than Th2 cells (Chen et al., 1994; Seder et al., 1998). Suppressor of cytokine signalling (SOCS3) - deficient $CD4^+$ T cells infected with *L. major* were shown to produce more TGF-β1 and IL-10 and are thought to be more likely to exhibit a Th3-like phenotype (Kinjyo et al., 2006).

7.2.4. Th17 response

Another subset of $CD4^+$T cells that produce IL-17, Th17 cells, and to a lesser extent, TNF and IL-6, has been identified as a highly pro-inflammatory lineage and induces severe autoimmunity (Cua et al., 2003; Langrish et al., 2005). Whereas IL-23 serves to expand previously differentiated Th17 cell populations (Langrish et al., 2005), IL-6 and TGF-β induce the differentiation of Th17 cells from naive precursors (Veldhoen et al., 2006; Mangan et al., 2006). It has pleiotropic activities, one of which is to coordinate tissue inflammation by inducing the expression of pro-inflammatory cytokines (such as IL-6 and TNF), chemokines (such as KC, MCP-1 and MIP-2) and matrix metalloproteases, which mediate tissue infiltration and tissue destruction (Kolls et al., 2004). IL-17 is also involved in the proliferation, maturation and chemotaxis of neutrophils (Fossiez et al., 1996). Of particular interest is recent data suggesting a dichotomy between $Foxp3^+$Treg cells and Th17 cells. T cells exposed to TGF-β upregulate *Foxp3* and become iTreg cells; however, when cultured with TGF-β and IL-6, naïve T cells generate Th17 cells with highly pathogenic activities

(Betelli et al., 2006). Therefore, during the steady state, when the immune system is not activated, TGF-β favours the generation of iTreg cells, which suppress inflammation and prevent the development of autoimmunity. However, after infection, IL-6 produced by the innate system inhibits the generation of iTreg and, together with TGF-β, induces the differentiation of Th17 cells. These results suggest that Th17 and iTreg cells may arise from the same precursor in distinct cytokine milieus.

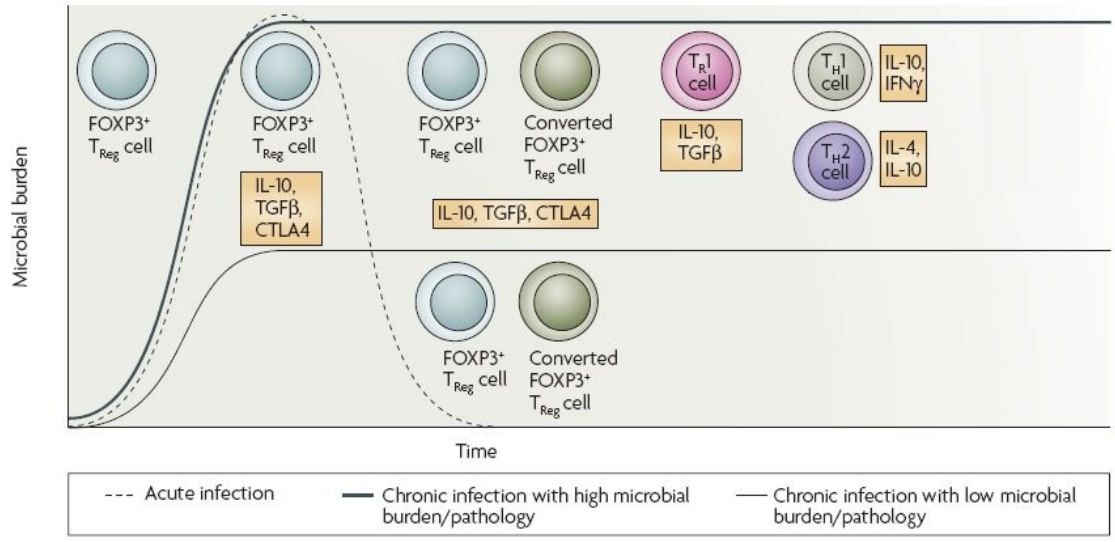


Figure 5. Summary of the nature of regulatory T cells and the mechanism of suppression that depends on the strength and stage of the pathological process. During an acute infection, polyclonal natural Treg cells may contribute to the control of the inflammatory process. During chronic infections with sustained Th1 or Th2 cell responses, several regulatory processes may contribute to immune regulation. Natural Treg cells, expressing CTLA-4, could produce cytokines, such as IL-10 or TGF-β, to prevent tissue damage; those that accumulate at the site of infection may be enriched in pathogen-specific Treg cells. Tr1 cells can be induced because of the effect (deactivation and induction of cytokine production) of pathogens on APCs or chronic exposure to microbial antigen. $FOXP3^-$ T cells could be converted into $FOXP3^+$ regulatory T cells at sites enriched in TGF-β, such as the skin or the gut. During infections that induce a sustained Th1-cell response, Th1 cells themselves may also contribute to the limitation of immune responses and tissue damage through the release of IL-10 (adapted from Belkaid et al., 2007).

IL-6 and probably other pro-inflammatory cytokines may act as 'pivots', determining whether an immune response is dominated by protective Treg cells or highly pro-inflammatory Th17 effector T cells (Betelli et al., 2007). Currently, there is limited information on the role of IL-17 in *Leishmania* infection. Given the chronic inflammation and neutrophil/macrophage migration during *Leishmania* infection, it is logical to speculate that IL-17 may play a role in leishmaniasis. Xin, (2007) observed that *L. amazonensis*-infected DCs preferentially induce $CD4^+$ T cells with an IFN-γ^{low} IL-10^{high} IL-17^{high} phenotype although the specific condition that induces IL-17 expression remained unclear.

7.3. Immunoregulation

Pathogens can favour regulatory T cell function. Because nTreg cells generate favourable conditions for the persistence of microorganisms, it is conceivable that the induction, maintenance and function of natural Treg cells could also be manipulated by microorganisms. In addition to the TCR recognition of specific antigens, nTreg cells can also respond to microbial products independent of TCR signals. New evidence has demonstrated that Treg cells can also sense pathogens directly through Toll-like receptors (TLR) and, consequently, modify their behaviour (Sutmuller et al., 2006). TLR signalling may directly or indirectly regulate the immunosuppressive function of $CD4^+CD25^+$ Treg cells in immune responses and shift the balance between $CD4^+$ Th cells (effector T cells) and Treg cells, and subsequently influence the outcome of the immune response (Liu and Zhao, 2007).

The family of TLRs is a major class of receptors (Lemaitre et al., 1996; Medzhitov et al., 1997) that recognize pathogen-associated molecular patterns (PAMPs). These molecules, found in bacteria, viruses, fungi and protozoa, present highly conserved molecular structures. Signalling pathways activated by TLRs result either in the activation of the transcription nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), with subsequent production of pro-inflammatory

cytokines, chemokines, antimicrobial peptides and additional defense molecules, or in the activation of mitogen-activated protein kinases p38 and JNK (Kawai et al., 2006). Between the activation of TLR and the release of NF-κB, there is an important step that is mediated by adaptor molecules. MyD88 is the most common adaptor molecule for the activation of NF-κB and is present in most TLR.

The first study evaluating TLRs and the MyD88 dependent pathway in *Leishmania* infection was undertaken by Hawn, (2002). This study evaluated experimental *L. major* infection and cytokine expression from macrophages in MyD88$^{-/-}$ deficient mice. MyD88$^{-/-}$ C57BL/6 mice were highly susceptible to *L. major* and developed non-protective Th2 response (Debus et al., 2003; Muraille et al., 2003). Other studies showed that the activation of NF-κB in macrophage cell lines by the parasite derived molecule LPG was mediated by TLR-2 (de Veer et al., 2003). Becker, (2003) had similar results *in vitro* using NK cells, suggesting that three molecules of LPG aggregate with one molecule of TLR-2. In VL, NK cells are associated with a good prognosis and TLR9 was also shown to be required for their activation as it is essential for the production of IL-12 by DCs (Schleicher et al., 2007). Ribeiro-Gomes, (2007) recently determined the role of neutrophils elastase. This enzyme produced by neutrophils induces the leishmanicidal activity of macrophages through TLR4 activation.

It is commonly accepted that TLR-mediated recognition of specific structures of invading pathogens initiates innate as well as adaptive immune responses via DCs or other APCs. However, emerging evidence of TLR expression on T cells (Kabelitz et al., 2006), including nTreg cells (Caramalho et al., 2003) suggests the potential involvement of TLR signalling in adaptive immune regulation. Consistent with a direct role for TLRs, human nTreg cells express TLR-5 at levels that are comparable to APCs, and co-stimulation with the TLR-5 ligand flagellin increases their suppressive capacity and enhances their expression of Foxp3 (Crellin et al., 2005). This feature could offer certain pathogens an opportunity to enhance immunosuppression. Although some ligand interactions with TLRs have been proposed to increase nTreg-cell suppressive capacity,

others have been shown to limit their function (Sutmuller et al., 2006). For example, TLR2 signalling temporarily abrogates the suppressive phenotype of nTreg cells and decreases their Foxp3 expression (Sutmuller et al., 2006a), Following removal of the TLR-2 trigger, the *in vitro*-expanded Treg cells fully regained their phenotype and suppressive capabilities. Thus, TLR ligands have the capacity to act directly on Treg cells through TLR and, ultimately, modulate the immune response (Liu et al., 2006).

Recently, Peng, (2005) have shown that human Treg cells express high levels of TLR-8, and that the TLR-8 triggering of Treg cells also prevents their suppressive phenotype. It is not known exactly how the TLR triggering of Treg cells modulates their suppressive effects, and whether the triggering of different TLRs occurs through the same pathway or has the same effect on Treg-cell function (Sutmuller et al., 2006). One explanation could be the up-regulation or down-regulation of Foxp3 expression following the different TLR stimulations (Crellin et al., 2005; Liu et al., 2006), but how TLR-signalling affects Foxp3 expression is still unclear. Experimental data support the idea that TLR-2 signalling on murine Treg cells might function as a strong co-stimulatory trigger. This would force Treg cells into the proliferative pathway, which might be paralleled by the reversal of their suppressive capabilities (Sutmuller et al., 2006a). It is tempting to hypothesize that *Leishmania*-derived TLR-2 ligands could help to expand Treg cells and abrogate suppression during the acute phase of the infection. During late chronic phases of infection, accompanied by decreases in the presence of TLR2 ligands, Treg-cell suppressive activity would be expected to increase again in order to prevent immune-mediated pathology and enable long-term parasite persistence.

CHAPTER II. MATERIALS AND METHODS

1. Phenotypic characterization of regulatory T cell populations during *L. infantum in vivo* infection

The first part of the study involved direct identification of typical regulatory T cell populations through the use and detection of several known cell surface molecules. To confirm their presence and behaviour within the host infected with *L. infantum* parasites, host cells were isolated, phenotypically characterized and followed-up in terms of cell dynamics and cytokine response during the course of *in vivo* infection.

1.1. Experimental design

Treg cell frequency and phenotype was studied in pooled lymph node (LN) and spleen (SP) cells isolated from susceptible mice infected with *L. infantum*. Samples were collected from lymph nodes and spleen for isolation of total leukocytes, single-cell separation, cytokine *in vitro* assays and gene expression as well as parasite detection in spleen and liver (LIV), the main target organs of *L. infantum*. The experiments were repeated three times.

1.2. Animals and parasites

1.2.1. Mice

Six- to eight-week-old male BALB/c mice (*Mus musculus*), specific pathogen-free (SPF), were purchased from the Instituto Gulbenkian de Ciência and maintained at Instituto de Higiene e Medicina Tropical (IHMT) animal facilities. Standard commercial feed (Harlan Ibérica, Spain) and water were provided *ad libitum.* Upon arrival the mice were divided into two groups, control and infected. Animals were infected via intraperitoneal (i.p.) route with a parasite suspension of

virulent *L. infantum* promastigotes. Five to seven animals were sacrificed for each studied time point. The experiments were conducted according to EU requirements (86/609/CEE), recognized by the Portuguese law (DR DL129/92 and Portaria 1005/92).

1.2.2. Parasites

The *L. infantum* MON-1 (MHOM/PT/89/IMT151) strain was maintained by regular passage through BALB/c mice and amastigotes were isolated from infected spleens. After *in vitro* transformation, virulent promastigotes were collected from spleen homogenates after two weeks of culture at +24ºC in a solid phase composed of NNN (Novy, McNeal, Nicolle) growth medium (Santos-Gomes and Abranches, 1996) plus Schneider's insect liquid medium (Sigma, USA), with L-glutamine, 100 $U.ml^{-1}$ penicillin, 100 $\mu g.ml^{-1}$ streptomycin and supplemented with 10% foetal calf serum (FCS) (Invitrogen, USA), previously inactivated for 30'at +56ºC.

In order to obtain a high number of promastigotes, primary isolated parasites were expanded in Schneider 10% FCS. Stationary phase parasites with less than five subculture passages were used for mice inoculation. Promastigotes were harvested by centrifugation (1800 *g*, 15', 4ºC) and washed once with phosphate buffered saline (PBS) (Cambrex, USA). Parasites were subsequently counted in Neubauer hemocytometer and resuspended in the appropriate volume of PBS. Infection was performed i.p with $5x10^6$ promastigotes in a final volume of 100 μl per mouse.

1.2.3. Antigen preparation

Promastigote lysate for use in cell stimulation assays was prepared from stationary phase promastigotes. Parasites were harvested by centrifugation (1800 *g*, 15', +4ºC) and washed twice with isotonic PBS / 2 mM EDTA (Sigma). The supernatant was discarded and pellet resuspended

in 500 ml of distilled water. The promastigotes were disrupted by repeated freeze thawing cycles [-70ºC and room temperature]. Cell debris was spun down (15 000 *g*, 15' +4ºC) and the protein content of the supernatant was determined by spectrophotometry (Gene Quant II; Pharmacia Biotek, Sweden). The lysate was stored in aliquots at -70ºC until use.

1.3. Sample collection

Animals were sacrificed by cervical dislocation at days 2, 7, 14, 28 and 56 days post-infection (pi). The organs were aseptically dissected from animals and maintained at +4ºC in RPMI 1640 (Gibco-Brl, USA) containing 2 mM L-glutamine, 2 mM sodium pyruvate, 25 mM Hepes, $5x10^{-2}$ mM β-mercaptoethanol, 100 $U.ml^{-1}$ penicillin, 100 $\mu g.ml^{-1}$ streptomycin and 10% FCS. Cervical superficial, illiary, axillary, brachial, inguinal, superior mesenteric and popliteal LN were extracted together with whole spleen and liver. Individual portions of the spleen and liver of each animal was separated and maintained in Schneider's 10% FCS for parasite detection. LN and the remaining spleen samples were processed and pooled according to infected and non-infected animal group in order to further achieve optimal lymphocyte concentration for use in follow-up immunological assays.

1.4. Parasite detection

Parasite burden in the spleen and liver was determined by limiting dilution assay (LDA) (Rodrigues et al., 2009). This method estimates the number of viable parasites within an infected organ by using geometrical dilutions of homogenized organ and detecting live viable promastigotes through light microscopy. After, aseptic collection, spleen and liver were weighed and homogenized individually in a known volume of Schneider 10% FCS (1.5 ml and 3.5 ml, respectively). The homogenized organ suspensions were diluted 1:2 for spleen and 1:30 for liver, and an initial

suspension volume of 200 µl was placed into the first four wells (quadruplicates of each sample) and four-fold serial dilutions (50 µl suspension + 150 µl medium) of the infected tissues were distributed in 96-multiwell plates (Nunc, Germany). After 10-15 days at +24ºC, a sample of each well was examined by light microscopy and defined as positive or negative depending on the presence or absence of promastigotes in the well. The final titre was set as the highest dilution for which the well contained at least one parasite and the number of parasites per gram of homogenized organ was calculated as follows: (Reciprocal titer of the last positive well per total volume of homogenized organ x dilution factor) divided by the weight (gram) of the homogenized organ. The viable parasitic load (VPL) was expressed as the number of *Leishmania* per gram of homogenized organ:

$$VPL = \frac{(4)^{\text{last positive titre}} \times [\text{volume of homogenized organ} / 0.2\ (\text{ml})] \times \text{dilution factor}}{\text{weight of homogenized organ (g)}}$$

1.5. Phenotypic characterization of regulatory T cells

The identification of regulatory T cell subsets and the analysis of cell dynamics during *L. infantum* infection were carried out using a technology called flow cytometry. Immunophenotyping is one of the largest clinical and research applications of flow cytometry. Usually, immune cells such as lymphocytes are the main cells under study where the primary measurements are of fluorochrom-conjugated antibodies bound to cellular receptors. A complex system of receptor identification has been developed within immunology using specific monoclonal antibodies (Ab) able to recognize surface cell markers, which are referred to as Cluster of Differentiation (CD) antigens of which at the time of writing and for human cells there are 320 such classifications. By conjugating fluorescent molecules to antibodies that recognize specific receptors, a population of cells binding that antibody and therefore that fluorescent molecule can be identified and level of expression

quantified by flow cytometry.

1.5.1. Isolation of total leukocytes

After collection, LN were placed in ice-cold RPMI 10% FCS. Fatty tissues in suspension were discarded and LN transferred to cold medium and gently macerated with a syringe knob through a sterilized plastic strainer onto a Petri dish. The released cell suspension was aspirated and gently resuspended several times through sterilized nylon mesh (Cadisch, UK). The suspension was washed three times, by centrifuging (10', 300 *g*, +4ºC) until no cloudy supernatant (fatty aggregates) were visible and resuspended in RPMI 10% FCS for cell separation. Cell numbers and viability were assessed by Trypan blue (Sigma) exclusion.

Spleens were firstly disrupted through a stainless steel sieve in cold Hanks balanced salt solution (HBSS) (Sigma) 10% FCS. After one centrifugation (10', 300 *g,* +4ºC), the pellet was resuspended in incomplete RPMI and gently layered onto Ficoll gradient density solution (Biochrom AG, UK) (2 ml of homogenized organ per 4 ml of separating solution). The mixed suspension was then centrifuged (20', 925 *g*, +20ºC). Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erythrocytes which are aggregated by the Ficoll and, therefore, sediment completely through the Ficoll. The layer immediately above the erythrocyte layer contains mostly granulocytes which at the osmotic pressure of the Ficoll solution attain a density great enough to migrate through it. Because of their lower density, the lymphocytes are found at the interface between the plasma and the Ficoll with other slowly sedimenting particles (platelets and monocytes). The lymphocytes were then recovered from the interface and subjected to three washing steps (5', 300 *g*, +4ºC) with incomplete HBSS to remove any contaminating platelets, plasma proteins and excess Ficoll. Cell numbers and viability were assessed by Trypan blue exclusion.

1.5.2. Flow cytometry - basic concepts

The essential principle of flow cytometry is that single particles or cells suspended within a stream of liquid are interrogated individually in a very short time as they pass at high speed through a light source focused at a very small region defined as the interrogation point. The optical signals generated are mostly spectral bands of light in the visible spectrum, which represent the detection of various chemical or biological components, mostly fluorescence. Flow cytometry is defined by the measurement of multiple physical and chemical characteristics of particles or cells. By analyzing single particles/cells in flow, it is possible to separate them into populations based upon a group of variables that can be measured on each particle/cell. These populations can be separated electronically and identified using multivariate data analysis techniques.

The most common detection system in flow cytometry uses fluorescent molecules that are attached by one means or another to the particle of interest. If the particle is a cell, such as a white blood cell, for example, the fluorescent probe might be membrane bound, cytoplasmic, or attached to nuclear material. Monoclonal or polyclonal antibodies that recognize specific receptors on cells are commonly used for this purpose. By conjugating fluorescent molecules to these antibodies, it is possible to monitor both the location and number of these conjugated antibodies as they bind to cell receptors (Figure 6).

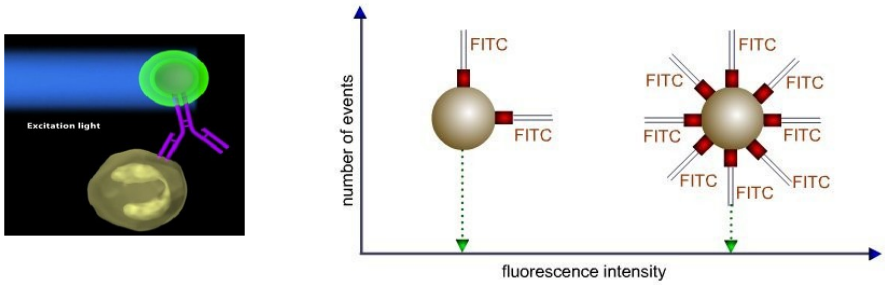


Figure 6. Fluorophore-labeled antibody binding to specific molecule on the cell surface or inside the cell. Intensity of fluorescence detected depends on the number of specific receptors for each fluorophore-labeled antibody (http://www.invitrogen.com/site/us/en/home/support/Tutorials.html).

Basically flow cytometry studies cell properties in terms of light scattering and fluorescence. Forward scatter (FSC), or low-angle light scatter, is the amount of light that is refracted or scattered in the forward direction as the laser light source strikes the cell (Figure 7). The light is then quantified by a detector that converts intensity into voltage and the latter into a numerical value. The magnitude of the voltage pulse recorded for each cell is proportional to the cell size.

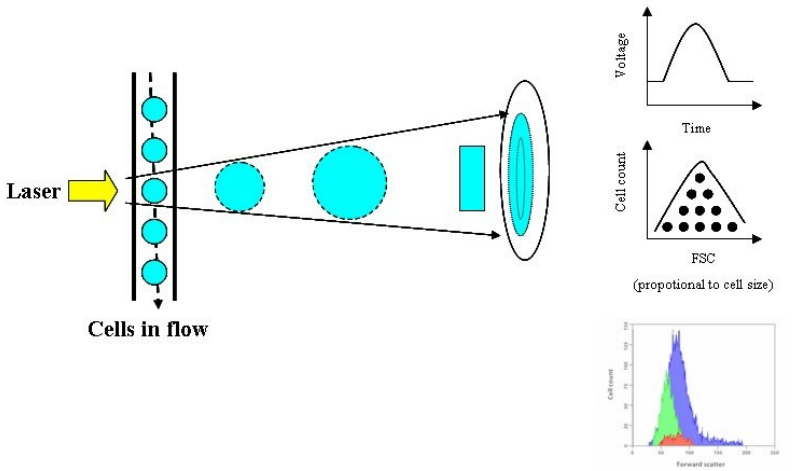


Figure 7. Forward scatter (FSC) of light generated at forward low angle as the cell passes through the laser is proportional to cell size.

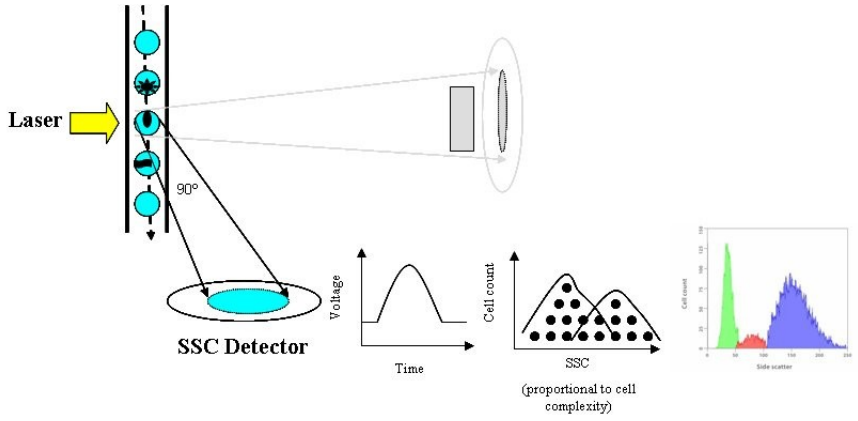


Figure 8. Side scatter (SSC) light generated at large angles as the cell passes through the laser is proportional to granularity and internal structural complexity of the cell.

Light scattering at larger angles for example to the side is caused by granularity and structural complexity inside the cell. This side-scattered light (SSC) is collected at a separate detector, usually located 90º from the laser's path. The signals collected by the FSC and SSC detectors can be plotted individually on a one-dimensional histogram or two-dimensional histogram that's gives information on both cell size and internal cell complexity (Figure 8).

Fluorescence is the third parameter used to study cellular characteristics. When laser light of the right wavelength strikes the fluorophore-labeled antibodies that have been used to mark a cell population, a fluorescent signal is emitted and detected along the same path as the side scatter signal. Fluorescence data is collected in generally the same way as FSC and SSC data. Multiple fluorescence parameters can be used simultaneously in order to be able to detect multiple characteristics within a single cell population. The analysis of fluorescence data in addition to that generated by FSC and SSC, is what is defined in flow cytometry as multiparametric analysis.

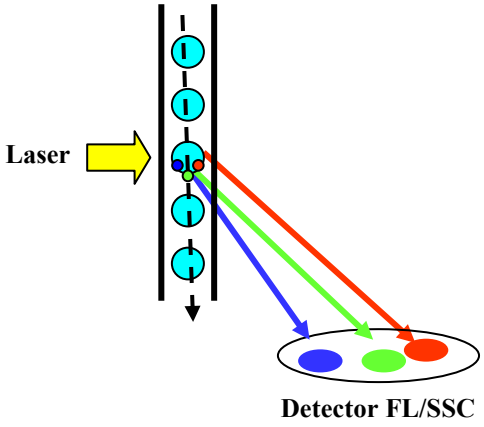


Figure 9. Fluorescence signals (FL) generated as the cells labeled with fluorophore-antibodes passes through the laser.

As mentioned above, data collected can be graphically represented by histograms or dot plots. Histograms present only one-dimensional data and show the number of cells or events according to cell distribution to the parameter of choice. A dot plot presents two-dimensional data where two

parameters can be studied at once in a single cell population. Each dot on the dot plot represents a cell, and each cell has been associated with all of the scatter and fluorescence data collected at the time it passed through the interrogation point. By directing the analysis software to consider only a certain population of interest, a region or gate can be drawn around different populations and selected for restricted analysis, excluding all cells outside the gate (Figure 10).

Data analysis on a two parameter dot plot allows the distinction of four populations. Dividing the dot plot into four quadrants, cells with positive fluorescence for both parameters are double positive and appear in the upper right quadrant. Cells positive for only one of the parameters appear either in the upper left or lower right quadrants and double negative cells in the lower left quadrant (Figure 11).

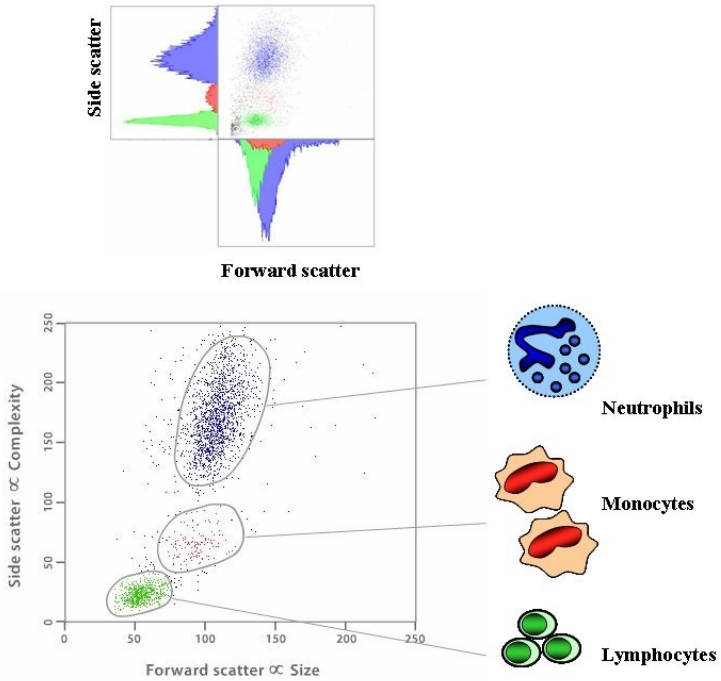


Figure 10. A dot plot representing two-dimensional data, forward and side scatter, is able to distinguish several cells subsets within in a single cell population.

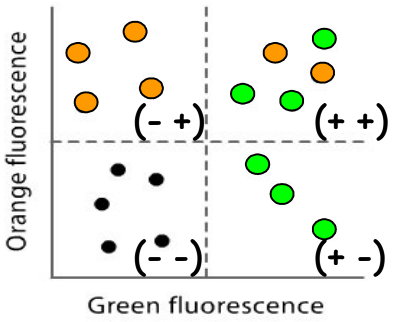


Figure 11. A two parameter dot plot orange and green fluorescences and the distinction of four different sub-populations.

If the fluorescence emission profiles for each different individual fluorophore used in an assay overlap, it becomes very difficult to identify which fluorophore is the real emitter of fluorescence for the cell population of interest. To achieve this, it is necessary to perform spectral compensation whereby a percentage of signal from one detector is subtracted from the other in order to correct for spectral overlap. Depending on the instrument and software used, compensation is set in the instrument hardware before the sample is run or within the software after data collection.

1.5.3. Cell preparation and surface staining

$CD4^+$ T pre-enriched cell fractions were centrifuged (5', 300 *g*, +4ºC) and re-suspended with PBS, 2% FCS, 0.01% NaN_3 at 10^6 cells ml^{-1}. Aliquots of $5x10^5$ cells were incubated in the dark (30', +4ºC) with 20 µl of the following fluorochrom-conjugated anti-mouse Ab: Ab FITC-labeled anti-CD3 (clone 145-2C11), PerCp-labeled anti-CD4 (clone RM4-5), PE-labeled anti-CD25 (clone PC61.5), FITC-labeled anti-CD103 (clone M290), FITC-labeled anti-CD45RB (clone 16A), FITC-labeled anti-GITR/TNFRSF18 (clone 108619), (BD Pharmingen, USA). The antibodies used were titrated for optimal performance and concentration where a set of serial antibody dilutions (1:2) is

used for a constant volume and number of cells. As the cells are stained with increasingly diluted concentrations of antibody, the intensity of fluorescence decreases. The ratio of the mean fluorescence of the positive cells by the negative cells was calculated and defined as the signal-to-noise ratio (S/N) and plotted as a function of the dilution to produce an inverted parabolic shaped curve whose zenith is the ideal titre. For discrete populations this concentration produces the best distinction between positive and negative cells. For heterogeneous populations, the titre is determined as the titre point where saturation has occurred. Optimal dilutions are shown in Table 2.

Table 2. Fluorophore-labeled antibodies used for immunophenotyping of cell populations.

Anti-mouse monoclonal antibody	**Antigen**	**Specificity**	**Final concentration ($\mu g.ml^{-1}$)**	**Dilution (v:v)**
Anti - CD3 - FITC	T-cell receptor-associated CD3 complex	Mature T cells	5	1:100
Anti – CD4 - PerCP	L3T4 differentiation antigen	MHC class II-restricted T cells including Th cells and Treg	0.5	1:400
Anti – CD25 - PE	IL-2 receptor α chain, p55	Early T cell activation	1	1:200
Anti – CD103 - FITC	α chain of αIELβ7 integrin	Peripheral T cell interaction with epithelia	1	1:100
Anti – CD45RB -FITC	Exon B-dependent epitope of CD45 glycoprotein	Activation of naïve cells into memory cells express low levels	1.25	1:400
Anti – GITR - FITC	TNFRSF18 glycoprotein	Activated T, B cells, co-stimulatory receptor on Treg	2.5	1:20

To characterize regulatory T cell populations, various T cell subsets were detected with 3-color combinations of fluorochrom-conjugated Ab directed towards antigens of interest as follows:

- CD4-PerCP / CD25-PE / CD3-FITC

- CD4-PerCP / CD25-PE / CD45RB-FITC

- CD4-PerCP / CD25-PE / CD103-FITC

- CD4-PerCP / CD25-PE / GITR-FITC

Cells were washed with PBS and then fixed (20', +4ºC) with 2% paraformaldehyde in the dark (20', +4ºC), then washed again and resuspended in 200 μl of PBS, 2% FCS, 0.01% NaN_3 and kept in dark at +4ºC until analyzed by flow cytometry. Compensation was set for every different combination of fluorophores used in each assay. Data acquisition was performed on a FACSCalibur flow cytometer and analyzed using BD FACSDiva Software (BD Biosciences, USA).

1.6. Cytokine in vitro assay of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations were isolated by magnetic cell separation and cultured in RPMI 10% FCS. 10^5 cells per 150 μl per well were cultured in U-bottom 96-well plates and incubated (72h, 5% CO_2) under different conditions, 50 μl of: (1) complete medium; (2) equal numbers of antigen-presenting cells (APC); (3) soluble crude *Leishmania* antigen (Ag); (4) APC and Ag (10 $\mu g.ml^{-1}$); (5) APC and soluble anti-CD3ε Ab (1 $\mu g.ml^{-1}$) (BD Biosciences) and (6) APC, anti-CD3ε and Ag, and (7) *L. infantum* infected APC and anti-CD3ε, at a ratio of 1 lymphocyte per 1 APC.

1.6.1. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell isolation

$CD4^+$ T cells were subsequently pre-enriched from pooled lymphocytes from spleen and LN. Total $CD4^+$ T cells were purified by using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Germany). Mouse $CD4^+$ T cells were isolated by depletion of non-$CD4^+$ T cells (negative selection). Non-$CD4^+$ T cells were magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies (Ab), as primary labeling reagent, and anti-biotin monoclonal Ab conjugated to microbeads, as secondary labeling reagent. The magnetically labeled non-$CD4^+$ T cells were depleted by retaining them on the MACS® column (Miltenyi Biotec) in the magnetic field of a MACS Separator

(Miltenyi Biotec), while the unlabeled $CD4^+$ T cells passed through the column.

Briefly, the lymphocyte suspensions were resuspended in ice cold degassed PBS buffer, pH 7.2, containing no Ca^{2+} or Mg^{2+}, supplemented with 0.5% bovine serum albumin (Boehringer Mannheim, Germany) and 2 mM EDTA and passed through pre-separation filters with 30 µm nylon mesh to remove cell aggregates. Cells were centrifuged (10', 300 *g*, +4ºC), the supernatant completely pipetted off and the pellet resuspended in 40 µl buffer per 10^7 total cells. 10 µl of biotin-antibody cocktail was added per 10^7 total cells and left to incubate (25', +4ºC) in the dark. 30 µl of PBS 0.5% BSA, EDTA buffer and 10 ml of anti-biotin microbeads cocktail per 10^7 total cells were then added and cells left to incubate (25', +4ºC). Cells were washed by adding 10-20 X labeling volume of buffer and centrifuged (10', 300 *g*, +4ºC). The pellet was then resuspended in 500 µl buffer per 10^8 cells and passed through the LS^+ column in the magnetic field of the MACS separator. The effluent, the negative unlabeled fraction representing the enriched $CD4^+$ T cells, was collected and then used for flow cytometry analysis and further $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell separation. Cells retained in the column, the magnetically labelled (non-$CD4^+$ T cell) fraction were eluted by removing the column from the magnetic field of the MACS separator. Cell purity which was determined by flow cytometry was > 95% for separated $CD4^+$ T cell populations.

The $CD4^+$ T cell-enriched fraction was centrifuged (10', 300 *g*, +4ºC), the supernatant discarded and the pellet resuspended in 100 µl PBS 0.5% BSA, EDTA buffer per 10^7 total cells. Cells were stained with 20 µl of phycoerythrin (PE) -anti-CD25 Ab (BD Pharmingen) per 10^7 total cells and left to incubate 15', +4ºC on ice and in the dark and then washed with 2 ml buffer per 10^7 total cells and centrifuged (10', 300 *g*, +4ºC). The pellet resuspended in 100 µl buffer per 10^7 total cells. 10 µl of anti-PE-microbeads (Miltenyi Biotec) per 10^7 total cells was added and the cells left to incubate (15', +4ºC). The pellet was then resuspended in 500 µl buffer per 10^8 cells and passed through the MS^+ column in magnetic field of the MACS separator. The effluent, the negative unlabeled fraction representing the enriched $CD4^+CD25^-$ T cells, was collected. Cells retained in the column, the magnetically labelled ($CD4^+CD25^+$ T cell) fraction were eluted. Cell purities which were

determined by flow cytometry were approximately ~ 80% and 96% for $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets, respectively. $CD4^+CD25^+$ and $CD4^+CD25^-$ subsets were then used for *in vitro* cytokine assaying and gene expression analysis.

1.6.2. APC Preparation

APC were obtained from spleen and LN of syngeneic naïve mice. Non-$CD4^+$ T cell fractions, containing CD4 negative T cells and CD4 negative non-T cells including antigen-presenting cells such as dendritic cells, macrophages and B cells (Xu et al., 2003) were obtained through positive selection using the $CD4^+$ T cell isolation kit (see section 1.6.1.). APC were treated with 1 $mg.ml^{-1}$ of mitomycin C (Sigma) and incubated (~120', +37ºC, 5% CO_2). Mitomycin C inhibits DNA synthesis and consequently unable cells to proliferate but they can still function as APC and induce T cell proliferation. APC were then washed three times with RPMI 10% FCS before culture.

1.6.3. Detection of *in vitro* cytokine production

Cytokines were determined in supernatants collected from co-cultures of $CD4^+$ T cells subsets. IFN-γ and IL-4 levels by effector $CD4^+CD25^-$ T cells and IL-10 and TGF-β by regulatory $CD4^+CD25^+$ T cells were determined by enzyme–linked immunosorbant assay (ELISA). The sandwich ELISA or double antibody technique begins with an antibody bound to a solid phase support. An unknown amount of the antigen from a sample to be measured is added to the well. At the same time, serial dilutions of known amounts of purified recombinant antigen are added so as to construct a standard curve. An enzyme conjugate is then added to the well with bound antigen-antibody or immune complexes. A substrate is added to the enzyme conjugate which is bound to the immune complex. In the presence of the enzyme conjugate bound to the immune complex, the substrate is converted into a detectable signal that will show as a positive test or color change.

ELISA assays for all cytokines were performed using ELISA sets (BD Biosciences). Briefly, 100 µl per well of diluted capture monoclonal Ab were added to a 96-well plate and incubated (overnight, +4°C). Unbound Ab was aspirated and the wells washed 3X with PBS 0.05% Tween-20 (Sigma-Aldrich, USA). To avoid non-specific binding, the reaction was blocked with 200 µl per well of assay diluent (PBS, FCS, 10%, pH 7), incubated (1h) and later aspirated and washed once again three times. 100 µl of standard recombinant protein or of the sample to be measured were then added to each well and incubated (2h) and later aspirated and washed five times. 100 µl of working detector, containing the detection biotinylated monoclonal Ab and streptavidin-horseradish peroxidase conjugate (SAv-HRP) was added to each well and left to incubate (60'), aspirated and washed seven times (30''- 1' soaks). The substrate solution (100 µl, tetramethylbenzidine) was added to each well, and the plate was left to incubate (30') in the dark. Finally 50 µl of the stop solution (1 M H_3PO_4) was distributed to each well and the absorbance read at 450 nm within 30' on a microplate ELISA reader (Titer Multiskan Plus MK II; Labsystems, USA). The limit of detection of the recombinant cytokines are listed in Table 3.

Table 3. Concentrations of recombinant proteins used for standard dilutions of each cytokine detected by ELISA.

Cytokine	Limit of Detection ($pg.ml^{-1}$)
IFN–γ	2000 – 31.3
IL-4	500 – 7.8
IL-10	2000 – 31.3
TGF-β	4000 – 62.5

1.7. Gene expression analysis of Treg selective marker – Foxp3

Expression of the transcription factor Foxp3 (forkhead box P3) has been implicated as a key element for $CD4^+CD25^+$ T regulatory cell function in mice (Hori et al., 2003). In order to determine whether *foxp3* gene expression is confined or not to $CD4^+CD25^+$ T cells with a regulatory phenotype during *L. infantum* infection, $CD4^+CD25^+$ and $CD25^-$ cell fractions were analyzed *ex vivo* for *foxp3* mRNA expression.

1.7.1. RNA extraction and reverse transcriptase reaction

Total RNA was extracted from separated $CD4^+CD25^+$ and $CD25^-$ cell populations by using the RNeasy Mini kit (Qiagen, Germany) according to the manufacturer's recommendations. This method combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 μg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. A DNase treatment follows to ensure removal of any DNA contamination. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in 40 μl water.

RNA samples were then denatured for 5' at +65ºC before being reverse transcribed into cDNA using 200U M-MLV RT (Promega), at +37ºC for 60' in the presence of 3 mM 5X M-MLV RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl and 15 mM $MgCl_2$) (Promega, USA), 10 mM BSA (Boehringer Mannheim), 0.5 mM dNTPs (Life technologies, Gibco Brl), 40U rRNAsin

ribonuclease inhibitor and 0.1 μg of Oligo (dT)15 (Promega) per 1 μg of RNA. The samples were then heated 5' at +95ºC for RT inactivation cooled and stored at -20ºC.

1.7.2. Molecular cloning of *foxp3*

In order to obtain large quantities of stock *foxp3* for absolute quantitation of target DNA, *foxp3* gene was cloned using PCR fragments generated by a selected pair of primers, into a pGEM-T Easy Vector cloning system (Promega). The recombinant plasmid containing the sequence of interest was used to transform *E. coli* competent cells, extracted and purified for construction of standard curves and quantification of *foxp3* mRNA expression by real-time PCR.

1.7.2.1. PCR of *foxp3* gene

The *foxp3* primer sequences were described by Zelenay, (2005) and PCR conditions further adapted and optimized (Table 4). For the PCR reaction, a final concentration of 0.3 μM was used for sense and anti-sense primers, 1.5 μM of $MgCl_2$, (Bioline, Germany), 0.2 mM of dNTPs (Bioline) and 0.04 $U.\mu l^{-1}$of Taq polymerase (Bioline) with A-tailing activity that optimizes cloning efficiency. PCR amplification of *foxp3* used the following conditions: initial denaturation for 4' at +94ºC, followed by a total of 35 cycles (thermal profile for each cycle: 30''at +94ºC, 30'' at +61ºC, 30'' at +72ºC).

Correction for inefficiencies in RNA input of reverse transcriptase reaction was performed by normalization to a "housekeeping gene". An ideal "housekeeping" gene should be expressed at a constant level among different tissues of an organism, at all stages of development, and should not be affected by the experimental treatment itself. A commonly used endogenous control gene was selected for this purpose; hypoxanthine guanine phosphoribosyl transferase (HPRT). The sense

and anti-sense primer sequences (Table 4) were designed on Primer Express software (Perkin-Elmer/Applied Biosystems) (Rosa et al., 2005; Rodrigues et al., 2006). For the PCR reaction, a final concentration of 0.4 μM was used for sense and anti-sense primers, 2.5 μM of $MgCl_2$, 0.25 mM of dNTPs and 0.01 $U.\mu l^{-1}$of Taq DNA polymerase. PCR amplification of *hprt* used the following conditions: initial denaturation for 5' at 94ºC, followed by a total of 30 cycles (thermal profile for each cycle: 20''at +94ºC, 30'' at +59ºC, 30' at +72ºC).

Table 4. Primer sense and anti-sense sequences for housekeeping genes hypoxanthine guanine phosphoribiosyl transferase (HPRT) and Foxp3.

Target genes	Nucleotide Sequences	Size of PCR product (nº base pairs)
HPRT – Sense primer	5'- GCTCGAGATGTCATGAAGGAGAT - 3'	91
HPRT – Anti-sense primer	5'- CCAGCAGGTCAGCAAAGAACT - 3'	
FOXP3 – Sense primer	5'- TTCATGCATCAGCTCTCCACT – 3'	100
FOXP3– Anti-sense primer	5'- AAGGTGGTGGGAGGCTGA - 3'	

1.7.2.2. Transformation of *E. coli* with recombinant plasmid DNA

Recombinant plasmid DNA was obtained by cloning PCR products of target genes in the pGEM®-T Easy Vector cloning system (Promega). Linearized pGEM®-T Easy vector contains 3' terminal thymidine nucleotides to both ends. These single 3'-T overhangs promote the ligation with PCR fragments through binding to the 3´-A overhangs added previously by the Taq DNA polymerase during PCR reaction. The insert:vector ratio was optimized to 9:1 and the amount of insert needed for the ligation reaction calculated according to the following formula:

$$\text{ng insert} = \frac{\text{ng vector} \times \text{kb size of insert} \times \text{insert : vector molar ratio}}{\text{kb size of vector}}$$

Briefly, for every standard reaction, 5 μl of 2X Ligation buffer, T4 DNA Ligase, 1 μl of pGEM®-T Easy vector, 1 μl of T4 Ligase (3 Weiss units/ μl) were added to the appropriate volume of PCR product insert (corresponding to the quantity calculated above) to obtain a final volume of 10 μl per reaction and incubated overnight at +4ºC.

E. coli JM109 efficiency competent cells were transformed by adding 50 μl of bacterial suspension to 2 μl of the ligation reaction and incubated for 20' on ice. Cells were heat-shocked for 45''-50'' at exactly 42ºC and immediately returned to ice for 2'. Highly enriched medium (950 μl SOC) (Promega) was added to the ligation reaction transformations and incubated 90', at +37ºC with shaking (150 rotations per minute). 100 μl of each transformation culture was plated overnight at +37ºC onto duplicate Luria broth (Lennox, Scharlau Microbiology, Spain) containing 100 $\mu g.ml^{-1}$ amplicillin (Sigma), 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) (Sigma) and 80 $\mu g.ml^{-1}$ 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) (Sigma) for colour screening and further selection of recombinant clones containing the PCR product. These colonies are in most cases white as a consequence of the interruption of the coding sequence of β-galactosidase gene when the PCR product is correctly cloned in-frame with the *lacZ* gene within the cloning region of the pGEM®-T Easy vector.

1.7.2.3. Plasmid DNA extraction and restriction analysis

Plasmid DNA was extracted from bacteria by using the High Pure Plasmid Purification kit (Roche Applied Science, Switzerland) according to the manufacturer's recommendations. Alkaline lysis

releases plasmid DNA from bacteria and RNases removes all RNA in the lysate. Then, in the presence of a chaotropic salt (guanidine HCl), plasmid DNA binds selectively to glass fiber fleece in a special centrifuge tube. The DNA remains bound while a series of rapid "wash-and-spin" steps remove contaminating bacterial components. Finally, low salt elution removes the DNA from the glass fiber fleece.

In order to confirm the presence of the target sequence within the purified recombinant DNA plasmids, the latter were subject to restriction analysis by using restriction enzymes that cuts at specific sites of the pGEM®-T Easy vector generating a known number of fragments with specific expected sizes. Recombinant plasmid DNA (4 μl) was incubated 120' in a water bath +37ºC with 1 μl of *Nci* I (MB Fermentas, Germany), 1 μl of 10X Tango™ buffer and 4 μl of distilled water. The restriction fragments obtained (10 μl) were run 90', 120 mV on a 1% agarose gel stained with ethidium bromide (EtOH) to confirm for the presence of insert within the plasmid nucleotide sequence. Normal PCR reaction, using conditions described in section 1.7.2.1., was also carried out for *foxp3* and *hrpt* using recombinant plasmid DNA as PCR template for mouse genes. PCR bands reconfirming presence of PCR product in question were run on a 3% agarose gel for 90' at 120 mV.

1.7.3. Real-time PCR assay

A reliable quantification of cytokine gene expression is a valuable technique for analyzing immune responses. The study of cytokine profiles can help to clarify functional properties of immune cells and inflamed tissues both for research and for clinical diagnosis. Real-time PCR offers a powerful tool for the quantitation of target nucleic acids (Holland et al., 1991, Higuchi et al., 1992). Being a very high throughput technique that is both accurate and sensitive enough, real-time PCR can be performed on very small samples with low levels of gene expression. It monitors the progress of the PCR as it occurs (i.e., in real time) and reactions are characterized by the point

in time during cycling when amplification of a target is first detected rather than when the amount of target accumulates after a fixed number of cycles (e.g. end-point PCR assay).

Figure 13. shows a graphical display of fluorescence signal versus cycle number. In the initial cycles of PCR, there is no significant change in fluorescence signal. This predefined range of PCR cycles is called the “baseline”. A baseline subtracted amplification plot is generated by calculating a mathematical trend using fluorescence values (Rn) corresponding to the baseline cycles of amplification. Then, an algorithm searches for the point on the amplification plot at which the delta Rn value crosses the threshold, the level set above the baseline and sufficiently low to be in the exponential growth region of the amplification curve. The fractional cycle at which the fluorescence passes the threshold is defined as C_T.

One of the applications of this technology is quantitative real-time PCR using SYBR Green - a highly specific, double-stranded DNA (dsDNA) binding dye used to detect PCR product as it accumulates during PCR cycles. This method enables simple and rapid measurement of PCR product accumulation during the exponential reaction phase combining amplification and detection in one single step. It also obviates the need for expensive hybridization probes. SYBR Green dye binds to all dsDNA, the result is an increase in fluorescence intensity proportional to the amount of PCR product produced.

The advantages of the SYBR Green dye is that it can be used to monitor the amplification of any dsDNA sequence, no probe is required, which reduces assay setup and running costs and due to multiple dye binding the fluorescent signals produced are much stronger which increases sensitivity. SYBR Green dye provides a convenient and cost effective technique to screen a large number of samples and targets. The primary disadvantage is that it may generate false positive signals; i.e., because the SYBR Green dye binds to any dsDNA, it can also bind to non-specific dsDNA sequences. To circumvent this, RNA samples were pre-treated, prior to cDNA synthesis, with DNase to eliminate any contaminating DNA. DNA melting curves of post-PCR of product

were analyzed individually for non-specific amplification with distinct melting profiles curves to those of the target product and for the formation of primer-dimers. dUTP replacement of dTTP in SYBR Green PCR Master mixes and enzyme treatment with AmpErase UNG, that eliminates dUTP, prevents re-amplification of PCR carry-over products in SYBR Green assays.

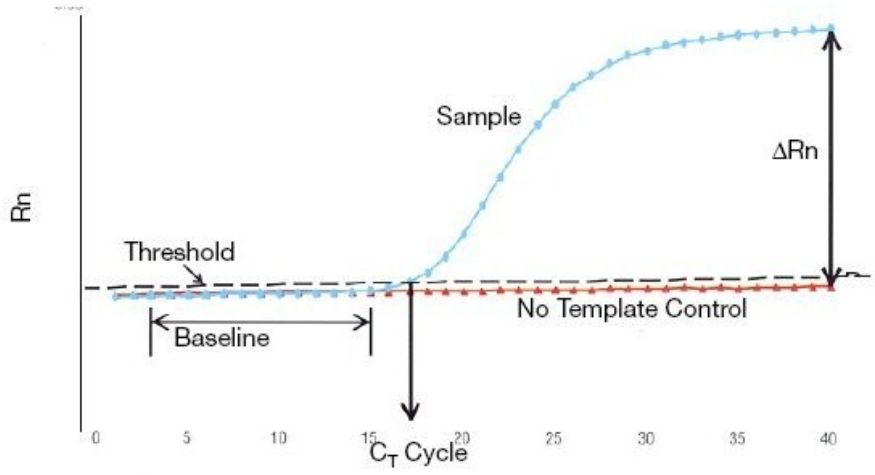


Figure 13. PCR amplification plot. Fluorescence emission is measured continuously during the PCR reaction and Rn (increase in fluorescence emission, from which the background fluorescence signal is subtracted) is plotted against cycle number. The standard deviation is determined from the data points collected from the base line of the amplification plot. The threshold cycle (Ct) is the cycle at which the fluorescence exceeds a chosen threshold limit (usually 10 times the standard deviation of the base line) (adapted from: http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_041440.pdf).

In this study, absolute quantification was performed to determine levels of gene expression. Absolute quantification determines the input copy number of the template of interest by relating the PCR signal to a standard curve. In order to measure the cytokine copy number in unknown samples, the measurement to a standard curve of known cytokine copy numbers is used as comparison. The basis for measurement is a standard curve with known quantities of plasmid

cytokine DNA generated as described in section 1.7.2.2. The corresponding copy numbers were calculated according to the following equation (Hein et al., 2001):

$$\text{Copy number} = 9.1 \times 10^{11} \text{ [}\mu\text{g of plasmid standard/size (plasmid + insert) in kb]}$$

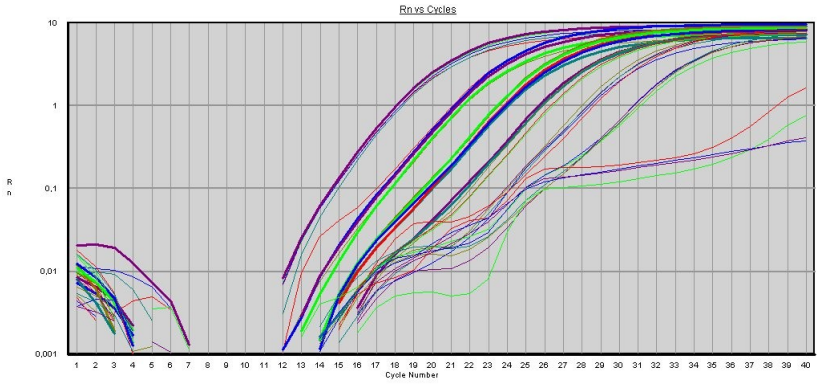
The DNA content of each sample was determined using a standard curve obtained by processing, in parallel, samples containing fixed DNA concentrations. Determination of DNA concentrations was performed by spectrophotometry, and molar concentrations were determined using the following formula: 1 μg of 1,000 bp DNA = 1.52 pmol = 1.52×10^{-12} mol x N molecules (where N stands for Avogadro's number or 6.023×10^{23} molecules / mol). Serial dilutions from the resulting clones were used as standard curves, each containing a known amount of input copy number. Final results were expressed as the copy number of each cytokine per 1000 copies of *hprt*.

Amplification was carried out in a total volume of 20 μl, containing 2 μl of cDNA sample or plasmid DNA standards, 10 μl of 2X SYBR® Green I dye PCR Master Mix (Applied Biosystems, USA) and *foxp3* primers (0.2 μM). PCR amplification was performed in the ABI GeneAmp 5700 (Perkin-Elmer/Applied Biosystems), in duplicate wells, using the following conditions for *foxp3*: 15' at 95ºC for AmpliTaq Gold activation, followed by a total of 45 cycles (thermal profile for each cycle: 15'' at +95ºC, 20'' at +61ºC, 10'' at +72ºC) and for *hprt* (0.3 μM): 10' at +95ºC followed by 40 cycles (15''at +95ºC, 1' at +60ºC)**.**

PCR amplification efficiency is the rate at which a PCR amplicon is generated and is expressed as a percentage value. If a particular PCR amplicon doubles in quantity during the geometric phase of its PCR amplification then the PCR assay has 100% efficiency. The slope of the standard curve is used to estimate the PCR amplification efficiency of a real-time PCR reaction. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of C_T value vs. log of input nucleic acid (Figure 14). A standard curve slope of –3.32 indicates a PCR reaction with 100% efficiency. A calculation for estimating the efficiency (E) of a real-time PCR assay is:

$$E = (10^{-1/slope} - 1) \times 100$$

(A)



(B)

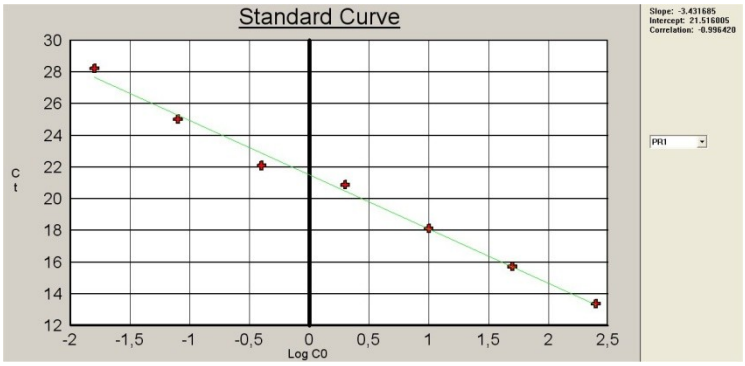


Figure 14. Quantitative analysis of gene expression by real-time PCR. (A) Amplification plots of standard curves from titration of recombinant plasmid DNA ranging from $1.5x10^8$ to $9.4x10^4$ copies of each target gene. (B) Amount of plasmid DNA used for the determination of gene expression was plotted against the mean Ct value.

Efficiencies of amplification for *hprt and foxp3*, were 93.1% and 97.8% respectively. Intra-assay coefficient of variation of copy numbers ranged from 0.12% – 1.64% for *hprt* and 0.12 – 2.02% for *foxp3*. Inter-assay coefficient of variation ranged from 1.8 – 5.6% for *hprt*, 1.2 – 3.5% for *foxp3*.

1.8. Gene expression analysis of Toll-like receptor-2 on $CD4^+CD25^+$ T cell subsets

In order to detect the expression of Toll-like receptor-2 by $CD4^+CD25^+$ T cell subsets, real-time PCR was performed and samples evaluated for *tlr2* mRNA expression. Gene expression analysis and molecular cloning of *tlr2* was done using the same procedures as previously described for *foxp3* in section 1.7.

1.8.1. PCR of *tlr2* gene

The *tlr2* primer sequences were described by Sutmuller, (2006) and PCR conditions further adapted and optimized (Table 5). For the PCR reaction, a final concentration of 0.2 μM was used for sense and anti-sense primers, 1.5 μM of $MgCl_2$, 0.2 mM of dNTPs and 0.04 $U.\mu l^{-1}$of Taq polymerase. PCR amplification of *tlr2* used the following conditions: initial desnaturation for 5' at +94ºC, followed by a total of 30 cycles (thermal profile for each cycle: 20''at +94ºC, 30'' at +60ºC, 30'' at +72ºC).

1.8.2. Real-time PCR assay

Amplification was carried out in a total volume of 20 μl, containing 2 μl of cDNA sample or plasmid DNA standards, 10 μl of 2X SYBR® Green dye PCR Master Mix and *tlr2* primers (0.2

μM). PCR amplification was performed in duplicate wells, using the following conditions for *tlr2*: 10' at 95ºC for AmpliTaq Gold activation, followed by a total of 40 cycles (thermal profile for each cycle: 15'' at +95ºC, 1' at +60ºC). Efficiencies of amplification for *tlr2* were 99.4% and intra-assay and inter-assay coefficients of variation ranged from and 0.21 - 2.11% and 0.4 – 6.9% respectively.

Table 5. Primer sense and anti-sense sequences for TLR-2.

Target genes	Nucleotide Sequences	Size of PCR product of amplification (nº base pairs)
TLR2 – Sense primer	5'- AACCTCAGACAAAGCGTCAAATC - 3'	65
TLR2 – Anti-sense primer	5'- ACCAAGATCCAGAAGAGCCAAA - 3'	

2. Evaluation of the effect of TLR-2 modulation on Treg populations during *L. infantum in vivo* infection

The second part of the study involved the evaluation of the role of the pathogen pattern recognition receptor TLR2 on the modulation of regulatory T cells during *in vivo L. infantum* infection and its' effect on protective immune response and consequent control of infection. To investigate this, gene disrupted TLR2$^{-/-}$ knock-out mice were infected with *L. infantum* parasites and compared to wild-type mice regarding Treg kinetics, cytokine response, liver histopathology and evolution of parasite burden.

2.1. Experimental design

Treg cell frequency and phenotype were separately studied in spleen cells, lymph node cells, and in

liver and spleen tissues isolated from *L. infantum* infected - TLR2$^{-/-}$ knock-out mice on a C57BL/6 genetic background and compared to C57BL/6 wild type (WT) mice. Parasite detection was carried out in spleen and liver organs. Two independent experiments were performed.

2.2. Animals and parasites

TLR2$^{-/-}$ knock-out male mice, 12 to 14-week-old, obtained from S. Akira, University of Osaka, Japan, were bred in the IBMC animal facility and maintained at IHMT animal house. C57BL/6 littermate mice were purchased at IBMC. Upon arrival, each mice strain was divided into two groups, control and infected. Sterilized commercial feed (SAFE Scientific animal food & engineering) and water were provided *ad libitum* and animals kept in HEPA filtered sterilized cages with weekly replacements. Animals were infected via intravenous (i.v.) route with a parasite suspension of 10^7 virulent *L. infantum* promastigotes prepared as described in section 1.2.2. All experiments were conducted according to EU requirements (86/609/CEE), recognized by the Portuguese law (DR DL129/92 and Portaria 1005/92).

2.3. Sample collection

Four animals per each group were sacrificed by cervical dislocation at days 7 and 28 days pi. The organs were aseptically dissected from each individual animal and maintained at +4ºC in RPMI 10% FCS. Cervical superficial, illiary, axillary, brachial, inguinal, superior mesenteric and popliteal LN were extracted together with whole spleen and liver. Portions of the spleen and liver tissues of each individual animal was separated and maintained either in Schneider's 10% FCS for parasite detection, in RNA stabilizing solution (Qiagen) for RNA extraction and in 3.7% buffered formaldehyde for histopathology. Samples were processed for use in follow-up immunological assays.

2.4. Parasite detection

Parasite burden in the spleen and liver for each individual animal was determined by LDA as previously described in section 1.4.

2.5. Phenotypic characterization

Spleen cells of each animal were stained with the same surface markers as described in section 1.5.3. although cells underwent intracellular staining for the FOXP3 proteins and prior fixation.

2.5.1. Isolation of total leukocytes

Total leukocytes were isolated from LN and spleen from each individual animal according to section 1.5.1..

2.5.2. Cell surface markers and intracellular staining of FOXP3

For FOXP3 intracellular staining, the APC anti-mouse/rat FOXP3 Staining Set was used according to manufacturer's recommendations (eBioscience, USA). Briefly, 10^6 cells were re-suspended with PBS, 2% FCS, 0.01% NaN_3. Aliquots of 10^6 cells were incubated in the dark (20', +4ºC) with 20 µl of the following fluorochrom-conjugated anti-mouse Ab: Ab FITC-labeled anti-CD3, PerCp-labeled anti-CD4, PE-labeled anti-CD25, FITC-labeled anti-CD103, FITC-labeled anti-CD45RB, FITC-labeled anti-GITR/TNFRSF18. Cell were washed once in PBS, 2% FCS, 0.01% NaN_3 by centrifuging (370 *g*, 5', +4ºC) and fixed in 250 µl Fix/Perm solution (eBioscience) for FOXP3 intranuclear staining for overnight at +4ºC. The cells were washed twice and resuspended in 250 µl of Permeabilization buffer (eBioscience), blocked (to avoid non-specific antibody binding) with 50

μl rat serum diluted 1:50 in 1X Permeabilization buffer for 30'. Without washing after blocking step, allophycocyanin (APC)-labeled conjugated anti-mouse/rat FOXP3 FJK-16s was used for staining. This antibody has specificity for FORKHEAD BOX P3 or SCURFIN, a 4955 kDa protein and member of the forkhead/winged helix family of transcriptional regulators and that was identified as the gene defective in 'scurfy' (sf) mice. The Ab was diluted (1:100) in 1X Permeabilization buffer and 30 μl added to suspension. The cell suspension were then incubated for 30' at +4°C in the dark. After washing twice in 250 μl of 1X Permeabilization buffer, cells were resuspended in PBS, 2% FCS, 0.01% NaN_3 and analyzed by flow cytometry.

To characterize regulatory T cell populations, various T cell subsets were detected with 4-color combinations of fluorochrom-conjugated antibodies directed towards antigens of interest as follows:

- CD4-PerCP / CD25-PE / CD3-FITC / FOXP3-APC

- CD4-PerCP / CD25-PE / CD45RB-FITC / FOXP3-APC

- CD4-PerCP / CD25-PE / CD103-FITC / FOXP3-APC

- CD4-PerCP / CD25-PE / GITR-FITC / FOXP3-APC

Compensation was set for every different combination of fluorophores used in each assay. Data acquisition was performed on a FACSCalibur flow cytometer and analyzed using BD FACSDiva Software.

2.6. Detection of cytokine transcripts

Immediate RNA stabilization of harvested LN and spleen cells, spleen and liver tissues was performed by immersing overnight at +4ºC in RNA later and frozen at -80ºC for further analysis of cytokines gene expression (IFN-γ, IL-4, IL-10 and TGF-β) profiles. Gene expression analysis

and molecular cloning of cytokines were done using the same procedures as previously described in section 1.7. The sense and anti-sense primer sequences were designed on Primer Express software (Table 6). For the PCR reaction, a final concentration of 0.4 μM was used for sense and anti-sense primers, 2.5 μM of $MgCl_2$, 0.25 mM of dNTPs and 0.01 $U.\mu l^{-1}$of Taq DNA polymerase. PCR amplification of IFN-γ, IL-4, IL-10 and TGF-β used the following conditions: initial denaturation for 5' at 94ºC, followed by a total of 30 cycles (thermal profile for each cycle: 20''at +94ºC, 30'' at +59ºC, 30'' at +72ºC) (Rosa et al., 2005; Rodrigues et al., 2006).

Real-time PCR assay was carried out using the same procedure as described for *hprt* in 1.7.3.. Efficiencies of amplification for IFN-γ, IL-4, IL-10, TGF-β were 99 %, 94 %, ~100 % and 98.6 % respectively. Intra-assay coefficient of variation ranged from 0.04 to 2.17 % for IFN-γ, 0.04 to 1.79 % for IL-4, 0.06 – 2.69 % for IL-10 and 0.04 – 5.94 % for TGF-β. Inter-assay coefficient of variation ranged from 0.4 to 1.33 % for IFN-γ, 0.15 to 1.49 % for IL-4, 1.7 – 6.5 % for IL-10 and 1.0 – 4.5 % for TGF-β

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Table 6. Primer sense and anti-sense sequences for cytokines IFN, IL-4, IL-10 and TGF-β.

Target genes	Nucleotide Sequences	Size of PCR product (nº base pairs)
IFN-γ – Sense primer	5'- CAATGAACGCTACACACTGCATC - 3'	72
IFN-γ – Anti-sense primer	5'- CGTGGCAGTAACAGCCAGAA - 3'	
IL-4 – Sense primer	5'- GACGCCATGCACGGAGAT – 3'	81
IL-4 – Anti-sense primer	5'- GCCCTACAGACGAGCTCACTCT - 3'	
IL-10 – Sense primer	5'- CAAGGCAGCCTTGCAGAAA - 3'	71
IL-10 – Anti-sense primer	5'- CAGTAAGAGCAGGCAGCATAGC - 3'	
TGF-β – Sense primer	5'- CACCTGCAAGACCATCGACAT - 3'	71
TGF-β – Anti-sense primer	5'- ACAGGATCTGGCCACGGAT - 3'	

2.7. Histopathology

The host tissue response in terms of immunopathology was assessed by analyzing the granulomatous response in the liver. Liver inflammation was evaluated by estimating the total number of infiltrating cells per square millimeter and the granuloma size in histological liver tissue sections.

For tissue processing, the livers were removed, cut into small pieces, and immersed in fixative, 3.7 – 4 % formaldehyde. The volume of fixative versus the volume of tissues was 10:1. Dehydration was performed with increasing concentration of graded ethanol up to absolute alcohol. After dehydration, tissue specimens were cleared with xylene and embedded in paraffin. Tissue sections, 5 to 6 μm in thickness was prepared from the tissue blocks and mounted on standard glass slides.

2.8. H&E staining

The distribution of inflammatory cell infiltrates were examined in slides of paraffin-embedded livers stained with hematoxylin and eosin. Glass slides with the tissue sections were immersed 10' in xylene to deparaffinated and hydrated in decreasing concentrations of graded ethanol (100%, 96%, 70%), placed under running tap water for 2' and immersed in filtered Harris' hematoxilin (Sigma) for 10'. Slides were then placed again under running tap water, 1% HCl (Sigma) added for a few seconds and washed with water for 10 min. Slides were then immersed in 1% Eosin Y (w/v) (Sigma) and washed. Dehydration was performed with increasing concentration of graded ethanol up to absolute alcohol. Slides were incubated 5', +65ºC, dried and mounted with resin embedding agent – Entellan (Sigma) and observed using light microscopy for granuloma formation. Size and number were expressed as the number of infiltrating cells per area (cm^2).

3. Statistical analysis

The non-parametric Mann–Whitney *U* test was used to compare variables of two independent samples from infected and non-infected mice, $TLR2^{-/-}$ and WT mice, in relation to all parameters studied and to compare the same variables between different time points. Results are represented as mean values ± standard error (SEM) or mean values ± standard deviation (SD) according to samples in each study. Differences were considered significant with a 5% significance level ($p<0.05$). Statistical analysis was performed with the SPSS 13.0 for Windows software (SPSS Inc.).

CHAPTER III. RESULTS

1. Phenotypic characterization of regulatory T cell populations during *L. infantum in vivo* infection

1.1. Parasite detection

Parasite burden in the spleen and liver was evaluated during the course of infection. Viable *Leishmania* parasites that were detected in the spleen at two days pi, showed a considerable increase in number at 28 days pi and a reduction at 56 days pi. Except for day 7 pi, the spleen showed higher levels of parasitism than liver. In the latter, parasites were only detected 7 days pi. Parasite load in liver showed some variations in number, with increases at 28 days pi and a slight decrease at 56 days pi (Figure 15). Follow-up studies beyond the experimental period defined, revealed the presence of high levels of viable parasites in the spleen after 112 days pi.

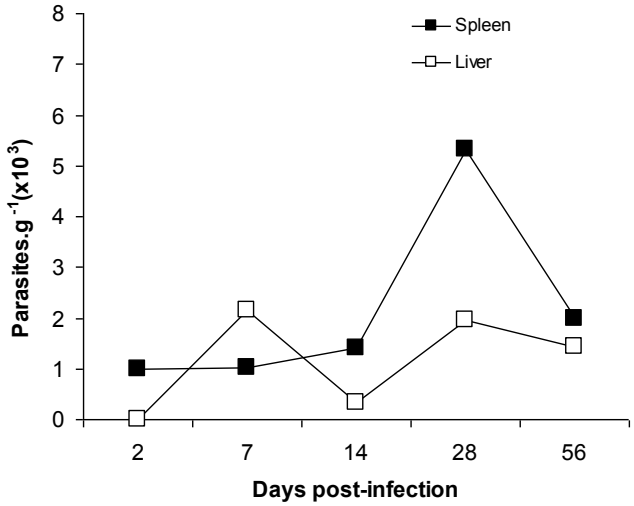

Figure 15. Evolution of parasite burden in spleen and liver of BALB/c mice infected with *L. infantum* promastigotes. Parasite load was determined at 2, 7, 14, 28 and 56 days post-infection (pi) by limiting dilution assay. Results are expressed as the number of parasites x 10^3 per gram (g) of homogenized organ.

1.2. Phenotypic characterization of regulatory T cells

The frequencies of expression of various Treg markers on the surface of freshly isolated and pooled leukocytes from spleen and lymph nodes of *L. infantum* – infected and control BALB/c mice were analyzed in order to identify potential subsets of Treg cells induced in response to the parasite (Figure 16).

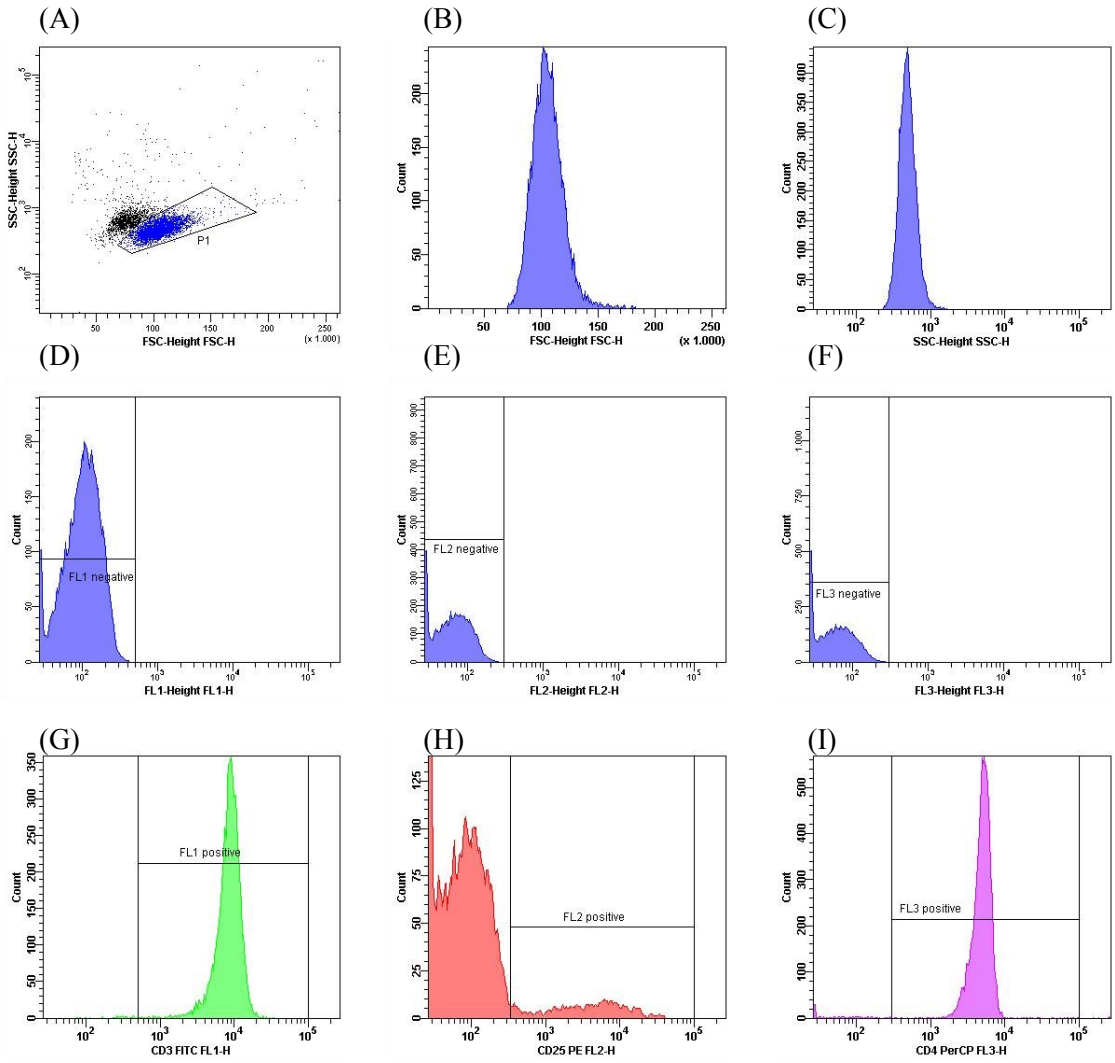

Figure 16. Typical FSC/SSC plot of the population of isolated total $CD4^+$ T cells from BALB/c mice. P1 represents the subpopulation of viable lymphocytes gated on for further immunophenotyping (A). Total number of cells characterized on the basis of size and granularity and structural complex is shown in B and C. Non-stained samples were used as negative controls for FL1, FL2 and FL3 (D-F) in comparison to samples positive for each fluorescence detector (G-I).

Data was collected and analyzed on a two parameter dot-plot in order to measure the percentage of cells positive for each fluorophore-labeled antibody used. Shown below are four representative dot plots for $CD4^+CD25^+$ and $CD25^-$ cells, $CD4^+CD25^+GITR^+$ and $CD4^+CD25^+CD103^+$, $CD4^+CD45RB^{low}$ and $CD45RB^{high}$ T cells (Figure 17).

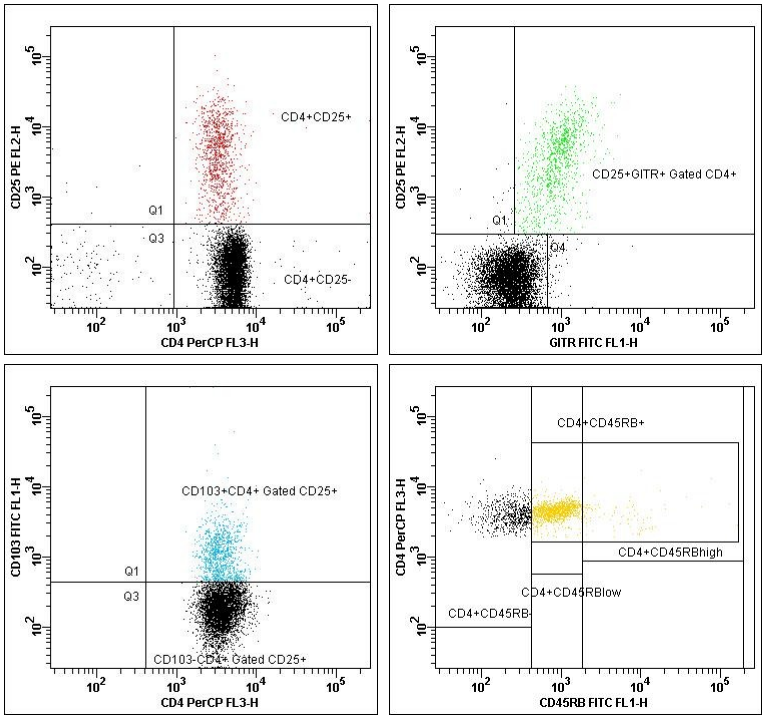

Figure 17. Representative plots of $CD4^+$ T cells for CD25, GITR, CD103 and CD45RB markers.

After 7 days of infection with *L. infantum*, a transient expansion of $CD3^+CD4^+CD25^+$ (p=0.010, Figure 18A), $CD4^+CD25^+GITR^+$ (p=0.017, Figure 18B), and $CD4^+CD25^+CD103^+$ (p=0.010, Figure 18C) T cells was detected. Cell frequencies returned to steady state levels at 14 days pi. $CD4^+CD25^+GITR^+$ and $CD103^+$ T cells again showed increases at 28 days pi (p=0.027) (Figure 18B, C) and $CD3^+CD4^+CD25^+$, $CD4^+CD25^+GITR^+$ and $CD4^+CD25^+CD103^+$ T cells at 56 days pi (p=0.002) (Figure 18A, B and C). $CD3^+CD4^+CD25^-$ effector T cells of infected animals maintained

lower than non-infected animals throughout the infection period being significantly reduced at 7 (p=0.027) and 56 days pi (p=0.002) (Figure 18D). These results indicate that $CD4^+CD25^+$ T cell subsets that also express GITR and CD103 are induced and readily expand in response to *L. infantum in vivo* infection and are in fact recruited to the sites of parasite infection.

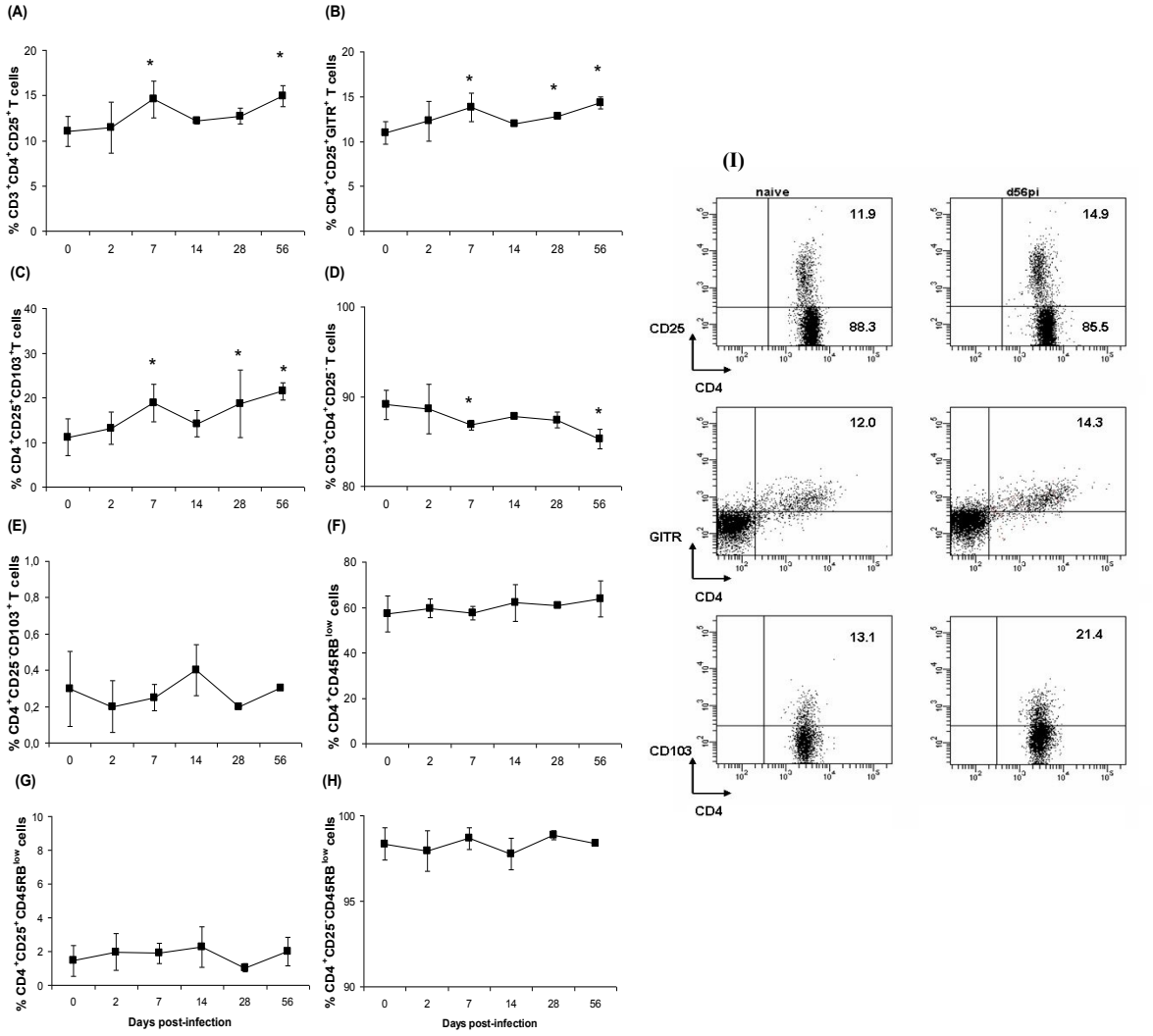

Figure 18. Dynamics of $CD4^+CD25^+$ and $CD25^-$ T cell populations during *L. infantum* infection. Frequency of pooled draining lymph nodes and spleen cells from BALB/c mice at 2, 7, 14, 28 and 56 days pi. Cells were analyzed for surface expression of CD3, CD4, CD25, GITR, CD103, CD45RB (A-H). Representative plots of cells from naïve and day 56 infected BALB/c mice show staining and percentage for CD25 gated on $CD4^+$ T cells, for GITR and CD103 of $CD4^+CD25^+$ gated cells (I). Results are mean values ± SD. *(p<0.05) indicates statistically significant differences in comparison to naïve cells (day 0).

Cell frequencies of $CD45RB^{low}$ between infected *vs.* naïve cells within the $CD4^+$ showed slight variations throughout the period of study (Figure 18F). $CD45RB^{low}$ frequency within the $CD4^+CD25^+$ (Figure 18G) and $CD4^+CD25^-$ (Figure 18H) fractions were also analyzed in this study, as well as, $CD103^+$ numbers within the $CD4^+CD25^-$ fraction however, no significant differences were found.

The expression of *foxp3* was used as a discriminatory intra-cellular marker for regulatory T cells. Therefore, the expression of *foxp3* was analyzed *ex vivo* in cells isolated from spleen and LN, that were magnetically separated into $CD4^+CD25^+$ and $CD25^-$ defined subsets.

Increased gene expression levels of transcription factor *foxp3* by $CD4^+CD25^+$ T cells, was observed at days 2 (p=0.029), 7 (p=0.029) and 28 pi (p=0.016) in *L. infantum*-infected mice compared to naïve mice, confirming the presence of cells with regulatory phenotype (Figure 19A). In fact, *foxp3* expression by $CD4^+CD25^+$ T cells reached higher levels at 7 and 28 days pi coincidently with higher frequencies of $CD4^+CD25^+GITR^+$ and $CD103^+$. *foxp3* levels by $CD4^+CD25^-$ effector cells of infected mice were low throughout the experimental period, with statistical significance at 14 (p=0.010) and 28 (p=0.012) (Figure 19B) days pi.

These data show clearly that *foxp3* is selectively expressed in the $CD4^+CD25^+$ T cell fraction with enhanced expression in cells from infected animals.

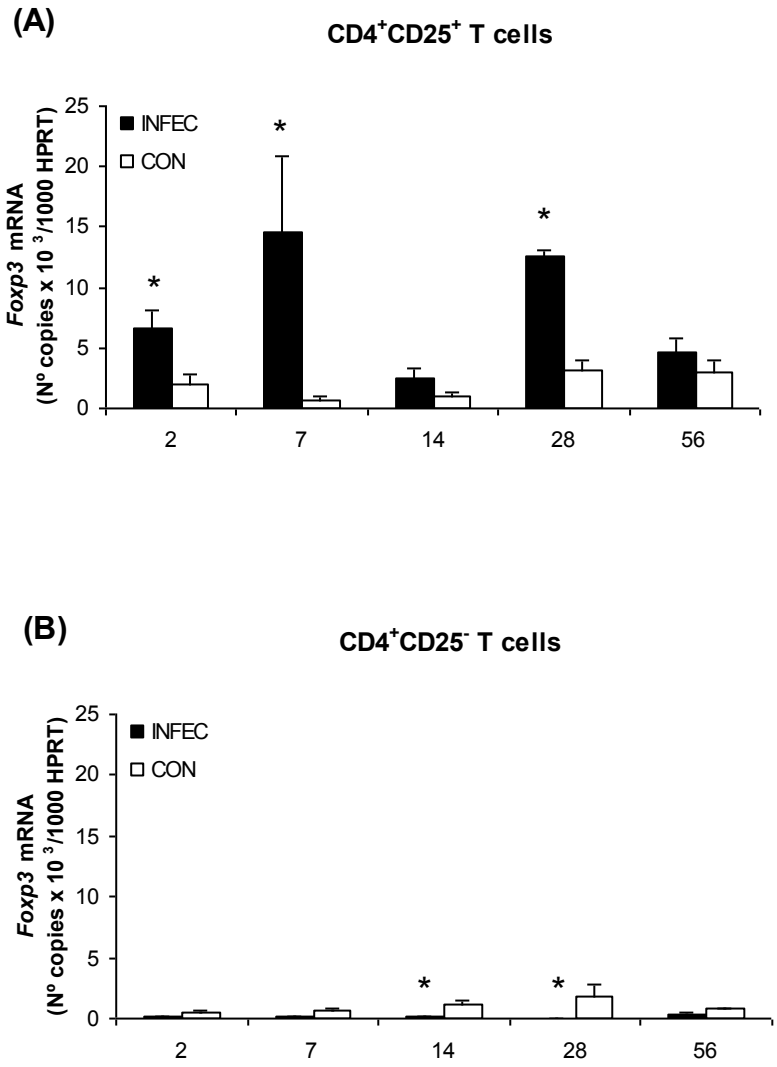

Figure 19. Expression of *foxp3* mRNA by $CD4^+CD25^+$ (A) and $CD25^-$ (B) cell fractions from pooled draining lymph nodes and spleen cells of *L. infantum*-infected (■) and naïve (□) BALB/c mice at 2, 7, 14, 28 and 56 days pi. Results are analyzed by real-time PCR and data are mean values ± SEM and expressed as number of copies x 10^3/1000 copies of housekeeping gene HPRT. *(p<0.05) indicates statistically significant differences in comparison to naïve cells (CON).

1.3. Detection of *in vitro* cytokine production

1.3.1. $CD4^+CD25^+$ T cell subset

After providing evidence that $CD4^+CD25^+$ T cells express *foxp3,* their functional activity was characterized with regard to production of Treg-associated cytokines such as IL-10 and TGF-β.

In vitro cytokine production was evaluated in the separated $CD4^+CD25^+$ fractions isolated from pooled spleen and LN cells. Significant results presented correspond to the following conditions of stimulation: cells with medium; cells with APC and anti-CD3ε and cells with APC, anti-CD3ε and antigen. $CD4^+CD25^+$ T cells showed increased IL-10 production when stimulated with APC and anti-CD3ε at days 14 (p=0.013) and 56 pi (p=0.029) (Figure 20A). Stimulation with *Leishmania* antigen increased production of this cytokine at 2 and 14 day pi, however significant differences were not observed. No significant differences were observed with other stimulatory conditions mentioned in section 1.6 of Material and Methods.

TGF-β concentrations produced by $CD4^+CD25^+$ T cells were found to be significantly increased when in the presence of APC and anti-CD3ε at 28 (p=0.007) and 56 (p=0.034) days pi and also when additionally stimulated with antigen earlier at day 7 pi (p=0.048) and later at 28 and 56 days pi (p=0.005) (Figure 20B).

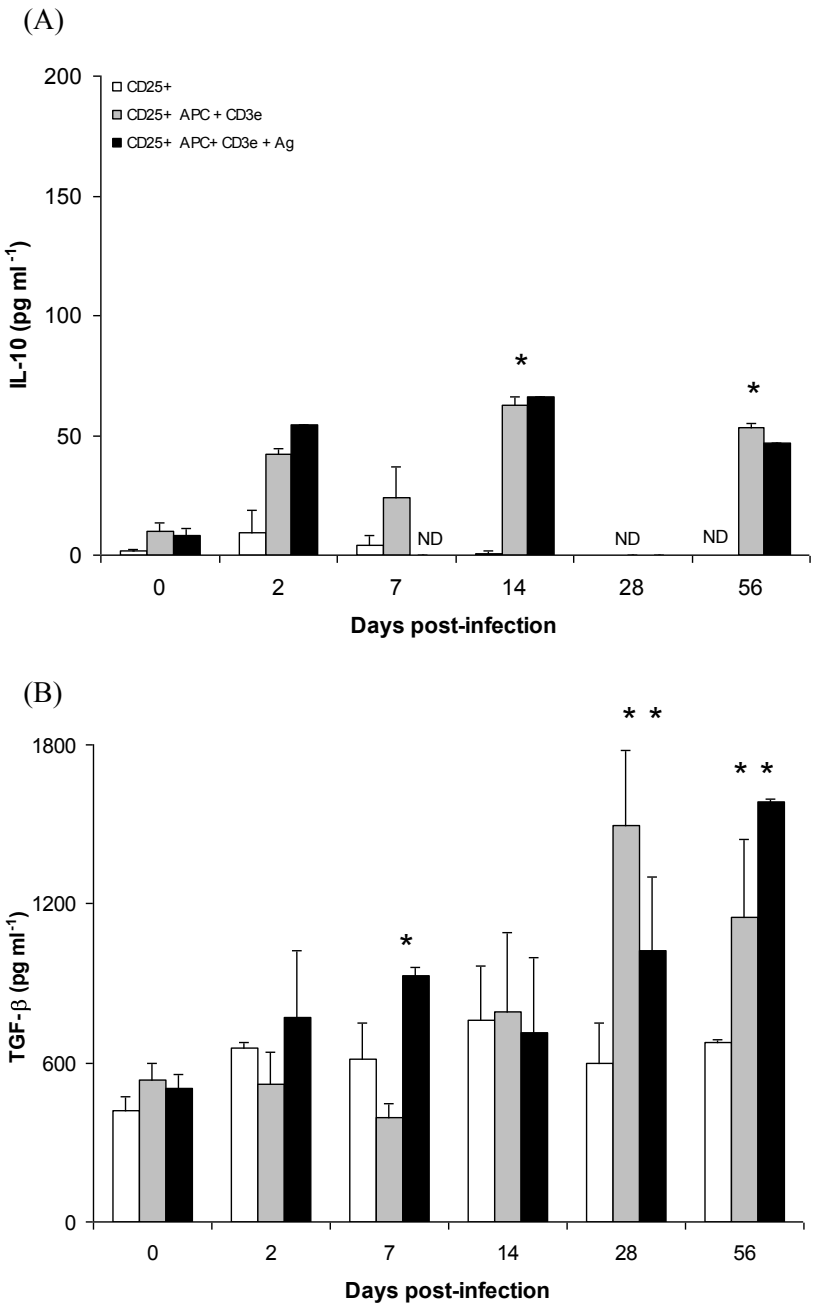

Figure 20. *In vitro* production of IL-10 and TGF-β by $CD4^{+}CD25^{+}$ T cells fractions (A, B), purified from pooled draining lymph nodes and spleen cells of *L. infantum*-infected and naïve BALB/c mice, and cultured *in vitro* alone (□), in the presence of mitomycin C-treated antigen presenting cells (APC) at a ratio of 1 lymphocyte per 1 APC activated with soluble anti-CD3ε (■) or with *Leishmania* antigen (■) were evaluated. Cytokines were quantified by ELISA in culture supernatant after three days of activation. Results are mean values ± SEM expressed in pg..ml^{-1}. *(p<0.05) indicates statistically significant differences in comparison to naïve cells (day 0). ND, not detectable.

1.3.2. $CD4^+CD25^-$ T cell subset

Th1 and Th2 cytokines were also evaluated in effector T cell populations in order to determine the type of immune response induced with disease outcome. Although effector cells produced IFN-γ, no significant differences were found when comparing to naïve cells, either alone, in the presence of APC and soluble anti-CD3ε or in the presence of APC and soluble anti-CD3ε and antigen (Figure 21A).

Concentrations of IL-4 produced by $CD4^+CD25^-$ effector T cells from infected animals maintained reduced in comparison to naïve cells until day 28 pi (Figure 21B). An early specific inhibition of IL-4 was observed at day 7 pi when stimulated with antigen in the presence of APC and anti-CD3ε (p=0.019). Effector cells were only able to produce significant levels of IL-4 at day 56 pi when stimulated with antigen (p=0.030) and with APC and anti-CD3ε (p=0.033).

Interestingly, $CD4^+CD25^-$ effector T cells produced large quantities of IL-10, without additional stimulation soon after 2 (p=0.001) and 7 days pi (p=0.027) and also when stimulated with APC and anti-CD3ε at 2 (p=0.032), 7 and 56 days ($p_{\text{day 7 and 56}}$<0.001) of infection (Figure 22). In presence of *Leishmania* antigen, effector cells responded with even higher levels of IL-10 at day 7 pi (p<0.001), reaching peak values at 56 days pi (p<0.001). However, inhibition of IL-10 production occurred at days 14 (p=0.037) and 28 pi, in non-stimulated cells, (p=0.024), in cells stimulated with APC (p=0.032) and anti-CD3ε (p=0.018) and with antigen at day 28 pi (p=0.006).

Th1 and Th2 responses to *L. infantum* infection do not seem to really contribute to infection control or disease progression since IFN-γ and IL-4 were produced in reduced quantities to be biologically significant and affect parasite evolution. On the other hand, Treg-associated cytokine secreting cells, such as TGF-β–producing $CD4^+CD25^+$ T cells and IL-10-producing $CD4^+CD25^-$ T cells are actively induced in response to infection.

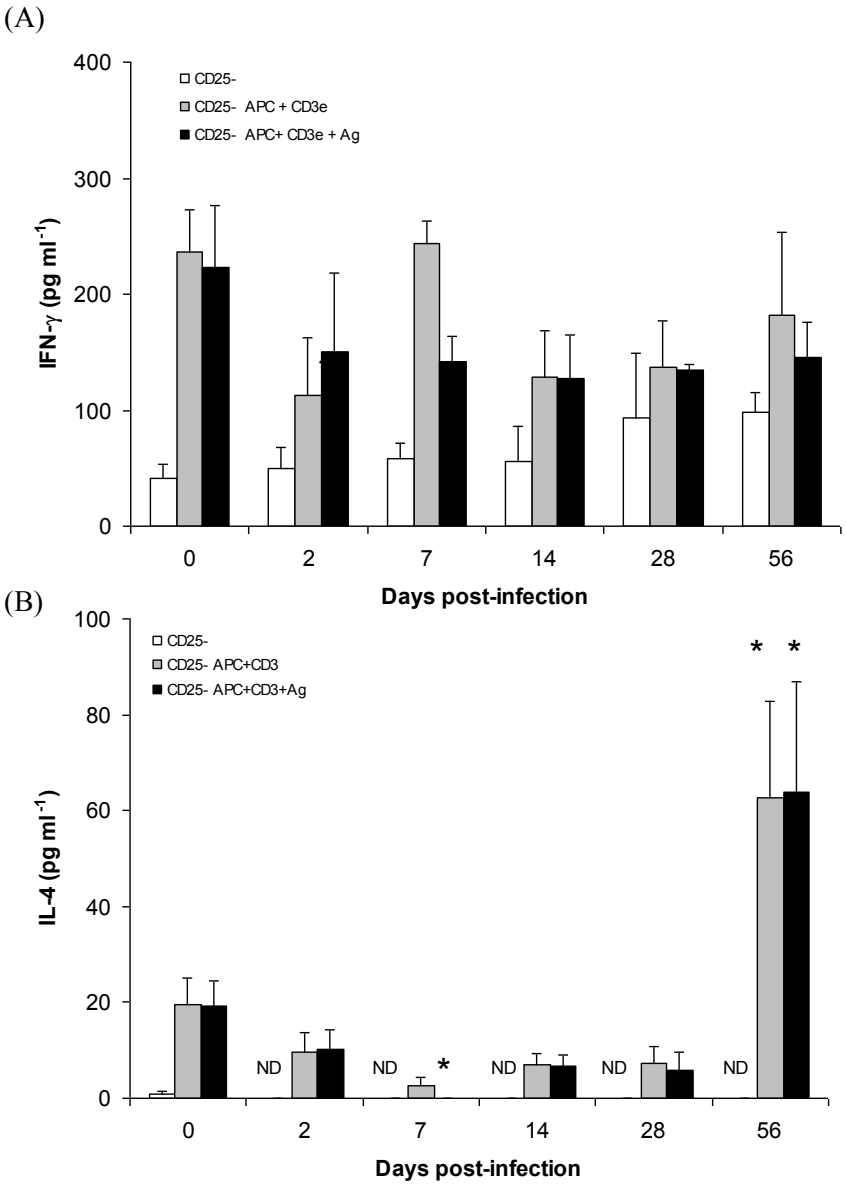

Figure 21. *In vitro* production of IFN-γ and IL-4 by $CD4^{+}CD25^{-}$ T cell fractions (A, B), purified from pooled draining lymph nodes and spleen cells of *L. infantum*-infected and naïve BALB/c mice, and cultured *in vitro* alone (□), in the presence of mitomycin C-treated antigen presenting cells (APC) at a ratio of 1 lymphocyte per 1 APC activated with soluble anti-CD3ε (■) or with *Leishmania* antigen (■) were evaluated. Cytokines were quantified by ELISA in culture supernatant after three days of activation. Results are mean values ± SEM expressed in $pgml^{-1}$. *($p<0.05$) indicates statistically significant differences in comparison to naïve cells (day 0). ND, not detectable.

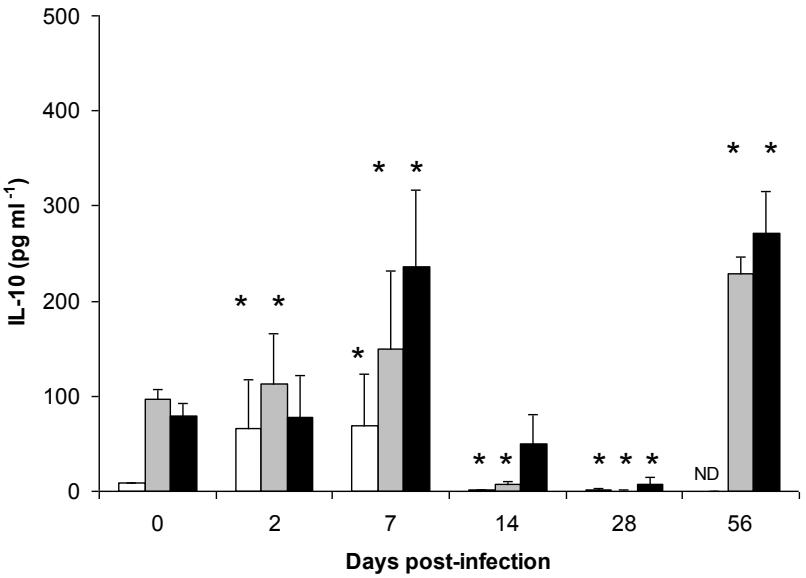

Figure 22. *In vitro* production of IL-10 by $CD4^+CD25^-$ T cell fractions, purified from pooled draining lymph nodes and spleen cells of *L. infantum*-infected and naïve BALB/c mice, and cultured *in vitro* alone (□), in the presence of mitomycin C-treated antigen presenting cells (APC) at a ratio of 1 lymphocyte per 1 APC activated with soluble anti-CD3ε (■) or with *Leishmania* antigen (■) were evaluated. Cytokines were quantified by ELISA in culture supernatant after three days of activation. Results are mean values ± SEM expressed in $pg.ml^{-1}$. *($p<0.05$) indicates statistically significant differences in comparison to naïve cells (day 0). ND, not detectable.

1.4. Gene expression analysis of *tlr2*

To test whether the $CD25^+$ T cell fraction isolated expressed TLR-2 and to compare levels of induction during *Leishmania* infection, murine TLR-2 gene expression was monitored by real-time PCR.

$CD4^+CD25^+$ T cells isolated and separated from spleen and LN cells did indeed express detectable levels of murine TLR-2 (Figure 23). In response to *L. infantum* infection TLR-2 expression was

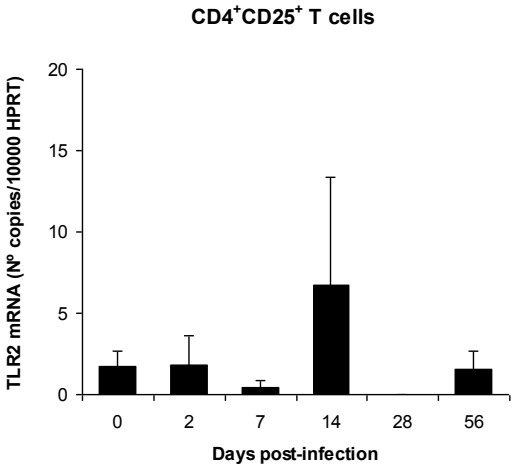

Figure 23. Expression of *tlr2* mRNA by $CD4^+CD25^+$ cell fractions from pooled draining lymph nodes and spleen cells of *L. infantum*-infected BALB/c mice at 2, 7, 14, 28 and 56 days pi. Results were analyzed by real-time PCR and data are mean values ± SEM expressed as number of copies x 10000 copies of housekeeping gene HPRT.

increased in relation to non-infected animals at days 2 and 14 pi and reduced at days 7, 28 and 56 pi, although these differences were not statistically significant.

2. Evaluation of the effect of TLR-2 modulation on Treg populations during *L. infantum in vivo* infection

2.1. Parasite detection

The number of parasites was detected in spleen and liver of TLR-2 deficient (TLR2-/-) and wild-type C57Bl/6 (WT) mice by limiting dilution assay.

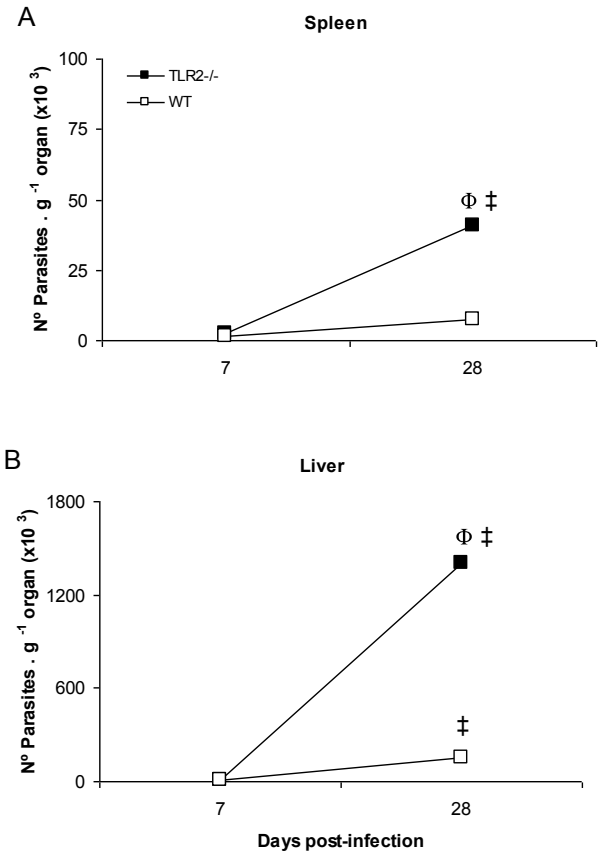

Figure 24. Determination of parasite burden in spleen and liver of TLR-$2^{-/-}$ and WT mice on a C57Bl/6 background infected with *L. infantum* promastigotes. Parasite load was determined at 7 and 28 days post-infection (pi) by limiting dilution assay. Results are expressed as the number of parasites per gram (g) of homogenized organ. Φ (p<0.05) and ‡ (p<0.05) indicate statistically significant differences when comparing TLR-$2^{-/-}$ vs WT mice and days 7 vs. 28 pi, respectively.

TLR-2$^{-/-}$ mice showed pronounced parasite expansion and seemed to be more susceptible than WT mice to *L. infantum* (Figure 24).

Higher rates of parasite multiplication, at both time points were observed in spleen and liver of TLR-2$^{-/-}$ mice, showing evidence of non-healing infection. Significant high values were observed on day 28 pi in the spleen (p=0.003) and in the liver (p<0.001). The liver showed higher rates of parasitization than spleen regardless of type of mice or observational time point. Increases in parasite burden with infection time was found significant in both spleen and liver of TLR-2$^{-/-}$ mice (p=0.002) but only in liver of WT mice (p=0.001).

2.2. Phenotypic characterization of regulatory T cells

To evaluate the influence of the TLR-2 gene in parasite-induced regulation of Treg and protective immunity to *L. infantum* infection in mice with resistant phenotype, *in vivo* Treg subpopulations were phenotypically characterized in both gene disrupted TLR-2$^{-/-}$ knock-out and WT mice.

In contrast to the first part of this study, FOXP3 protein expression was evaluated in the same T cell subsets by flow cytometry, through intracellular detection of FOXP3 nuclear transcription factor together with other Treg surface markers (Figure 25).

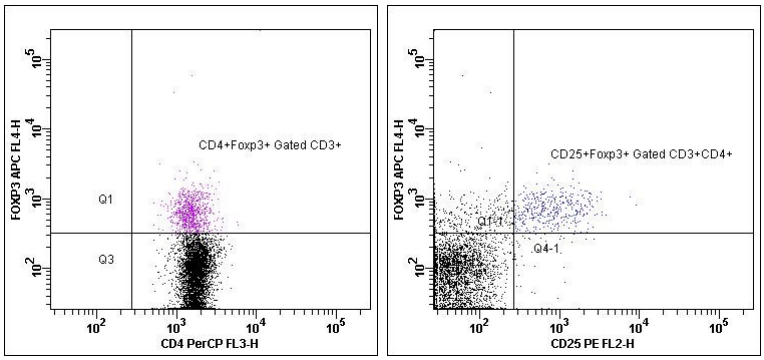

Figure 25. A typical dot plot of the population of CD3$^+$CD4$^+$CD25$^+$FOXP3$^+$ T cells from C57Bl/6 mice.

Non-infected TLR-2$^{-/-}$ mice showed reduced numbers of CD3^{+}CD4^{+}CD25^{+}FOXP3^{+} T cells in relation to their WT counterparts although no significant differences were observed (Figure 26). However, when looking specifically to other Treg markers, such as CD4^{+}CD25^{+}GITR^{+}FOXP3^{+} T cells, the number of cells in infected TLR-2$^{-/-}$ mice were significantly reduced (p=0.008) when compared to WT mice during the initial phase of infection (day 7 pi) (Figure 26B). But in WT mice, CD4^{+}CD25^{+}GITR^{+}FOXP3^{+} T cells actually decreased significantly with infection ($p_{\text{day 28 } vs. \text{ day 0}}$=0.026; $p_{\text{day 28 } vs. \text{ day 7}}$=0.024).

In WT mice, CD4^{+}CD25^{+}CD103^{+}FOXP3^{+} T cells also decreased upon initial infection ($p_{\text{day 7 } vs. \text{ day 0}}$=0.006) but recovered cell numbers ($p_{\text{day 28 } vs. \text{ day 7}}$=0.016) (Figure 26C). In TLR-2$^{-/-}$ mice, significant increases in CD4^{+}CD25^{+}CD103^{+}FOXP3^{+} T cells were detected after 28 days pi ($p_{\text{day 28 } vs. \text{ day 0}}$=0.001; $p_{\text{day 28 } vs. \text{ day 7}}$=0.003). Also, *L. infantum* induced significantly higher levels of CD103^{+} Treg in TLR-2$^{-/-}$ mice than in WT at both times pi ($p_{\text{day 7 } vs. \text{ day 0}}$ =0.006; $p_{\text{day 28 } vs. \text{ day 7}}$=0.008). The same was observed at day 28 pi for Treg with memory phenotype CD4^{+}CD25^{+}CD45RB^{low}FOXP3^{+} T cells when comparing with cells from non-infected TLR-2$^{-/-}$ and day 7 pi (p =0.006) and when comparing both type of mice (p=0.004) (Figure 26D).

So the results indicate that in WT mice reductions of GITR^{+} and CD103^{+} FOXP3^{+} Treg occur with *L. infantum* infection while gene disrupted TLR-2$^{-/-}$ mice show clear induction of CD103^{+} CD45RB^{low} memory Treg with late infection. This may imply that WT mice are perhaps more capable of surmounting a more robust effector response against the parasite since the presence of immunosuppressive Treg seems hampered. The absence of TLR-2 seems to favour Treg accumulation and retention in infected spleen which is confirmed in TLR-2$^{-/-}$ mice. So TLR-2 could play a role in regulating Treg since in WT mice retention is not evident.

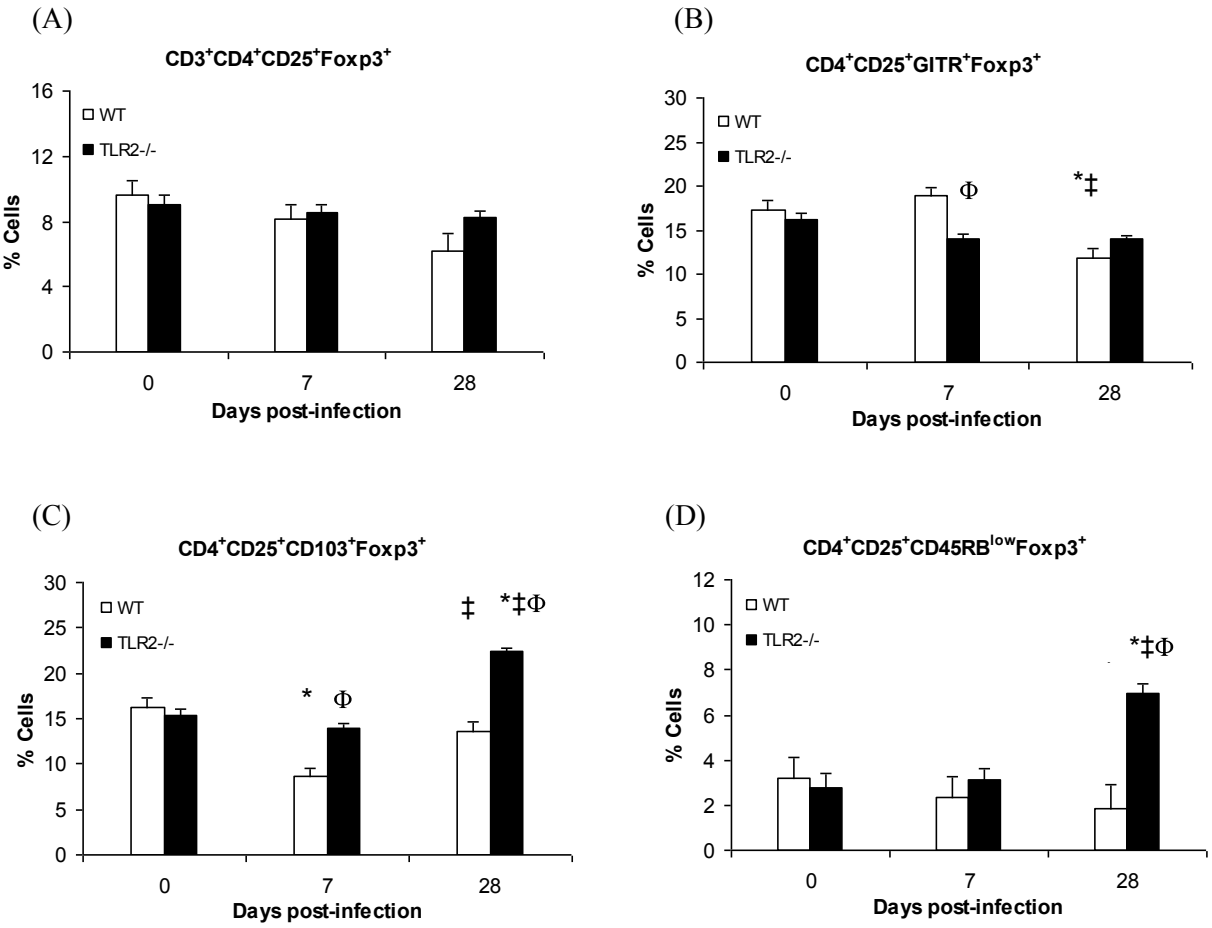

Figure 26. Characterization of Treg cell populations during *L. infantum* infection. Frequency of cells isolated from spleen of WT and TLR-2$^{-/-}$ on a C57BL/6 background at days 7 and 28 pi. Cells were analyzed for surface and intracellular expression of CD3, CD4, CD25, GITR, CD103, CD45RB and FOXP3 (A-D). Results are mean values ± SEM, representative of two independent experiments. * (p<0.05), ‡ (p<0.05) and Φ (p<0.05) indicate statistically significant differences when comparing samples from control (day 0) vs. infected animals, days 7 vs. 28 pi and TLR-2$^{-/-}$ vs. WT mice, respectively.

Several effector T cell subsets were also analyzed. Significant increases in $CD3^+CD4^+CD25^-$ $FOXP3^-$ T cells from both TLR-2$^{-/-}$ and WT mice were only seen in response to late infection ($p_{\text{TLR2-/- day 28 } vs. \text{ day 0}}$=0.006; $p_{\text{WT day 28 } vs. \text{ day 0}}$=0.047) (Figure 27A). So different mouse genotype does not seem to have different outcomes in the number of splenic effector cells induced with infection.

It is now generally accepted that $CD4^+FOXP3^+$ T cells with low amounts of CD25 or even lacking CD25 expression show potent suppressive capacity (Lehmann et al., 2002; Fontenot et al., 2005). For this reason, $FOXP3^+$ cells within the $CD25^-$ and $CD103^+$ cell fraction has also been analyzed. Similarly to $GITR^+$ Treg (Figure 26B), TLR-$2^{-/-}$ showed lower number of $CD4^+CD25^-FOXP3^+$ T cells when compared to WT mice (p=0.001) (Figure 27B). In WT mice, these cells decreased at day 28 pi ($p_{\text{day 28 } vs. \text{ day 0}}$=0.033; $p_{\text{day 28 } vs. \text{ day 7}}$=0.009). On the other hand, $CD4^+CD25^-CD103^+FOXP3^+$ T cells of WT mice behaved in the same fashion as regulatory $CD4^+CD25^+CD103^+FOXP3^+$ T cells, having decreased numbers ($p_{\text{day 28 } vs. \text{ day 0}}$=0.008) and significantly lower than TLR-$2^{-/-}$ mice (p=0.038) (Figure 27C).

WT mice weren't initially able to induce effective effector response upon *L. infantum* infection even though there were reduced Treg numbers in these mice. C57BL/6 mice experimentally infected with VL strains are known to be initially susceptible to infection, showing rapid increases in liver parasite burden until 4 weeks of infection. After reaching a plateau, the number of parasites decline and the mice develop self-curing phenotype and low levels of chronic infection (Murray et al., 2001). In fact, increased effector response was only observed later during infection ($CD3^+CD4^+CD25^-FOXP3^-$).

Even though, TLR-$2^{-/-}$ mice showed more Treg retention, this did not seem to affect effector T cell response against *L. infantum*. So, immunosuppression is not evident, regarding influence on effector response, in the absence of TLR-2. Other mechanisms may be involved in eliciting effector response and by-passing the action of immunosuppressive Treg.

Regarding $FOXP3^+$ populations within the CD25 negative subset, again in TLR-$2^{-/-}$ mice, the numbers are not increased in relation to WT. However, in WT mice this $FOXP3^+$ population and the $CD103^+FOXP3^+$ population within the CD25 negative cell fraction do still decrease with infection as expected, the latter being more reduced in relation to TLR-$2^{-/-}$ as well.

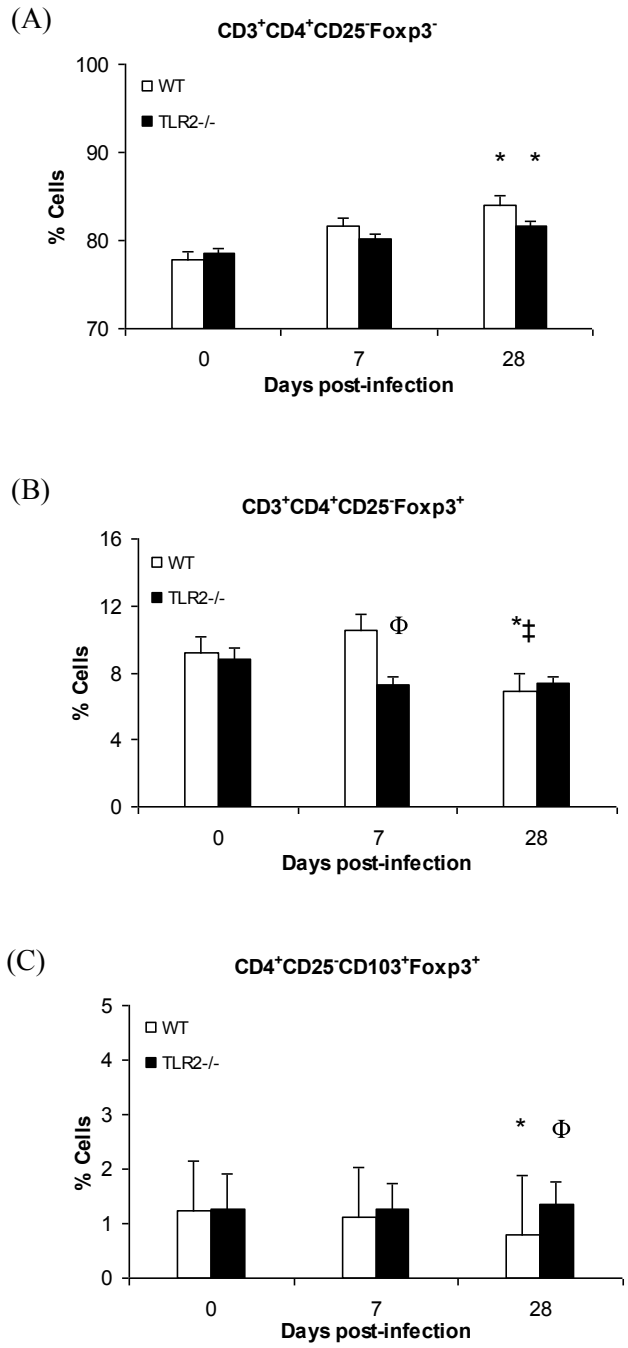

Figure 27. Characterization of CD25 negative $CD4^+$ populations during *L. infantum* infection. Frequency of cells isolated from spleen of WT and $TLR\text{-}2^{-/-}$ on a C57BL/6 background at days 7 and 28 pi. Cells were analyzed for surface and intracellular expression of CD3, CD4, CD25, CD103 and FOXP3 (A-C). Results are mean values ± SEM representative of two independent experiments. * (p<0.05), ‡ (p<0.05) and Φ (p<0.05) indicate statistically significant differences when comparing samples from control (day 0) vs. infected animals, days 7 vs. 28 pi and $TLR\text{-}2^{-/-}$ vs. WT mice, respectively.

2.3. Detection of cytokine transcripts

In order to evaluate whether the absence of TLR-2 affects protective immune response and consequent outcome of *L. infantum* infection, cytokine response was analyzed at each observational time point as well as in both TLR-$2^{-/-}$ and WT mice and in separate cell and tissue samples so as to study the influence of this gene on specific organ response.

2.3.1. IFN-γ

The spleen, a peripheral lymphoid organ, is regarded as one of the centers of activity of the reticuloendothelial system which is part of the immune system, consisting of phagocytic cells located in reticular connective tissue, primarily monocytes and macrophages. The spleen is also the organ where B cells that migrate from the bone marrow mature and where mature T lymphocytes circulate and differentiate into effector and memory lymphocytes upon exposure to antigen. Taking all this into consideration, it is also important to evaluate total splenic tissue response and not just single-cell response.

An initial IFN-γ response to *L. infantum* infection was transiently elicited in TLR-$2^{-/-}$ mice by total mononuclear cells isolated from the spleen at day 7 pi (p=0.048) (Figure 28A). Total lymph node cells from WT mice showed an early Th1 response with significant expression of IFN-γ in relation to control (p=0.003). However, during the course of infection this ability was significantly lost (p=0.001) (Figure 28B). TLR-$2^{-/-}$ mice were less capable of expressing IFN-γ at day 7 pi (p=0.043) in comparison to WT, maintaining at day 28 pi, low expression levels (p=0.003; p=0.001). When looking at total spleen tissue, the same response occurs later showing increased but late expression of IFN-γ at day 28 pi ($p_{\text{day 28 } vs. \text{ day 0}}$=0.013) when compared to non-infected mice and to day 7 pi ($p_{\text{day 7 } vs. \text{ day 28}}$=0.020) and also in relation to TLR-$2^{-/-}$ mice (p=0.019) (Figure 28C). Liver tissue of both types of mice did not respond with significant variations in IFN-γ expression (Figure 28D).

Results show that spleen cells of TLR-2$^{-/-}$ mice initially produced weak IFN-γ response to infection and showed significantly lower levels during early infection in LN and later in total spleen tissue than WT mice.

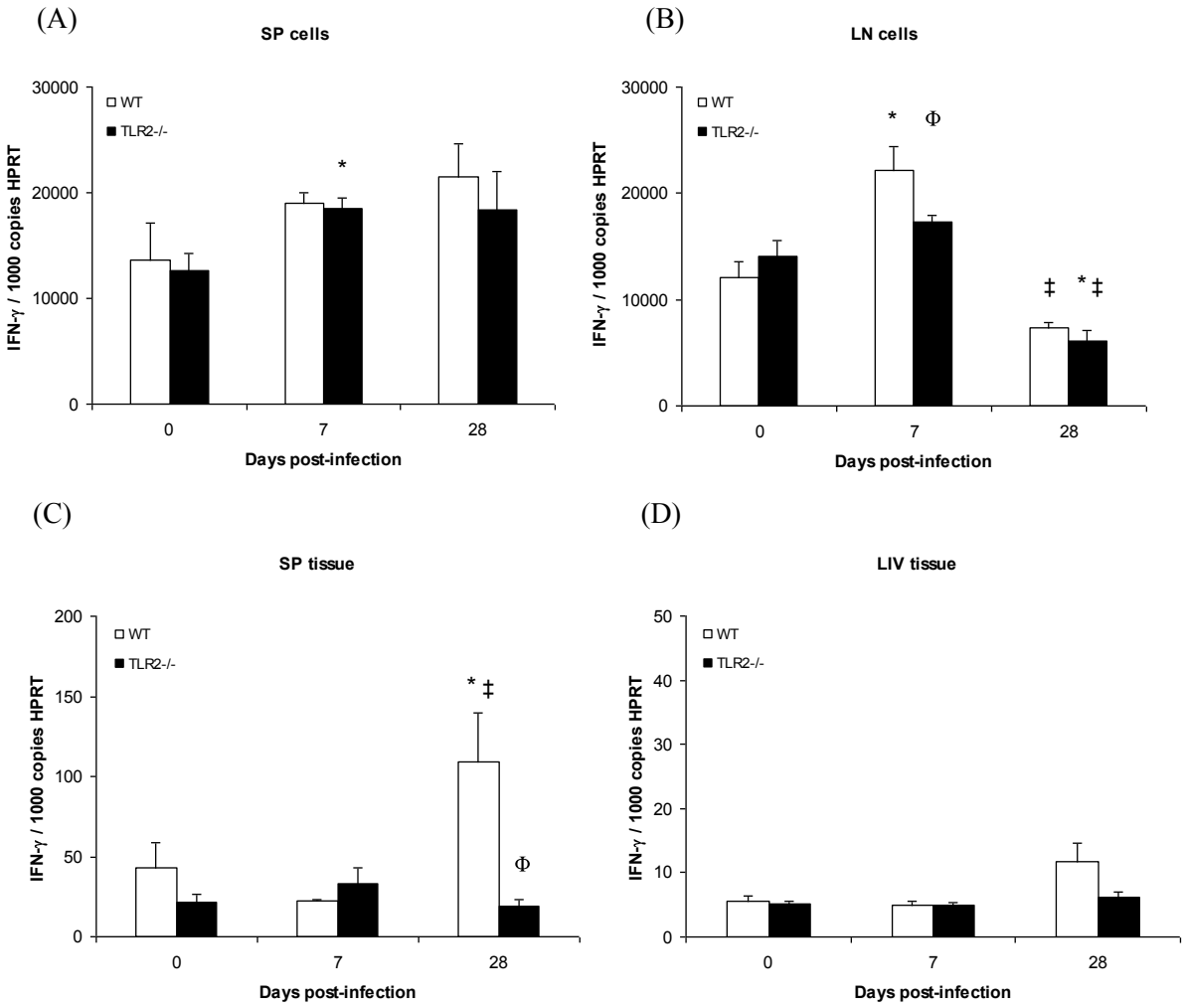

Figure 28.*Ex vixo* mRNA expression of IFN-γ by isolated mononuclear spleen (SP) cells (A), total SP tissue (B), isolated lymph node cells (LN) (C) and liver (LIV) tissue (D) from *L. infantum*-infected wild-type (WT) and TLR-2$^{-/-}$ knock-out mice (TLR2-/-). Results were analyzed by real-time PCR and data are mean values ± SEM representative of two independent experiments and expressed as the number of copies per 1000 copies of housekeeping gene HPRT. * (p<0.05), ‡ (p<0.05) and Φ (p<0.05) indicate statistically significant differences when comparing samples from control (day 0) vs. infected animals, days 7 vs. 28 pi and TLR-2$^{-/-}$ vs. WT mice, respectively.

This associated to increases observed in effector T cell populations late during infection in WT mice might explain better control of parasite load in WT mice. In addition, $GITR^+$ Treg were also found to be low in number later during infection. However, in LN an early IFN-γ response was more evident in WT mice, indicating differential organ response perhaps due to other circulating immune cells also capable of producing this cytokine (e.g. $CD8^+$ T cells, NK cells).

Overall, Th1 response mediated by IFN-γ was not strong or clearly evident in TLR-$2^{-/-}$ mice, perhaps a reflection of the presence of high levels of accumulating Treg.

2.3.2. IL-4

In spleen cells of TLR-$2^{-/-}$ mice, IL-4 was found reduced after day 28 pi ($p_{\text{day 28 } vs. \text{ day 0}}$=0.005; $p_{\text{day 28 } vs. \text{ day 7}}$=0.001) and significantly less in relation to WT mice (p=0.001) (Figure 29A). In addition, in spleen tissue IL-4 response also increased significantly in WT mice early upon infection ($p_{\text{day 7 } vs. \text{ day 0}}$=0.001) but then decreased ($p_{\text{day 28 } vs. \text{ day 7}}$=0.001) with the course of infection (Figure 29C). The same occurred in TLR-$2^{-/-}$ mice ($p_{\text{day 7 } vs \text{ day 0}}$=0.002) where it was significantly higher than WT mice (p=0.046). At day 28 pi a decrease in IL-4 expression was detected in TLR-$2^{-/-}$ mice ($p_{\text{day 28 } vs. \text{ day 7}}$=0.021).

Similar pattern was observed by LN cells, having increased significantly IL-4 expression in WT mice at day 7 pi ($p_{\text{day 7 } vs. \text{ day 0}}$=0.010) and then decreased later ($p_{\text{day 28 } vs. \text{ day 0}}$=0.012; $p_{\text{day 28 } vs. \text{ day 7}}$ <0.001) (Figure 29B). Again this was seen in TLR-$2^{-/-}$ mice ($p_{\text{day 7 } vs. \text{ day 0}}$<0.001) but in contrast to WT mice, IL-4 maintained elevated in relation to control at day 28 pi ($p_{\text{day 28 } vs. \text{ day 0}}$=0.001) and was significantly higher than WT (p<0.001).

In liver, high levels of IL-4 expression was only detected at day 28 pi in TLR-2$^{-/-}$ mice ($p_{\text{day 28 } vs. \text{ day } 0} = 0.027$) (Figure 29D).

The results from spleen tissue and LN give evidence of an early IL-4 burst which occurs in both types of mice but is more accentuated in spleen tissue of TLR2$^{-/-}$ mice. After 28 days of infection, IL-4 expression subsides substantially in spleen of TLR2$^{-/-}$ mice. This strong IL-4 inhibition late during infection in TLR2$^{-/-}$ mice is interesting since one would expect susceptible mice to have high levels of Th2 cytokines.

The fact that Treg levels considerably increase later during infection of these mice, may suggest that Treg in spleen may be mediating Th2 response, by inhibiting local IL-4 response as infection progresses. However, when considering other organs like LN and liver, a different pattern can be seen. IL-4 actually remains relatively higher in TLR-2$^{-/-}$ mice in relation to WT mice. So, differential organ IL-4 response may influence the outcome of immune response to *L. infantum* infection.

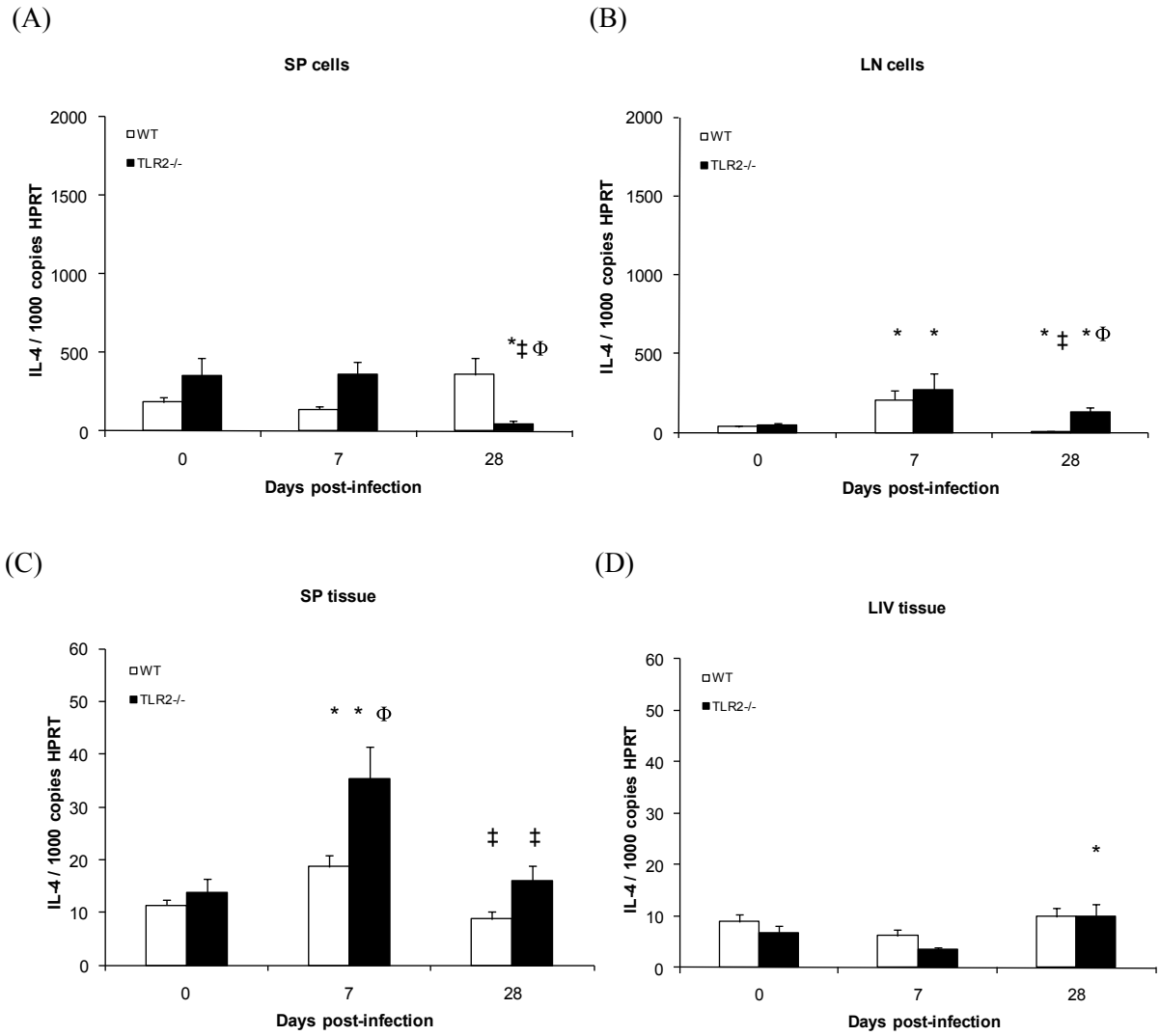

Figure 29.*Ex vixo* mRNA expression of IL-4 by isolated mononuclear spleen (SP) cells (A), total SP tissue (B), isolated lymph node cells (LN) (C) and liver (LIV) tissue (D) from *L. infantum*-infected wild-type (WT) and TLR-2$^{-/-}$ knock-out mice (TLR2-/-) Results were analyzed by real-time PCR and data are mean values ± SEM representative of two independent experiments and expressed as the number of copies per 1000 copies of housekeeping gene HPRT. * (p<0.05), ‡ (p<0.05) and Φ (p<0.05) indicate statistically significant differences when comparing samples from control (day 0) vs. infected animals, days 7 vs. 28 pi and TLR-2$^{-/-}$ vs. WT mice, respectively.

2.3.3. IL-10

In WT mice, IL-10 expression gradually increased with infection time at day 7 pi (p=0.012) and 28 pi ($p_{\text{day 7 vs. day 0}}$<0.001; $p_{\text{day 28 vs. day 7}}$<0.001) in SP cells (Figure 30A). TLR-2$^{-/-}$ mice behaved similarly with increases at day 28 pi ($p_{\text{day 28 vs. day 0}}$=0.002; $p_{\text{day 28 vs. day 7}}$ =0.004). However, total spleen tissue showed low IL-10 levels and no significant variations (Figure 30C).

LN cells from TLR-2$^{-/-}$ mice presented increased values at day 28 pi ($p_{\text{day 28 vs. day 7}}$<0.001) (Figure 30B). In liver although IL-10 values were lower than other organs, an early transient decrease was observed in WT mice ($p_{\text{day 7 vs. day 0}}$ =0.007), followed by increasing levels at day 28 pi ($p_{\text{day 28 vs. day 0}}$=0.046; $p_{\text{day 28 vs. day 7}}$<0.001) (Figure 30D). On the other hand, TLR-2$^{-/-}$ mice also increased IL-10 values after one month of infection ($p_{\text{day 28 vs. day 0}}$=0.027; $p_{\text{day 28 vs. day 7}}$<0.001). However, no differences between both types of animals were seen regarding IL-10.

In general, IL-10 expression is mainly observed during late infection, regardless of mice type, although in LN this is only evident in TLR-2$^{-/-}$ mice. Late IL-10 expression could be contributing to higher parasite multiplication rates observed in the liver when comparing to spleen. Spleen cells of WT mice showed some ability to express IL-10 earlier upon infection although in the liver, inhibition was observed in comparison to controls. This could indicate that liver shows some evidence of initial control of infection since the source of immunosuppressive IL-10 seems to be inhibited. This may be related to lower CD103^{+} Treg retention that was detected at the same time point, hence less immunosuppression, better effector response and lower parasite load in relation to TLR-2$^{-/-}$ mice. But the absence of TLR-2 and high Treg retention detected does not seem to correlate with IL-10 expression.

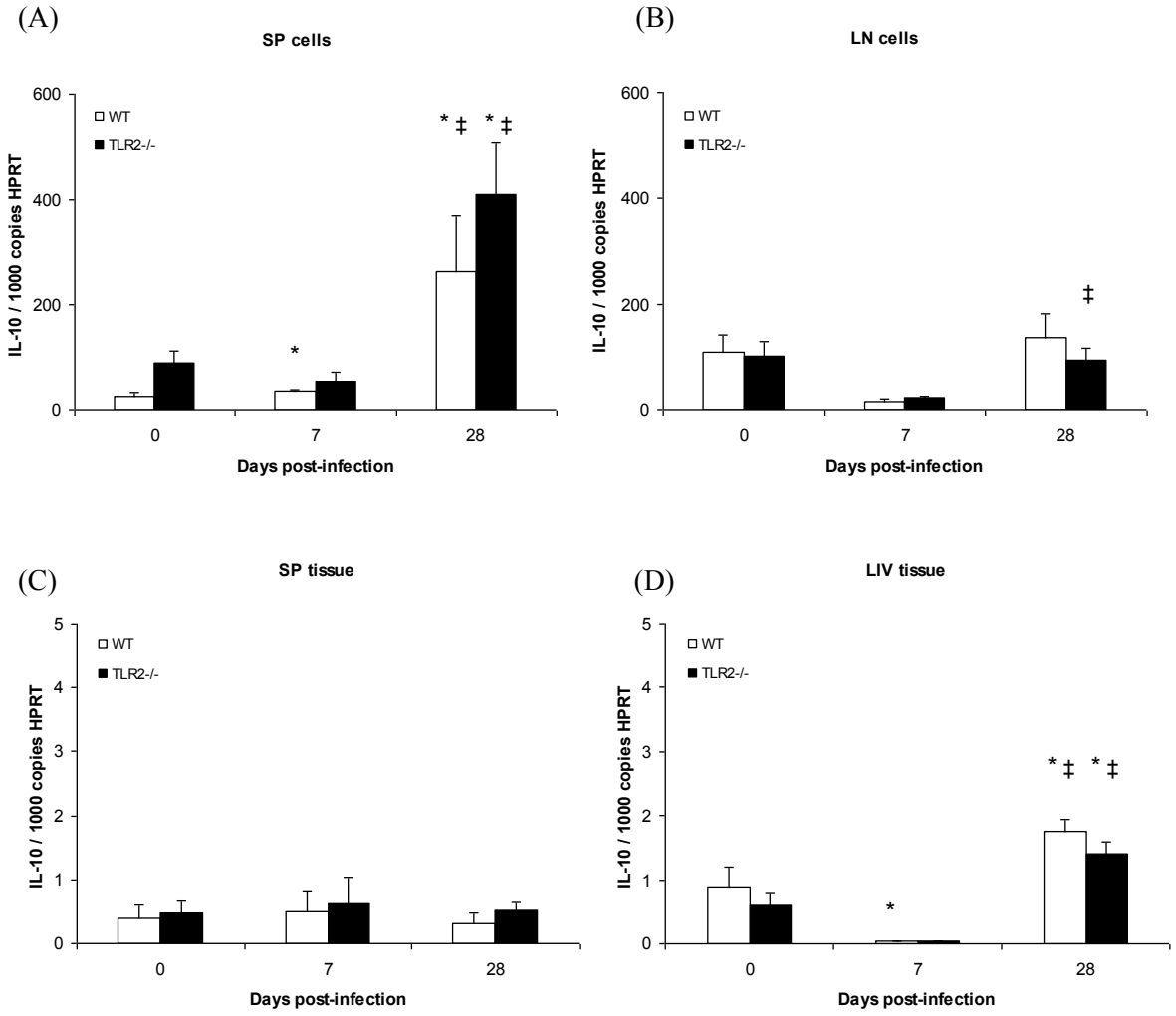

Figure 30. *Ex vixo* mRNA expression of IL-10 by isolated mononuclear spleen (SP) cells (A), total SP tissue (B), isolated lymph node cells (LN) (C) and liver (LIV) tissue (D) from *L. infantum*-infected wild-type (WT) and TLR-$2^{-/-}$ knock-out mice (TLR2-/-). Results were analyzed by real-time PCR and data are mean values ± SEM, representative of two independent experiments and expressed as the number of copies per 1000 copies of housekeeping gene HPRT. * (p<0.05) and ‡ (p<0.05) indicate statistically significant differences when comparing samples from control (day 0) vs. infected animals and days 7 vs. 28 pi, respectively.

2.3.4. TGF-β

At day 7 pi, SP cells from TLR-2$^{-/-}$ mice showed higher TGF-β levels in relation to their WT counterparts (p<0.001) (Figure 31A). However, infected WT mice showed fairly higher levels of

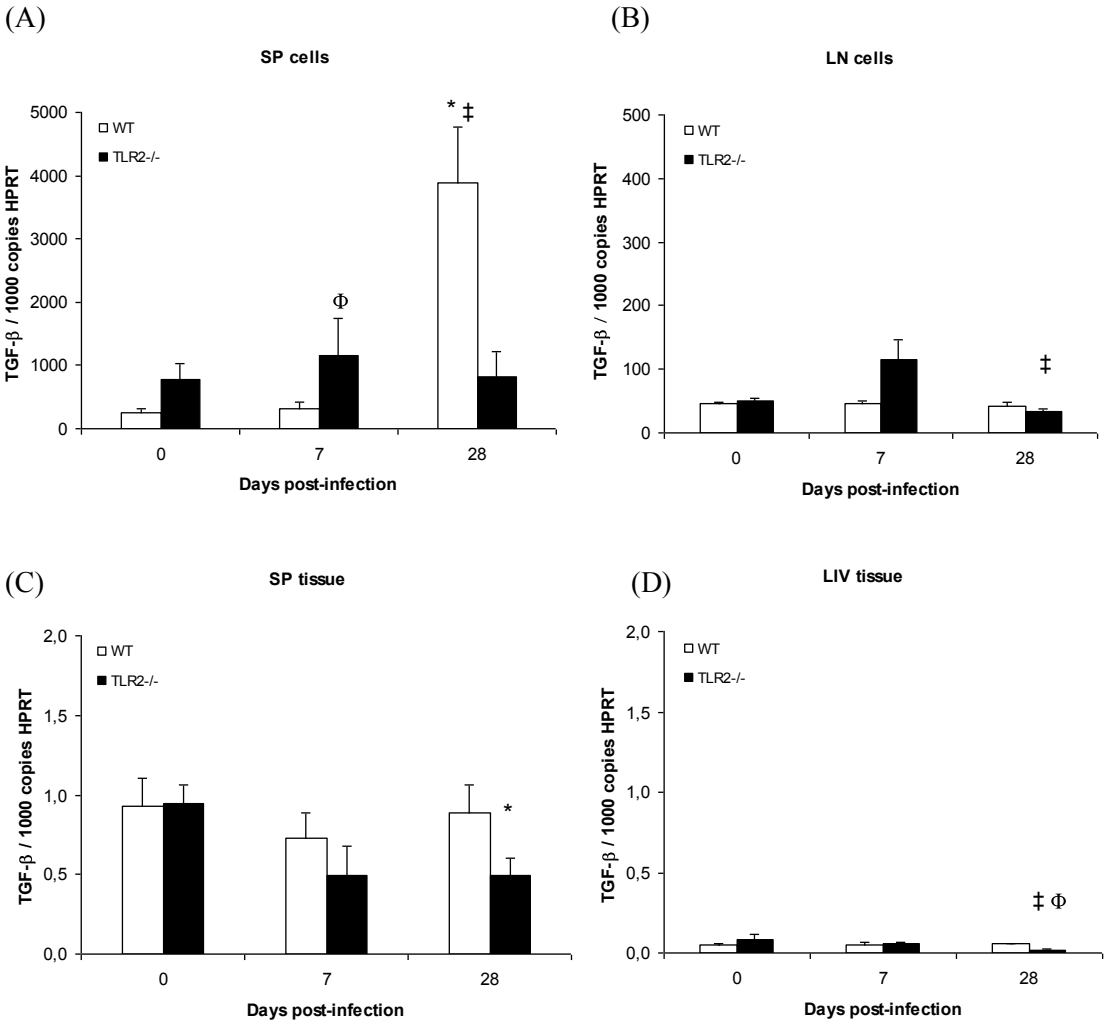

Figure 31. *Ex vixo* mRNA expression of TGF-β by isolated mononuclear spleen (SP) cells (A), total SP tissue (B), isolated lymph node cells (LN) (C) and liver (LIV) tissue (D) from *L. infantum*-infected wild-type (WT) and TLR-2$^{-/-}$ knock-out mice (TLR2-/-). Results were analyzed by real-time PCR and data are mean values ± SEM representative of two independent experiments and expressed as the number of copies per 1000 copies of housekeeping gene HPRT. * (p<0.05), ‡ (p<0.05) and Φ (p<0.05) indicate statistically significant differences when comparing samples from control (day 0) vs. infected animals, days 7 vs. 28 pi and TLR-2$^{-/-}$ vs. WT mice, respectively.

TGF-β with a significant increase during late infection ($p_{\text{day 28 } vs. \text{ day 0}}<0.001$; $p_{\text{day 28 } vs. \text{ day 7}}=0.006$).

With late infection, TLR-2$^{-/-}$ mice showed decreased TGF-β levels in SP tissue ($p_{\text{day 28 } vs. \text{ day 0}}=0.022$) (Figure 31C) as well as, in LN cells ($p_{\text{day 28 } vs. \text{ day 7}}=0.033$) (Figure 31B)**.** In liver, the same pattern was seen in TLR-2$^{-/-}$ mice with decreased TGF-β levels observed at day 28 pi ($p_{\text{day 28 } vs. \text{ day 7}}=0.006$) and in relation to WT mice ($p<0.001$) (Figure 31D).

Initially, spleen cells of TLR-2$^{-/-}$ mice expressed higher TGF-β levels than WT mice but later during infection, a strong reduction in the ability to express this cytokine was observed in spleen tissue, LN and liver suggesting a minor contributing role for TGF-β to susceptibility of TLR-2$^{-/-}$ mice to *L. infantum*.

Surprisingly, WT showed a strong TGF-β response to late infection that does not seem to contribute to increased parasite multiplication since these mice are effectively more resistant to *L. infantum*. This gives indication that although in the spleen, WT are able to develop a pro-inflammatory response with expression of IFN-γ, this occurs together with an anti-inflammatory response with detection of IL-10 and TGF-β. So, the Th1 response observed was not efficient and may have been hampered by clear evidences of immunosuppression in spleen of WT mice. However, these immunosuppressive cytokines do not seem to be involved in Treg-mediated suppression since they did not accumulate in WT mice. Despite this, these mice end up developing chronic infection and are not able to clear the parasite. On the other hand, TLR-2$^{-/-}$ mice were not able to develop a Th1 response and IL-10 seems to be the only contributing factor to immunosuppression in the spleen. However, the expression of IL-10 did not correlate with high Treg levels also detected in these mice suggesting both Treg and IL-10 of non-Treg origin may both be contributing to increased susceptibility of TLR-2$^{-/-}$ mice to *L. infantum*.

2.4. Histopathology

Initial control and eventual resolution of hepatic infection by visceralizing *Leishmania spp.* in normal mice (eg. C57Bl/6) are accomplished with well-formed mature tissue granulomas. In the liver, these immunologically active, inflammatory structures are assembled, upon antigen-specific response, around a core of fused, parasitized resident macrophages (Kupffer cells). The granuloma formation is normally associated to an influx of various effector cell populations (granulocytes, $CD4^+$ and $CD8^+$ T cells) that are known to target *Leishmania*-infected macrophages. Under optimal conditions, these functional structures limit infection, kill and remove the microbial target and then repair any accompanying tissue injury.

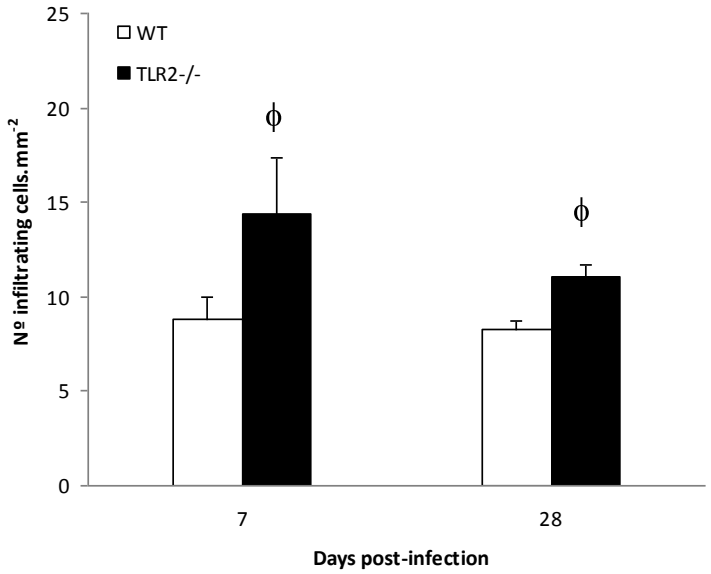

Figure 32. Liver tissue response to *L. infantum* in wild-type (WT) and TLR-$2^{-/-}$ knock-out mice (TLR2-/-) and the effect on the extent of granuloma formation. Inflammation in the liver was scored semi-quantitatively by estimating the total number of infiltrating cells per square millimeter per granuloma. Results were expressed as the mean number of infiltrating cells x mm^{-2} of granuloma for each infected mice group + SEM. $^{\phi}$ ($p<0.05$), indicate statistically significant differences when comparing TLR-$2^{-/-}$ vs. WT mice, respectively.

The overall antimicrobial efficacy of the granulomatous response appears to be variable, however, and depends upon host determinants, the pathogen and the criteria used to define efficacy. Although microbial eradication is the ideal outcome in infections which trigger granuloma assembly, it is not clear how often this objective is actually ever achieved. Thus, despite even an initially intense granulomatous response, a number of pathogens, including *L. donovani*, establish a well recognized state of chronic intracellular parasitism,

Significant differences between mice genotypes were observed in terms of hepatic inflammatory reaction at both time points of infection. TLR-$2^{-/-}$ mice showed granulomas with significantly larger infiltrates than WT mice ($p_{day\ 7}$= 0.034; $p_{\text{day } 28}$ =0.001) (Figure 32). Images of hepatic infiltration and granulomas are showed in Figure 33.

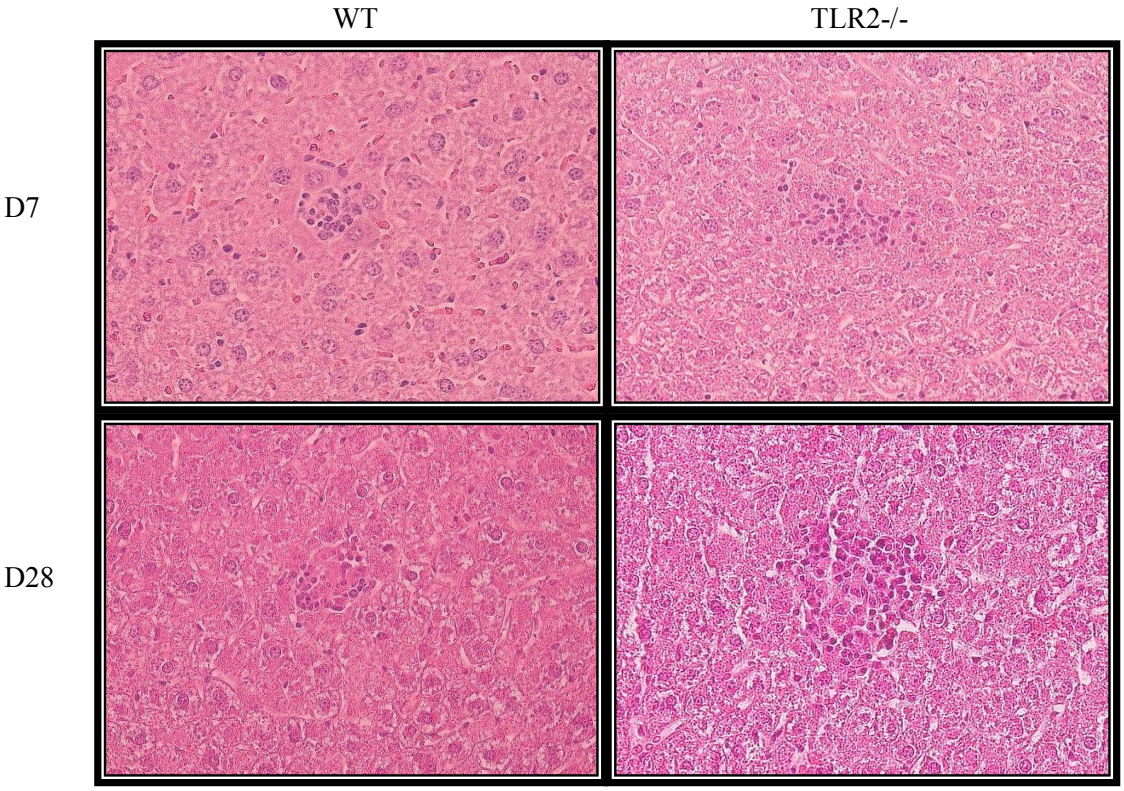

Figure 33. Liver histological response to *L. infantum* infection, 7 and 28 days after intravenous infection of *L. infantum* TLR-$2^{-/-}$ knock-out mice (TLR2-/-) (A) and wild-type (WT) C57BL/6 mice (B). Granuloma formation (arrows) was evaluated by examining fixed liver tissue sections stained with hematoxylin and eosin using light microscopy (magnification 200X)

CHAPTER IV. DISCUSSION

Infection with *Leishmania* protozoan parasite is a major human health issue worldwide, causing morbidity and mortality that, particularly in developing countries contributes to political, social and economic instability. There are currently no vaccines against any form of human leishmaniasis available to prevent this devastating infection. One of the underlying reasons for the lack of a successful vaccine is intrinsically linked to the complexity, as well as the incredible diversity of immune mechanisms involved in the pathogenesis caused by *Leishmania spp*. Surviving a given infection such as leishmaniasis requires the generation of a controlled immune response. Understanding these cell-mediated immunological mechanisms for controlling infection is essential for the development of effective prophylactic and therapeutic vaccines.

L. infantum is the causative agent of zoonotic visceral leishmaniasis (ZVL). It has been clearly established that *L. infantum/L. chagasi*-human interactions evolve under different forms: asymptomatic or subclinical infection, with few or reduced symptoms to typical symptomatic visceral leishmaniasis (VL) (Wilson et al., 1995). VL is characterized by marked parasitism in the spleen and bone marrow, fever, cachexia, hepatosplenomegaly and suppressed immune cell responses. Pathogenesis results from active parasite replication within macrophages and widespread dissemination to other organs and tissues of the mononuclear phagocytic system. Acute VL disease is associated with an ineffective parasite-specific T cell-mediated immune response since $CD4^+$ T cells, that are essential for acquired resistance to the invading parasite, can also contribute to the pathogenesis of leishmaniasis (Carvalho et al., 1994). Although, PBMCs of human VL patients respond to leishmanial antigen with the production of pro-inflammatory cytokines such as IL-2, IFN-γ and IL-12, these cells are unable to proliferate *in vitro* in response to antigen stimulation (Ghalib et al., 1995). In human and murine cutaneous leishmaniasis, non-healing response is normally associated with a Th2 type immune response with early and sustained production of IL-4. Elevated IL-4 levels have been observed in VL (Sundar et al., 1997; Nylen et al., 2007) however no association with the active disease has so far been described. High levels of IL-10 and TGF-β associated to general immunosuppression have been extensively described in

human and murine forms of ZVL (Ghalib et al., 1993; Karp et al., 1993; Rodrigues et al., 1998; Wilson et al., 1998; Gomes-Pereira et al., 2004; Caldas et al., 2005; Nylen et al., 2007). So, unfavourable VL disease outcome does not seem to be related to Th2 dominance nor a defective Th1 immune response *per* se. Other immunosuppressive or immune-evasion mechanisms may contribute to pathogenesis of VL.

Recently, there has been an explosion of interest in regulatory T cells (Treg), a particular subset of inhibitory immune cells involved in down regulating excessive immune effector response. Special focus has been given to the role that these cells may play during *Leishmania* infections and the control they may or may not exert on effective protection against these pathogens. $CD4^+CD25^+$ regulatory T cells were the first Treg to be shown to play a critical role in *Leishmania* persistence during infection (Belkaid et al., 2002). Whilst acting as a double-edged sword by exhibiting immunosuppressive properties and controlling the extent of immune-mediated pathology normally associated to *Leishmania* infections, Treg also invariably lead to incomplete parasite clearance and long-term pathogen persistence. Although the majority of the studies focus on Treg and cutaneous leishmaniasis (Belkaid et al., 2002; Ji et al., 2004; Karimini et al., 2005; Campanelli et al., 2006; Salhi et al., 2008; Bourreau et al., 2009), there are only three Treg studies in the murine *L. donovani* model and in human visceral leishmaniasis (Stager et al., 2006; Nylen et al., 2007; Saha et al., 2007).

To our knowledge, no studies up to this present work have been directed towards the participation of Treg during infection with *L. infantum*. Chronic *L. infantum* infections show neither evidences of a polarized Th cytokine response nor efficient parasite control. Regarding the immune response developed to infections with *L. infantum* and the underlying mechanisms elicited by this species in particular, no clear-cut response has until now been observed. Therefore, it is important to address the role for regulatory T cells in the control of immune response during ZVL. The purpose of this study is to evaluate whether Treg cells expand within sites of infection with visceralizing

Leishmania infantum in susceptible BALB/c mice and whether these cells are able to modulate effector immune response induced by this parasite, and contribute to maintain the balance between host immunity and pathology, resulting in chronic or persistent infection.

Several molecules characteristic of Treg were analyzed during *L. infantum* infection of BALB/c mice. An early expansion of $CD4^+CD25^+$ T cells also expressing GITR was observed after just one week of infection coinciding with increases in parasite burden in spleen. GITR is constitutively expressed at high levels on murine and human Treg cells. However, GITR must be used in combination with other Treg markers since it is not specific to Treg-like cells. It is also found in basal amounts on conventional $CD4^+$ T cells (McHugh et al., 2002; Shimizu et al., 2002). Activated T cells can also up regulate GITR expression (Shevach et al., 2006) which acts as a co-stimulatory molecule that upon triggering with GITR ligands, usually expressed on APCs, can control proliferation of effector responder T cells making them refractory to Treg suppression. But on Treg cells, stimulation via GITR works as a negative regulator of Treg function by directly blocking suppression.

Significant *foxp3* expression within the $CD25^+$ cell fraction coincided with $CD4^+CD25^+GITR^+$T cell accumulation, after one week and also later on after one month of *L. infantum* infection when high levels of parasitization were detected in the spleen, suggests the involvement of regulatory *foxp3* expressing - T cells in parasite expansion. The transcription factor FOXP3 appears to be critically important for Treg cell development (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003) and maintenance of their suppressive function (Williams and Rudenksy, 2007). Mice and human deficient for this transcription factor suffer from severe lymphoproliferative autoimmune disease (the IPEX syndrome in man and the scurfy phenotype in mice) and/or inflammatory disorder. Ectopic expression of FOXP3 in naïve T cells is sufficient to convert them into phenotypically and functionally Treg-like cells in mice. Retroviral transcription of FOXP3 suppresses cytokine gene transcription, confers *in vivo* and in vitro suppressive activity and

upregulates Treg-associated molecules including CD25 and GITR (Hori et al., 2003). FOXP3 is a repressor of IL-2, IL-4 and IFN-γ transcription through direct physical interactions with transcription factors, such as nuclear factor-κB (NF-κB) and nuclear factor of activated T cells (NF-AT) (Bettelli et al., 2005), blocking their ability to induce the endogenous expression of their target genes, thus inhibiting the production of pro-inflammatory cytokines and effector functions of primary T helper cells.

One mechanism by which microorganisms might manipulate regulatory T cell function is by creating an environment that favours the retention of regulatory T cells. Integrin $\alpha_E\beta_7$ (also known as CD103), is expressed at the surface of 25% of nTreg in lymphoid tissues (McHugh et al., 2002) and defines a subset of nTreg cells with enhanced suppressive properties and specific migratory patterns (Huehn et al., 2004). CD103 expression has been shown to favour natural Treg retention at dermal sites of *L. major* infection (Suffia et al., 2005). In fact, Treg that are known to respond to parasite antigen are restricted to sites of *Leishmania* infection (Suffia et al., 2006). Results in the present study confirm the presence and expansion of *foxp3* expressing - $CD4^+CD25^+CD103^+$ T cells within sites of *L. infantum* visceralizing infection, as is the case of the spleen and lymph nodes. This is particularly more evident after one month of parasite infection with *L. infantum*. In the model of *L. major* infection, CC-chemokine receptor 5 (CCR5) expression on Treg was also shown to be required for their migration to the infected sites (Yurchenko et al., 2006a). Interestingly, exposure of T cells to parasite-infected DC enhances CD103 expression and infection of APC with *L. major* and favours the production of ligands for CCR5 by APC (Sebastiani et al., 2001), suggesting that the pathogen itself manipulates its environment to favour Treg recruitment and retention so as to promote their own survival within the host.

Treg expansion followed by transient contraction could be related to host-parasite interactions. Higher parasite loads and increases in the amount of antigens present may affect the dynamic

equilibrium between effector and Treg cells. It has been shown that suppressive Treg activity can be selectively modulated (e.g. via toll-like receptors, discussed later in this section) during acute and chronic infections. In fact, during acute infection, bacterial antigens may rapidly induce effector function, boosting host adaptive immunity while attenuating suppressive Treg activity (Liu et al., 2006). In this study, Treg did not continue to expand at two weeks pi and may have temporarily lost suppressive activity since *foxp3* expression levels and CD25, GITR and CD103 were also found to be reduced.

In mice, most naïve $CD4^+$ T cells express high levels of CD45RB, whereas $CD4^+$ T cells that have previously encountered antigen have lower levels of CD45RB (Lee et al., 1990). Functional analysis of $CD45RB^{high}$ and $CD45RB^{low}$ $CD4^+$ T cells has shown that important interactions occur between these subsets *in vivo*, where regulatory cells seem to be enriched in the $CD4^+CD45RB^{low}$ population (Morrissey et al., 1993; Powrie et al., 1993; Hara et al., 2001). Immune suppression exerted by the $CD45RB^{low}$ subset was shown to be dependent on IL-10 and TGF-β, but independent of IL-4 (Powrie et al., 1996; Assessman et al., 1999). Further subdivision of the $CD45RB^{low}$ subset revealed that the regulatory T cells were enriched within the $CD25^+$ population (Read et al., 2000). In the present study, $CD4^+CD25^+$ T cells with $CD45RB^{low}$ phenotype failed to reveal important variations during infection. Gomes-Pereira, (2004a**)** observed only slight variations in the $CD4^+$ memory subsets $CD62L^{low}/CD45RB^{low}$ of splenic lymphocytes in mice of both "cure" and "non-cure" phenotype infected with *L. infantum*. However in the liver, the scenario was quite different, an imbalance between "protective" $CD45RB^{high}$ and "pathogenic" $CD45RB^{low}$ $CD4^+$ hepatic T cell subsets in "non-cure" animals was observed. This imbalance may have favoured Treg-mediated immunosuppression and the evolution of a non-healing infection. Together, these results give indication that perhaps CD45RB may not be a suitable marker for Treg subsets that expand in the spleen of BALB/c mice infected with *L. infantum*. However, this does not exclude the possibility that cells with this phenotype may be involved in suppressing effector immune responses against *L. infantum* in other infection models. Functional studies with purified

$CD4^+CD25^+CD45RB^{low}$ T cells from both spleen and liver were not carried out in this study although it would provide further insight on the role of this particular subset during *L. infantum in vivo* infection.

$CD4^+CD25^-$ effector T cell levels showed important decreases at the same time points of $CD4^+CD25^+$ regulatory T cells expansion. Although IFN-γ was secreted by effector cells, they were unable to respond adequately to specific stimulation throughout the entire experimental period of infection. There seems to be an inadequate Th1 response by effector cells to *L. infantum* infection that may perhaps be related to Treg cells and their immunosuppressive properties. Effector cells were only able to respond with IL-4 production after around two months of infection, and only when stimulated with antigen-presenting cells, coinciding with reduction in *foxp3* expression in Treg cells. Also, late IL-4 production failed to influence susceptibility of BALB/c to *L. infantum* since infection was characterized by a lack of major increases in parasite burden during the experimental period. However, Treg-mediated suppression of the Th2 response in BALB/c mice has in fact been seen during *L. major* infection (Aseffa et al., 2002; Xu et al., 2003). Together these results therefore suggest that Th1 and Th2 effector responses may be controlled by Treg expansion during *L. infantum* infection.

Regarding possible mechanisms of Treg suppression, $CD4^+CD25^+$ T cells were evaluated in relation to cytokine *in vitro* production upon *Leishmania* antigen stimulation. These cells were able to consistently produce TGF-β when specifically stimulated at day 7 and from day 28 of *L. infantum* infection onwards. It has also been suggested that TGF-β participates in parasite growth regulation, initially favouring its multiplication and preventing an inflammatory response (Gomes-Pereira et al., 2004). *In vivo*, $CD25^+$ Treg activity has been implicated to be dependent on TGF-β (Levings et al., 2002; Nakamura et al., 2004). It have been shown that these cells are *Leishmania*-specific (Suffia et al., 2006) and are involved in the control of effector immune response induced

during human mucocutaneous leishmaniasis (Campanelli et al., 2006). In this study, TGF-β producing-$CD4^+CD25^+$ T cells were seen to exhibit migratory phenotype expressing high levels of CD103 and were antigen-specific to *L. infantum* upon early and after one month of infection when parasite burden reached high levels in the spleen. CD103 expression is known to be positively regulated by TGF-β (Robinson et al., 2001), which is highly expressed at the vicinity of mucosal tissues and at sites of inflammation. Follow-up studies of the evolution of parasite burden, after the experimental observational period in this study, showed steadily increasing numbers of parasites. The presence of TGF-β producing-$CD4^+CD25^+$ T cells during *L. infantum* infection may contribute to immunosuppression (IL-4 inhibition and weak IFN-γ production) and parasite persistence while also allowing a better control of parasite-mediated-immunopathology.

IL-10 has been implicated as an immunosuppressive factor in both human and experimental leishmaniasis. It has pleiotropic, primarily deactivating effects on target cells. Elevated production of IL-10 is frequently found in human VL (Caldas et al., 2005) and anti-IL-10 treatment seems to revert antigen-specific unresponsiveness of PBMC in VL patients (Carvalho et al., 1994; Ghalib et al 1995). Based on experimental models, IL-10-deficient or anti-IL10 receptor treated mice display enhanced resistance and leishmanicidal activity against *L. donovani* (Murphy et al., 2001; Murray et al., 2005). Experimental models of cutaneous leishmaniasis have shown that IL-10 produced by nTreg is responsible for their suppressive activity *in vivo* and contributes to persistent *L. major* infection (Belkaid et al., 2002; Suffia et al., 2005). However, the extensive data generated fail to support a major role for nTreg in human VL. In this study, $CD4^+CD25^+$ T cells from *L. infantum*-infected BALB/c mice were capable of secreting some IL-10 but non-specifically and earlier during infection implicating a minor role for $CD4^+CD25^+$ IL-10 producing Treg during visceral *L. infantum* infection.

Several other different types of T regulatory cells have been described in the literature and shown

to exhibit regulatory activities (Shevach, 2006). Recent results implicate IL-10 producing-$CD25^-$ $FOXP3^-$ T cells in the pathogenesis of human visceral leishmaniasis and not naturally occurring $CD4^+CD25^+FOXP3^{high}$ T cells as the major source of IL-10 in the splenic aspirates (Nylén et al., 2007). $CD4^+CD25^-FOXP3^-$ cells were also shown to be the source of IL-10-mediated immune suppression in chronic cutaneous *L. major* infections (Anderson et al., 2007; Nagase et al., 2007) and in visceral *L. donovani* infection (Stäger et al., 2006). In the present study, the effector $CD4^+CD25^-$ T cell compartment unexpectedly showed to be an important source of IL-10 since they were in fact capable of specifically producing larger amounts of this cytokine, early and throughout the experimental period, except during two to four weeks of infection where specific inhibition was observed. This particular IL-10-secreting $CD4^+$ T cell subset seems to be distinct from nTreg since they did not express appreciable levels of *foxp3* gene and maintained residual IFN-γ production. Antigen-induced IL-10-producing $CD4^+$ T cells arising from $CD4^+CD25^-$ $FOXP3^-$ are currently referred to as Tr1 cells, adaptive Treg or inducible Treg that are involved in the down-regulation of immune responses through their ability of producing high concentrations of IL-10 and suppressing naïve and memory Th1 or 2 responses (Mills and McGuirck, 2004). In this study, IL-10 producing-$CD4^+CD25^-FOXP3^-$ T cells may well belong to the Tr1 regulatory subset that are being induced *in vivo* by *Leishmania* parasites, and that may be contributing to the evolution of non-healing phenotype in *L. infantum* infected-BALB/c mice.

Taken together, the information here presented identifies T cell subsets with phenotypic and functional characteristics of regulatory T cells during *L. infantum* infection of susceptible BALB/c mice. $CD4^+CD25^+CD103^+$and $GITR^+$ T cells readily expand, present high levels of *foxp3* gene expression and are antigen-specific. This suggests a predisposition for Treg retention within sites of *L. infantum* infection, consequently influencing local immune response. Th1 and Th2 effector immune responses seem inadequate perhaps related to Treg expansion during infection. *foxp3* expressing-$CD4^+CD25^+$ T cells are capable of specifically producing TGF-β. IL-10 producing-

$CD4^+CD25^-FOXP3^-$ T cells were also identified as an additional source of IL-10 and may represent a Tr1 regulatory type subset that is being induced upon encounter with infected APC or parasitic antigens. Together, these immunosuppressive T cells may be preventing parasite-mediated immunopathology therefore prolonging parasite persistence.

During the early stages of infection, the host innate immune system must rapidly detect and respond to protozoan parasite infection, and this is achieved through innate immune receptors. Toll-like receptors (TLR) have now emerged as a major receptor family that have a vital role in triggering innate immunity against initial infection, as well as orchestrating the development of acquired immune response, which is necessary for protection against re-infection.

Leishmania derived-molecules can be sensed by TLR molecules (Becker et al., 2003; de Veer et al., 2003; Schleicher et al., 2007) through several different immune cells. In fact, new evidence has demonstrated Treg can also sense pathogens directly through TLR and, consequently modify their behaviour. Does this bring an advantage to either the host or the parasite itself? It is now well known that Treg play a central role in the suppression of immune responses during infections to prevent tissue damage and in the prevention of autoimmune responses harmful to the host. However, during acute infection Treg might hinder effector immune response directed towards the elimination of the pathogenic challenge. So, Treg-mediated suppression needs to be tightly controlled or in other words the regulators need to be regulated. Since Treg express TLR and TLR sense pathogens, it is only natural to hypothesize that TLR may in fact modulate Treg-mediated suppression to the host's benefit. And this is what has proven to happen.

In mice and humans, 13 TLRs have now been identified that recognize distinct conserved pathogen- associated molecular patterns (PAMPs) (Akira et al., 2003; Pasare et al., 2004). Several TLR agonists derived from protozoans have also been identified in recent years (Gazzinelli et al., 2004). Glycosylphosphatidylinositol (GPI)-linked molecules or anchors that are composed of a

glycan core and a lipid component, function as anchors to the surface of eukarytotic cells, and are abundantly expressed by protozoan parasites. Specificity is conferred through variations in the carbohydrate branches and in the lipid inositol portion. Protozoan GPI anchors, such as of *Trypanossoma cruzi* (Campos et al., 2001), *Plasmodium falciparum* (Krishnegowda et al., 2005) and *Toxoplasma gondii* (Debierre-Grockiego et al., 2003), have been shown to activate both cells from both lymphoid and myeloid lineages and initiate host immune responses and inflammation. *Leishmania spp.* has GPI-linked molecules that can trigger TLR activation. Infectious metacyclic promastigotes have a repertoire of GPI-linked molecules on their surface. At this particular stage of development, the main GPI-linked molecules are lipophophosglycans (LPG), which contain long carbohydrate branches with repeating phosphoglycan units (McConville et al., 1995). Recent studies revealed that TLR-2 contributes to the recognition of *L. major* and to the subsequent immune response. LPG has been shown to stimulate mouse macrophages and human NK cells through TLR-2, enhancing IFN-γ, IL-12, TNF-α production and nuclear translocation of NF-κB (Becker et al., 2003; de Veer et al., 2003). Furthermore, the use of RNA interference to knock out the expression of various TLRs revealed that activation of macrophages by *L. donovani* is also, at least in part, dependent on TLR-2 (Flandin et al., 2006).

Generally speaking, pathogen recognition by TLRs triggers a rapid activation of innate immunity by inducing the production of pro-inflammatory cytokines. Once activated by microbial PAMPs, TLRs interact with adaptor proteins. TLR molecules have a cytoplasmic domain that is homologous to the IL-1 receptor, and is known as the TIR (Toll/interleukin-1 receptor) domain (O' Neil et al., 2003). The best-studied TIR-domain-containing adaptor protein is myeloid differentiation gene 88 (MyD88) and is common to signalling pathways of IL-1R, IL-18R, and TLRs to IL-1R-associated protein kinase. Several studies using MyD88-deficient mice in parasitic infection models have provided valuable indication on the role of TLR signalling in resistance. Resistant mice lacking MyD88 have been shown to be highly susceptible to infection with *L. major* (Muraille et al., 2003). This susceptibility is characterized by large non-healing lesions, marked

parasitism and the development of a polarized Th2 immune response, as opposed to the normal IL-12-dependent Th-1 protective immune response that develops in the wild-type C57BL/6 mice. Susceptibility of these MyD88$^{-/-}$ mice has been thought to be a consequence of impaired TLR signalling. Also *in vivo* maturation of dendritic cells induced by *L. donovani* seemed to be partially dependent on MyD88 signalling pathway (de Trez et al., 2004).

The first comparative study showed that TLR expression was related to functional states of different subtypes of T cells. Both Treg and effector T cells expressed TLR-1, 2 and 6. However, the Treg subset expressed significantly more TLR-4, 5, 7 and 8 than effector T cells, and increased suppressor function upon LPS activation (Caramalho et al., 2003). In fact, TLR-5 triggering of human Treg cells induced high levels of FOXP3 expression and enhanced Treg suppressive capacity (Crellin et al., 2005). But with respect to TLR-2 and TLR-5, effector cells also expressed significant amounts of these TLRs and when stimulated with their respective ligands showed increased proliferation and cytokine production (Komai-Koma et al., 2004; Crellin et al., 2005). TLR-2 expressed on the surface of activated and memory human T cells can serve as a co-stimulatory receptor for antigen-specific T cell development and help to maintain T cell memory (Komai-Koma et al., 2004) and increase immune response against pathogens. In this study, TLR-2 expression was in fact detected by real-time PCR in isolated CD4^{+}CD25^{+} T cell fractions from *L. infantum*-infected BALB/c mice. Interestingly, the highest level of TLR-2 expression was observed at the same time point (day 14) when Treg transiently decreased in number and vice versa when Treg expanded. This suggests possible TLR-2 regulation of Treg kinetics during *L.infantum* infection. However, replicate measurements of TLR-2 expression from higher number of isolated CD4^{+}CD25^{+} T cells should be performed to confirm statistical significance.

In fact, another study documented a crucial role for TLR-2 in regulating Treg expansion and suppression by acting directly on Treg themselves (Sutmuller et al., 2006). TLR-2 triggering on Treg cells combined with IL-2 treatment and TCR ligation resulted in the proliferation of the

otherwise anergic Treg cells both *in vitro* and *in vivo*. But the suppressive phenotype of Treg cells was in fact temporarily abrogated, thereby enabling the enhancement of the immune response *in vitro* against *Candida albicans* and in an acute *in vivo* infection model (Sutmuller et al., 2006). So, following removal of the TLR-2 trigger, the *in vitro*-expanded Treg cells fully regained their phenotype and suppressive capabilities. This was confirmed by a report from Liu, (2006) where FOXP3 expression was found to have decreased following TLR-2 stimulation of Treg, thus providing a further insight into the mechanism by which TLR-2 controls Treg-cell function. So it is important to stress that different TLR ligands may have the different abilities in regulating Treg cell activity, depending on the TLR signalling pathway in question. This ultimately may reflect on the modulation and type of immune response actually induced.

So there is evidence to a role for TLR-2 in regulating Treg immunosuppressive function whilst also being implicated in *Leishmania*-specific immune responses elicited in other cell types. In order to elucidate on immunosuppressive Treg function induced by *L. infantum* parasites and the underlying mechanisms involved in the direct host-parasite interactions and immune regulation, the second part of this study focuses on the role of TLR-2 on Treg kinetics during *L. infantum* experimental murine infection by investigating the influence of TLR-2 on Treg kinetics, immune response, parasite-associated pathology and the outcome of *L. infantum* infection. To achieve this, TLR-2 deficient mice and their wild type littermate control mice were infected with virulent *L. infantum* parasites and comparative analysis done to see whether or not the presence or absence of TLR-2 produces any differential effect on the host parameters related to Treg dynamics and protective immunity.

L. infantum parasites showed higher rates of multiplication in both spleen and liver of TLR-$2^{-/-}$ knock-out mice when compared to wild-type on the C57Bl/6 background, giving evidence of non-healing infection in the former. The liver was the organ that displayed higher parasitization levels regardless of type of mice or observational time point. Susceptible BALB/c mice infected with *L.*

donovani reveal initial internalization of parasites by liver Kupffer cells, and then influx of granulocytes (neutrophils and eosinophils) and $CD4^+$ cells, followed by $CD8^+$ cells (Wilson et al., 1996; McElrath et al., 2005). Parasite multiplication occurs up to four weeks after which there are reductions in parasite numbers and $CD4^+$ cells (Wilson et al., 1995). Progression of the granulomatous response should result ultimately in acquired anti-leishmanial immunity (McElrath et al., 2005). However, the overall antimicrobial efficacy of the granulomatous response appears to be variable and depends upon host determinants, the pathogen and the criteria used to define efficacy (Murray et al., 2001). Although microbial eradication is the ideal outcome in infections which trigger granuloma assembly, it is not clear how often this objective is actually ever achieved. Thus, despite even an initially intense granulomatous response, a number of pathogens, including *L. donovani*, establish a well recognized state of chronic intracellular parasitism (Murray et al., 2001).

In this study, TLR-$2^{-/-}$ mice infected with *L. infantum* developed higher liver parasitism with significant expansion after one month of infection. However, this was accompanied by a more intense granulomatous response in comparison to WT with increased number of infiltrating cells per area of tissue. High liver parasitism despite intense granulomatous response questions the efficacy of anti-leishmanial activity in the liver of these mice. Normal granuloma structure but no function may have given rise to ineffective granuloma during *L. infantum* infection. The presence of infiltrating immune cells does not seem to directly correlate with efficient parasite control.

Two possible underlying mechanisms should be considered: defective recognition of parasite ligands and initiation of innate immunity through TLR-2 signalling by influxing macrophages and other immune cells that are unable to activate basic microbicidal mechanisms despite the assembly of a structurally intact-appearing granuloma. In fact, the term, `ineffective granuloma' (Murray & Nathan 1999), has been applied to the setting in which macrophages within the granuloma remain heavily parasitized despite satisfactory kinetics of assembly and apparently intact cellular

composition and structure. In regard to this study in particular, a plausible cause for having observed higher numbers of infiltrating cells in liver granulomas in TLR-$2^{-/-}$ mice could be the fact that TLR are known to be highly expressed on several cells involved in innate immunity; the other possible mechanism is when the pathogen itself stimulates a suppressive host response which deactivates primary granuloma-inducing mechanisms. In this study, the presence of high levels of immunosuppressive Treg was in fact confirmed in TLR-$2^{-/-}$ mice giving indication that the absence of TLR-2 favours late Treg expansion that ultimately may have influenced effector immune response in the liver. To note that Treg expansion was evaluated in the spleen since this organ is considered as the source of *L. infantum*-specific T cells involved in the infection control (Gomes-Pereira et al., 2004a).

Until date, very little work has been done on the role of TLR in host response to infection with protozoan parasites. No studies have looked at the contribution of TLR-2 to acquired immune responses to *Leishmania* infection, let alone to what occurs in terms of parasitism and immune response in TLR-2-deficient mice. However, seminal studies have given strong evidences for a crucial role of the surface glycolipid LPG in activating immune signalling via TLR-2 (de Veer et al., 2003; Becker et al., 2003). LPG was considered as an important activator of pro-inflammatory cytokine synthesis in macrophages derived from WT and not MyD88-null mice, giving evidence that Toll pathway and a functional MyD88 adaptor protein is required for activation of cytokine synthesis and resistance to this pathogen. Also *L. major* LPG is recognized by human NK cells which are activated to produce IFN-γ, IL-12, TNF-α and NF-κB through TLR-2, thus increasing the effective destruction of the parasite. Studies performed with other members of the *Trypanosomatidae* family as is the case of *T. cruzi* showed that GPI anchors derived from these parasites induced TLR-2 expression and seemed to be essential for the release of IL-12, TNF-α and NO (Campos et al., 2001). However, when studying gene deficient knock-out models and their effect on host resistance to parasite infection, the phenotype observed in terms of parasitism and

immune response varies depending on the parasite and the TLR signalling pathway affected. *L. major* infected - MyD88$^{-/-}$ mice as mentioned already show a Th2 phenotype and increased susceptibility (de Veer et al., 2003; Muraille et al., 2003). This is also the case for *T. brucei* (Drennan et al., 2005) and *T. cruzi* (Campos et al., 2004) where decreased or impaired production of pro-inflammatory cytokines were observed, respectively, increasing susceptibility to infection. Similar results were seen with *T. gondii* where MyD88$^{-/-}$ mice die within 10 days of infection (Scanga et al., 2002). However, in malaria, MyD88$^{-/-}$ mice infected with *P. berghei* resulted in impaired cytokine production but in fact showed less pathology and better outcome (Adachi et al., 2001). Therefore, although signalling through MyD88 in innate immune cells has a protective role in most cases of protozoan infection by activating a Th1-associated immune response, in other situations decreased pro-inflammatory responses resulting from a lack of MyD88 signalling might be beneficial to the host.

The role of TLR-2 signalling in host resistance and pathogenesis of parasitic infections is still unclear. For example, lack of expression of TLR-2, did not affect the susceptibility of mice to infection with *T. cruzi*. No major phenotype is observed in terms of parasitism or immune responses when either TLR-2-deficient mice are infected with *T. brucei* (Drennan et al., 2005), *T. gondii* (Chen et al., 2002; Mun et al., 2003) or *P. berghei* (Adachi et al., 2001). Therefore, these studies seem to indicate that the lack of a specific functional TLR might not be sufficient to result in the dramatic enhancement of host susceptibility to infection that is seen with MyD88 deficiency. However, in the case of TLR-2-deficient mice infected with *L. infantum* there was a visible effect on outcome of infection. To further dissect this in terms of cell-mediated immunity, regulatory T cells and overall effector response were evaluated.

L. infantum-infected TLR-2$^{-/-}$ mice initially showed reduced numbers and no visible expansion of Treg that are FOXP3^{+}GITR^{+} when comparing with WT mice, implying that the absence of TLR-2 does not seem to favour immediate up-regulation of Treg as expected. In fact, Netea (2004) also

observed a 50% decrease of the total Treg population ($CD4^+CD25^+$) in TLR-$2^{-/-}$ mice infected with *Candida albicans* when compared to their wild-type littermates. However, the absence of TLR-2-mediated signalling resulted in increased resistance to disseminated candidiasis. Whereas production of TNF-α and IL-1 is normal, IL-10 synthesis is severely impaired in TLR-$2^{-/-}$ mice. Decreased production of IL-10 was associated with increased release of IFN-γ, to a reduction in the generation of Treg cells, and improved candidacidal function of macrophages. This implies that *C. albicans* evades host defence through TLR2-mediated signals.

Low Treg numbers in TLR-$2^{-/-}$ mice infected with *L. infantum* did not seem to be associated with better effector immune response during initial stages of infection in the spleen since relatively to WT mice there were no significant differences in parasite load, $CD3^+CD4^+CD25^-FOXP3^-$ effector T cell numbers and in total IFN-γ production. In fact, IL-4 was found to be strongly induced in spleen tissue of TLR-$2^{-/-}$ mice. During *L. major* infection, $CD4^+CD25^+$ T cells were been shown to regulate early IL-4 mRNA by inhibiting the magnitude and the subsequent development of a Th2 response (Aseffa et al., 2002). So, initially low Treg numbers in *L. infantum*-infected TLR-2-/- mice may allow for induction of higher levels of IL-4 in spleen tissue. However, IFN-γ response in LN cells of TLR-$2^{-/-}$ mice was significantly reduced in relation to WT and did not seem to be adequately induced despite low Treg numbers.

As already mentioned before, another potential marker for Treg cell subpopulation is the integrin (αEβ7, CD103) that is expressed on approximately 25% of mouse Treg cells, and that controls the adherence of conventional T cells to epithelium in the gut by binding to E-cadherin. The subset of $CD25^+CD103^+$ T cells appears to be expressed on Treg cells that circulate preferentially to inflammatory sites and to exert more potent suppressive effects *in* vitro (Huehn et al., 2004). Treg phenotype positive for CD103, used in this study gave a strong indication of cell migration to and retention within sites of infection in TLR-$2^{-/-}$ mice, since it showed to be significantly induced and

enhanced when compared to WT. In fact, in the first part of this work, $CD4^+CD25^+CD103^+$ Treg were also shown to be significantly increased in numbers in infected susceptible BALB/c mice. Furthermore CD103 expression has also been shown to favour natural Treg retention at dermal sites of *L. major* infection (Suffia et al., 2005). Higher levels of $CD4^+CD25^+FOXP3^+CD103^+$ Treg cells were observed during both early and late infection in TLR-$2^{-/-}$ mice, indicating increased Treg retention in addition to late induction of Treg with memory phenotype ($CD45RB^{low}$) in infected spleens. Alloantigen-specific regulatory T cells contained within the $CD45RB^{low}$ $CD4^+$ T cell population have been shown to be responsible for the maintenance of tolerance to donor alloantigens *in vivo* and require IL-10 for functional activity (Hara et al., 2001). In contrast to the first part of the work where CD45RB did not reveal important variations during *L. infantum* infection of susceptible BALB/c mice, when looking at TLR-$2^{-/-}$ mice on a C57BL/6 resistant background, this specific memory marker showed to be more strongly associated to Treg markers.

So high retention of memory Treg associated to significantly higher parasite loads in both spleen and liver during later stages of infection of TLR-$2^{-/-}$ mice and lower IFN-γ levels detected in total spleen tissue may also play a role in *Leishmania* pathogenesis in TLR-$2^{-/-}$ mice. IL-4 did not seem to be the cytokine particularly involved in the outcome of late *L. infantum* infection in the spleen. In fact, IL-4 was found to be significantly reduced in relation to WT during late infection. However, in the liver late IL-4 expression was detected in these mice that could have contributed to higher liver parasitism. Other Th2 signature cytokines, which were not under study in this current work, should also be considered so as to provide more information on cytokines that may be more directly involved in susceptibility to *Leishmania* (e.g. IL-5, IL-13). Also the presence of late immunosuppressive Treg cells in infected TLR-$2^{-/-}$ mice did not greatly influence differences that were expected in T cell effector populations. Effector T cell response was significantly induced with late infection regardless of mice type. Late effector response was elicited despite upregulation of the expression of immunosuppressive Treg in the absence of TLR-2. So, TLR-2 signalling in WT may be important in providing negative Treg regulation and more protective immunity, giving

rise to better control against *L. infantum* infection.

Regarding $FOXP3^+$ populations within the CD25 negative subset, again in TLR-$2^{-/-}$ mice, the numbers are not increased in relation to WT. However, in WT mice this $FOXP3^+$ population and the $CD103^+Foxp^+$ subset, within the CD25 negative population, do still decrease with infection as expected, the latter being more reduced than in TLR-$2^{-/-}$ mice. So again in WT mice, as observed previously in the $CD25^+$ Treg subsets, $FOXP3^+$ committed Treg populations also seemed to be more tightly controlled, thus favouring protective immunity against the parasite.

In retrospective, the most important differences observed between TLR-$2^{-/-}$ and WT mice infected with *L. infantum* are associated to levels of Treg retention with memory phenotype during late infection of the spleen. Although total Treg numbers seemed practically unaltered during infection, Treg showed increased migration to and retention at sites of infection, suggesting that the induction of immunosuppressive Treg function would favour *Leishmania* multiplication and parasite persistence in TLR-2 knock-out mice. In WT mice, thus in the presence of TLR-2 receptor and upon eventual recognition of parasitic ligands, abrogation of Treg function would be expected and accompanied by breakdown of immune tolerance, and enhanced immune response against infection. In this study, during initial infection, the levels of parasite ligands were probably not sufficient enough to exert an appreciable regulatory effect on Treg despite the presence of TLR-2 receptor. However, after one month of infection, significant reductions in infected WT mice of total $GITR^+$ Treg and $CD103^+$ Treg and in comparison to knock-out mice for $CD45RB^{low}$ Treg did in fact occur that could have favoured better immunity and parasite control.

So, antigen recognition by TLR-2 should render Treg less responsive to the presence of parasite ligands and a possible infection, thus allowing for an adequate immune response. In fact, it has been reported that TLR agonists can directly or indirectly inhibit Treg cell response (Pasare & Medzhitov et al., 2003, Peng et al., 2005). TLR-induced IL-6 production by DC indirectly blocks

the suppressive function of $CD4^+CD25^+$ Treg cells. Sutmuller (2006) showed that although the TLR-2 ligand PAM_3Cys directly induced proliferation of $CD4^+CD25^+$ T cells during *Candida albicans* murine infection, there was temporary loss of Treg suppressive activity, which was restored after removal of the TLR agonist. Similar findings were reported by Liu (2006) using a synthetic bacterial lipoprotein (BLP) PAM_3Cys-SK4, also a TLR-2 agonist that was able to induce the expansion of both $CD4^+CD25^+$ Treg and $CD4^+CD25^-$ T effector cells but also rendered the effectors more resistant to suppression by Treg cells and transiently suppressed Treg function. They suggested that the down-regulation of FOXP3 expression would be the putative mechanism for the abrogation of Treg suppressive function. In contrast to the previous studies, Zanin-Zhorov et al., 2006, used the endogenous TLR-2 ligand heat-shock protein 60 (hsp60) and showed that hsp60-activated Treg actually enhanced their suppressive capacity via both cell-contact dependent mechanism and IL-10 and TGF-β production. This discrepancy between several different studies could be possibly explained by the nature of the TLR-2 ligands used, the concentrations and the ways endogenous TLR-2 ligands interact with TLR-1/2 receptor dimer. Thus the effect of TLR-2 signalling on $CD4^+CD25^+$ Treg still remains somewhat controversial and most likely varies depending on the Treg subset in question, the nature of TLR-2 ligand and the type of pathogen under study.

For example, during acute *C. albicans* infection, Treg numbers ($CD4^+CD25^+$) in the blood circulation of TLR-$2^{-/-}$ mice are significantly reduced compared to their WT littermate controls (Netea et al., 2004). However, the TLR-$2^{-/-}$ mice were more resistant to disseminated *Candida* infection, and this was mainly associated to enhanced candidacidal macrophage activity and also to impaired IL-10 release by total spleen cells. These authors also showed that TLR-2 agonists (peptidoglycan) induced *in vitro* survival of Treg. So, in this model of acute infection TLR-2-derived signals seem to favour Treg survival, increase IL-10 production and thus contribute to fungal pathogenesis. In our model of infection with *L. infantum*, TLR-$2^{-/-}$ mice were in fact more susceptible and this was associated to increased Treg retention, apparently unefficient liver

granuloma formation and ultimately enhanced parasite persistence.

Anti-inflammatory response was also evaluated in this study. IL-10 was mainly observed during late infection, regardless of mouse phenotype. So, the presence or absence of TLR-2 did not seem to influence IL-10 expression, and it did not seem to correlate with $CD103^+$ Treg detected late during infection in TLR-$2^{-/-}$ mice. Other immune cells should be considered as potential sources of IL-10 that is being expressed. In fact, in the first part of this work the $CD4^+CD25^-$ effector T cell fraction isolated from infected susceptible mice were demonstrated *in vitro* to be an important source of IL-10. Since effector $CD3^+CD4^+CD25^-FOXP3^-$ T cells were also found to be expressed at significantly higher levels also later during infection it is possible that they may give rise to IL-10 that is being induced, thus contributing to anti-inflammatory milieu and perpetuation of chronic infection. Also a recent study in an experimental model of visceral leishmaniasis showed that NK cells were recruited to the spleen and into hepatic granulomas, where they inhibited host protective immunity against *L. donovani* in an interleukin-10 (IL-10)-dependent manner (Maroof et al., 2008).

Initial IL-10 response seems to be organ dependent since while it is detected early upon infection in spleen of WT mice it is significantly reduced in the liver. This could indicate that anti-inflammatory response in the liver is less evident in WT than in TLR-$2^{-/-}$ mice. Another factor that may be inducing better effector response and lower parasite load in WT mice.

To the present date, no studies have evaluated the phenotype and the type of Th responses that is induced in TLR-$2^{-/-}$ knock-out mice when infected with *L. infantum.* As mentioned above, de Veer (2003) and Muraille (2003) observed Th2 response and increased susceptibility to *L. major* but in $MyD88^{-/-}$ mice. Other authors have shown that TLR-2 promotes Th2 responses and increases the production of IL-10 (Agrawal et al., 2003). However, these studies evaluated how signalling via distinct TLR-2 agonists conditions DCs to elicit distinct Th responses. Activation of DCs via TLR-2 ligand PAM-Cys skewed toward Th2 responses via induction of extracellular MAP kinase

signalling and c-Fos. It would be expected that during an infection with *L. infantum*, increased concentrations of TLR-2 ligands (e.g. LPG) would limit Treg suppressive activity and promote the onset of immune response in WT mice. In fact, $CD4^+CD25^+CD103^+$ and $CD45RB^{low}$ Treg were found to be reduced in WT mice. Th1 response mediated by IFN-γ expression in WT was found to be higher during early infection in LN and in the spleen during late infection. However, Th2 response (IL-4) varied greatly according to the cells/organ in question. No evident differences in IL-10 expression were observed between mice although less TGF-β was initially detected in spleen cells of WT when compared to TLR-$2^{-/-}$ mice.

GPI anchors from *T. cruzi* parasites were found to be potent activators of TLR-2 and TLR-2 activation seems to be essential for initiating host innate response with induction of IL-12, TNF-α and NO. TLR-$2^{-/-}$ mice were able to mount a robust pro-inflammatory cytokine response (producing IFN-γ), during the acute phase of infection with *T. cruzi* parasites (Campos et al., 2004). However, deletion of the functional TLR-2 gene had no major impact on parasitemia nor on mortality. In contrast, the MyD$88^{-/-}$ mice had a diminished cytokine response and reactive nitrogen intermediates (RNI) production upon acute infection with *T. cruzi* and were more susceptible to infection, with high levels of parasitemia and accelerated mortality (Campos et al., 2004). These authors suggest that it is possible that TLR-2 may be playing an immunoregulatory role in wild type mice. Upon TLR-2 activation via GPI anchors, macrophages become refractory, cease to produce pro-inflammatory cytokines, leading to induced tolerance. The lack of TLR-2 could be preventing this tolerance state and induce more robust immune response. Alternatively, the lack of functional TLR-2 could also prevent stimulation of $CD4^+CD25^+$ T cells with immunoregulatory function, culminating in enhanced production of IFN-γ during *in vivo* infection with *T. cruzi*.

Increased susceptibility toward *T. brucei* observed for MyD88-deficient animals was shown to be due to defective TLR signaling. However, screening of several TLR-deficient animals revealed that

neither TLR-1, TLR-2, the combination of both TLR-2 and TLR-4, or TLR-9 were required by the host to control the first peak of parasitemia following a clonal *T. brucei* infection (Drennan et al., 2005). However, TLR-2-, TLR-4- and MyD88-deficient mice with the avirulent cyst-forming Fukaya strains of *T. gondii* showed high levels of IL-4 and IL-10 produced by peritoneal exudate cells and died within 8 days of infection (Mun et al., 2003). In this study higher levels of IL-10 were found in both spleen and liver although no differences were found between TLR-2 deficient and WT mice in response to *L. infantum*. However, an early expression of IL-4 in total spleen tissue, and later in LN was found significantly higher in TLR-2 deficient mice.

Visceral leishmaniasis has been frequently associated to TGF-β (Wilson et al., 1996; Gomes-Pereira et al., 2004). In susceptible BALB/c mice, the presence of regulatory TGF-β producing-$CD4^+CD25^+$ T cells were seen to exhibit migratory phenotype expressing high levels of CD103 and were antigen-specific to *L. infantum*. Other studies have also shown that, *in vivo,* $CD25^+$ Treg activity has been implicated to be dependent on TGF-β (Read et al., 2000, Levings et al., 2002; Nakamura et al., 2004), and these cells are *Leishmania*-specific (Suffia et al., 2006) and control effector immune response induced during human mucocutaneous leishmaniasis (Campanelli et al., 2006).

The expression of immunosuppressive cytokine TGF-β was evaluated in TLR-2 deficient mice infected with *L. infantum*, so as to determine whether there were any differences observed in the absence of TLR-2 and any association to increased levels of migrating regulatory cells to sites of infection. Initially, spleen cells of TLR-$2^{-/-}$ mice expressed higher TGF-β levels than WT mice but later during infection, a reduction in the ability to express this cytokine was shown, particularly more evident in liver. This may suggest a minor role for TGF-β in mediating parasite-induced immunopathology and susceptibility of TLR-$2^{-/-}$ mice to *L. infantum*.

Surprisingly, WT showed a strong TGF-β response to late infection that does not seem to contribute to increased parasite multiplication since these mice are effectively more resistant to *L. infantum*. This occured together with an anti-inflammatory response with detection of IL-10. However, Treg activity was not found to be elevated in response to infection in WT mice. Other immune cells such as Th3 cells (Weiner et al., 2001) or $CD8^+$ regulatory T cells (Cosmi et al., 2003; Pomié et al., 2008) should also be considered as potential producers of TGF-β and thus contribute to the development of chronic infection in WT mice. In conclusion, the migration to sites of *L. infantum* infection of suppressive Treg in TLR-2 deficient mice seem to be IL-10 and TGF-β independent, suggesting other mechanisms mediating parasite-induced pathology in these mice.

FINAL CONCLUSIONS

To summarize the results obtained in this present work, the following conclusions can be made regarding:

1. Phenotypic characterization of regulatory T cell populations during *L. infantum in vivo* infection

-Identification of T cell subsets with phenotypic and functional characteristics of regulatory T cells during *L. infantum* infection of susceptible BALB/c mice. Inducible $CD4^+CD25^+$ regulatory T cells that are also $GITR^+$ and $CD103^+$ readily expand upon *Leishmania* infection, present high *foxp3* gene expression and are antigen-specific, suggesting predisposition for Treg recruitment and retention within sites of *L. infantum* infection, consequently influencing local immune response.

-Treg expansion is transient perhaps due to selective modulation via host-parasite interaction. However, Treg expansion seems to be influence immune response since Th effector cells are unable to respond specifically to stimulation. Th1 is inadequate to control infection and Th2 is inhibited.

-*foxp3* expressing-$CD4^+CD25^+$ T cells are capable of specifically producing TGF-β. IL-10 producing-$CD4^+CD25^-FOXP3^-$ T cells were also identified as an additional source of IL-10 and may represent a Tr1 regulatory type subset that is being induced upon encounter with infected APC or parasitic antigens.

2. Evaluation of the effect of TLR-2 modulation on Treg populations during *L. infantum in vivo* infection

-Defective TLR-2 signalling had a visible effect on outcome of *L. infantum* infection. Higher rates of parasite multiplication were observed in both spleen and liver of TLR-$2^{-/-}$ knock-out mice, despite the ability in forming well-defined and structured liver granulomas. These granulomas were apparently ineffective in parasite clearance, when compared to wild-type mice.

-Defective TLR-2 signalling did induce during late infection high retention of memory Treg which seemed to be associated to high parasite load and low IFN-γ levels. TLR-2 signalling pathway may play a role in Treg modulation and consequently in *L. infantum* pathogenesis. Functional TLR-2 signalling in WT may be important in providing tight control over $FOXP3^{+}$ committed Treg populations, negative Treg regulation and more protective immunity, giving rise to enhanced immunity and more effective response against infection.

-The presence or absence of TLR-2 did not seem to influence IL-10 or TGF-β expression, and it did not seem to correlate with $CD103^{+}$ $FOXP3^{+}$ Treg detected late during infection in TLR-$2^{-/-}$ mice. Detection of high levels of suppressive Treg in *L. infantum* infected TLR-2 deficient mice is not accompanied by associated inductions of immunosuppressive cytokines.

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