CONTRIBUTIONS FOR THE IMPROVED CONTROL OF
*Leishmania infantum* INFECTION: ROLES OF IRON,
REACTIVE OXYGEN SPECIES AND AMINOQUINOLINES

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CONTRIBUTIONS FOR THE IMPROVED CONTROL OF *Leishmania infantum* INFECTION: ROLES OF IRON, REACTIVE OXYGEN SPECIES AND AMINOQUINOLINES

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List of abbreviations

AmB: amphotericin B
ABC: ATP-binding cassette
8-AQ: 8-aminoquinoline
BSA: bovine serum albumin
CarboxyPQ: carboxyprimaquine
CD163: hemoglobin scavenger receptor / hemoglobin-haptoglobin receptor
CD91: low-density lipoprotein receptor-related protein / heme-hemopexin receptor
CL: cutaneous leishmaniasis
CYBRD1: cytochrome b reductase 1
DC: dendritic cell
DCL: diffuse cutaneous leishmaniasis
DCT1: divalent cation transporter 1
DCYTB: duodenal cytochrome b
DFO: desferrioxamine
dLN: draining lymph node
DMEM: Dulbecco's modified eagle medium
DMT: divalent metal transporter
FAD: flavin adenine dinucleotide
FBS: fetal bovine serum
Fc: ferrocene
FLVCR: feline leukemia virus subgroup C cellular receptor
FPN: ferroportin
FT: ferrititin
HB: hemoglobin
HBSS: Hank's balanced salt solution
HEPES: hydroxyethyl piperazineethanesulfonic acid
HIV: human immunodeficiency virus
Ig: Immunoglobulin
IFN: interferon
IL: interleukin
IRE: iron responsive element
IREG1: iron-regulated transporter 1
IRP: iron regulatory protein
KC: Kupffer cell
LABCG5: *Leishmania* ATP-binding cassette, sub-family G, member 5
LCCM: L929 cell conditioned medium
LF: lactoferrin
LFR: *Leishmania* ferric iron reductase
LHR: *Leishmania* heme response
LIT: *Leishmania* iron transporter
LPG: lipophosphoglycan
LPS: lipopolysaccharide
MAA: medium for axenic amastigotes
MCL: mucocutaneous leishmaniasis
MHC: major histocompatibility complex
MTP: metal transporter protein
MZ: marginal zone
NADPH: nicotinamide adenine dinucleotide phosphate
NOS: nitric oxide synthase
NRAMP: natural resistance associated macrophage protein
ON: overnight
PALS: periarteriolar lymphoid sheaths
PBS: phosphate buffered saline
PKDL: post kala-azar dermal leishmaniasis
PMSF: phenylmethanesulfonyl fluoride
PQ: primaquine
PRX: peroxiredoxin
PV: parasitophorous vacuole
R•: carbon radical
RNS: reactive nitrogen species
RO₂•: peroxyl radical
ROS: reactive oxygen species
RPMI: Roswell park memorial institute
RS: reactive species
RT: room temperature
SLCXAX: solute carrier family X, member X
SOD: superoxide dismutase
STEAP: six transmembrane epithelial antigen of the prostate
t-bOOH: t-butyl hydroperoxide
TF: transferrin
TFR: transferrin receptor
TGF: tumour growth factor
TNF: tumour necrosis factor
TR: trypanothione reductase
Tₕreg: T regulatory cells
T(SH)₂: reduced trypanothione
TS₂: oxidized trypanothione
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling
UTR: untranslated region
VL: visceral leishmaniasis
Xaa – undefined amino acid residue
WT: wild-type
Abstract

*Leishmania infantum* is a parasite that maintains its life cycle by alternating between an extracellular flagellated promastigote in the gut of phlebotomine insects and an intracellular non-flagellated amastigote inside the macrophages of mammalian hosts. This protozoan causes visceral leishmaniasis in children and immunosuppressed individuals, a disease to which there are no adequate means of prevention and treatment. Hence, the improvement of our knowledge on the mechanisms of host resistance to *L.*infantum is imperative to contribute to the development of novel therapeutic strategies.

Recent studies have evidenced the key role played by iron metabolism in the host-pathogen interplay. Both the host and pathogen require iron to perform the most basic cellular functions, but are vulnerable to iron-induced toxicity. In this work, we studied the effects of iron supplementation or deprivation on the in vivo growth of *L.*infantum. We found that dietary iron deficiency did not affect *L.*infantum growth, whereas iron overload decreased its proliferation in the liver and spleen of a susceptible mouse strain. The anti-leishmanial action of iron was abrogated in mice genetically deficient in the NADPH oxidase or nitric oxide synthase 2, the phagocytic enzyme complexes responsible for the generation of microbicidal reactive oxygen and nitrogen species (ROS and RNS), respectively. This suggested that iron inhibited the growth of *L.*infantum in the mouse tissues via interaction with ROS and RNS. Iron overload did not significantly influence the mouse adaptive immune response to *L.*infantum. We also described that iron inhibited, in a dose dependent manner, the growth of axenic cultures of *L.*infantum promastigotes and amastigotes. So, from these results, we inferred that iron synergizes with the host’s oxidative mechanisms of defense against *L.*infantum.

Such observations prompted us to further characterize the relative contribution of those antimicrobial mechanisms to the control of *L.*infantum infection in the mouse. We established that NADPH oxidase, and hence ROS, were critical to restrain the initial establishment of *L.*infantum in the spleen and to control late infection in the liver. Contrariwise, nitric oxide synthase 2 and RNS did not confer protection against the parasite in both tissues at any stage of the infection. Hence, we provided evidence that ROS, but not RNS, are the critical host effector molecules involved in the restrain of *L.*infantum in the murine model.

In this connection, drugs that act by inducing the generation of pernicious ROS in target pathogens, such as the anti-malarial primaquine (PQ) and its analogues, might be
efficacious agents against *L.infantum*. Although PQ exhibits activity against visceral *Leishmania* species, it is not used in the clinical setting of visceral leishmaniasis. This is, in part, ascribed to the propensity of the aliphatic amine of PQ to undergo oxidative deamination *in vivo*, which consequently causes its inactivation. Such limitation can be overcome by the protection of the PQ's aliphatic amine with a peptidomimetic (imidazolidin-4-one) or organometallic (ferrocene) moiety. Here in, we tested the activity of PQ derivatives containing such substituents against both life stages of *L.infantum*. One of the peptidomimetic (3c) and one of the organometallic (7a) derivatives of PQ were active against the disease-causing intramacrophagic amastigote stage of the parasite. Furthermore, 3c and 7a were less cytotoxic towards host cells than reference drugs sitamaquine or miltefosine. Therefore, both these PQ derivatives constitute candidate leads for the development of new and safer anti-leishmanials.

In summary, the work presented in this PhD dissertation might contribute to a better understanding of the mechanisms of host resistance to *L.infantum* and to the development of improved therapeutic agents for the treatment of visceral leishmaniasis.
Resumo

*Leishmania infantum* é um parasita que mantém o seu ciclo de vida alternando entre a forma extracelular promastigota, presente no sistema digestivo de insectos flebotomíneos, e a forma intracelular amastigota, que reside em macrófagos dos hospedeiros mamíferos. Este protozoário causa leishmaniose visceral em crianças e indivíduos imuno-suprimidos, uma doença para a qual não existem meios adequados de prevenção e tratamento. Conhecer os mecanismos de resistência do hospedeiro à infecção por *L. infantum* poderá contribuir para o desenvolvimento de novas estratégias terapêuticas.

Estudos recentes têm evidenciado o papel crucial do metabolismo do ferro na relação entre os hospedeiros e os patogénios. Tanto o hospedeiro como o patogénio necessitam de ferro para executarem as suas funções celulares básicas, sendo no entanto vulneráveis à toxicidade induzida por este metal. Neste trabalho, estudámos os efeitos de suplementar ou privar o hospedeiro de ferro no crescimento de *L. infantum* no modelo ratinho. Verificámos que a deprivação de ferro não afetou o crescimento de *L. infantum*, enquanto que a sobrecarga de ferro diminuiu a proliferação deste parasita no fígado e no baço de uma estirpe de ratinho suscetível à infeção. O efeito inibitório da sobrecarga de ferro foi abolido em ratinhos geneticamente deficientes na oxidase do NADPH ou sintase do óxido nítrico 2, que são complexos enzimáticos dos fagócitos responsáveis pela síntese de espécies reativas de oxigénio e azoto (ROS e RNS), respetivamente. Este facto sugeriu que o ferro inibiu o crescimento de *L. infantum* nos tecidos do ratinho através da interação com ROS e RNS. Adicionalmente, demonstrámos que a sobrecarga de ferro não influenciou significativamente a resposta imune adaptativa do ratinho à infeção por *L. infantum*. Também mostrámos que o ferro inibiu, de um modo dependente da dose, o crescimento de culturas axénicas de promastigotas e amastigotas de *L. infantum*. Assim, estes resultados permitiram-nos inferir que o ferro atua em sinergia com os mecanismos oxidativos do hospedeiro usados na defesa contra *L. infantum*.

As observações anteriores incitaram-nos a caracterizar mais extensivamente a contribuição relativa destes mecanismos anti-microbianos para o controlo da infecção causada por *L. infantum* no ratinho. Estabelecemos que a oxidase do NADPH, e portanto as ROS, foram essenciais para restringir o estabelecimento da infeção por *L. infantum* no baço e para controlar a infecção tardia no fígado. Pelo contrário, a sintase do óxido nítrico 2 e as RNS não conferiram proteção contra o parasita em qualquer fase da infeção nos
dois tecidos. Concluímos pois que as ROS, mas não as RNS, são as moléculas microbicidas envolvidas na restrição da infeção por *L. infantum* no hospedeiro murino.

Tendo em conta esta conclusão, é possível que drogas que atuem nos patogénios alvo induzindo a formação de ROS, como o caso do anti-malárico primaquina (PQ) e seus análogos, possam ser agentes eficazes contra *L. infantum*. Apesar de a PQ exibir atividade contra espécies viscerais de *Leishmania*, não é utilizada como tratamento da leishmaniose visceral. Isso deve-se, em parte, à propensão da amina alifática da PQ para sofrer oxidação desaminativa *in vivo*, o que conduz consequentemente à sua inativação. Esta limitação pode ser contornada através da proteção da amina alifática da PQ com um grupo péptido-mimético (imidazolidin-4-ona) ou organometálico (ferroceno). Neste trabalho, testámos a atividade de derivados da PQ, contendo estes substituintes, em ambas as fases de *L. infantum*. Observámos que um derivado da PQ péptido-mimético (3c) e um organometálico (7a) foram ativos contra a fase clinicamente relevante do parasita, o amastigota intramacrófágico. Observámos ainda, que os compostos 3c e 7a foram menos tóxicos para as células hospedeiras do que as drogas de referência sitamaquina ou miltefosina. Concluímos assim, que ambos os derivados da PQ poderão constituir estruturas-base para o desenvolvimento de novos agentes anti-*Leishmania* com reduzida toxicidade.

Em suma, os resultados apresentados nesta dissertação de doutoramento poderão contribuir para a melhor compreensão dos mecanismos de resistência do hospedeiro à *L. infantum* e para a descoberta de agentes terapêuticos mais eficazes no tratamento da doença provocada por este protozoário.
Chapter I

General Introduction
Chapter I | General Introduction

1. Leishmaniasis

1.1. General considerations

The leishmaniases are a complex of mammalian diseases characterized by distinct clinical manifestations: cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL). These are caused by protozoa of the genus *Leishmania*, that belong to the Trypanosomatidae family and Kinetoplastida order [1,2]. Natural transmission of the parasite occurs by the bite of female insects of the genera *Phlebotomus* (Old World) or *Lutzomyia* (New World), from the Psychodidae family and Diptera order [1,3]. The transmission of *Leishmania* parasites mediated by the sandfly may be zoonotic - from animal reservoir to humans - or anthropoontic - between humans without involvement of an animal reservoir. In the former, reservoir hosts include rodents, dogs and wild canids [1,4]. *Leishmania* parasites have a digenetic life cycle, alternating between the promastigote stage in the insect gut and the amastigote stage in macrophages of mammalian hosts [5].

The World Health Organization estimates that leishmaniasis is endemic in 98 territories, where more than 350 million people are at risk of acquiring infection. Globally, the incidence of the disease is 2 million new cases per year, with 0.5 and 1.5 million cases of VL and CL, respectively [4]. VL alone is responsible for nearly 50,000 annual deaths, a rate exceeded only by malaria amongst parasitic diseases [4]. However, these figures are largely underestimated since official notification of the disease is not compulsory in many endemic countries and the disease is often either undiagnosed or misdiagnosed [6,7].

Leishmaniasis is generally regarded as a neglected tropical disease, being strongly associated to poverty. Within many endemic areas, increased infection risk is due to poor housing and sanitation conditions, household crowding and lack of personal protective measures. Migration driven by economic reasons or military conflicts can expose susceptible individuals to infected sandflies [8]. In addition, undernourishment [9,10] and co-infection with the human immunodeficiency virus (HIV) [11] increases the probability of developing clinically expressed VL. Lastly, diagnosis and treatment are expensive and often delayed due to difficult access to health care facilities [8].
1.2. Clinical presentations

CL is characterized by the appearance of skin lesions at the sites of parasite inoculation, which slowly evolve from papules to nodules and in some circumstances to ulcers. The latter have a central depression and an indurated border that heals spontaneously but leaves permanent disfiguring scars [2,4]. Numerous nodular or ulcerated lesions can also be found in disseminated CL [4]. While in the Old World, CL is caused by L.tropica, L.major and L.aethiopica (L.infantum and L.donovani are also implicated), in the New World it is caused by L.mexicana, L.braziliensis, L.panamensis, L.guyanensis, L.amazonensis, L.peruviana or L.infantum [2,4]. However, new papules can appear in the vicinity of healed lesions typically caused by L.tropica, a condition known as leishmaniasis recidivans [2,4]. Moreover, non-ulcerative and non-healing lesions are spread throughout the body in diffuse CL (DCL), with frequent relapses occurring after treatment [4,6]. This form of CL is caused by L.aethiopica in the Old World and L.mexicana and L.amazonensis in the New World [4]. Most CL cases occur in Central and West Asia, North and East Africa, as well as in South America [4,6,12].

MCL is caused by lymphatic or hematogenous dissemination of the parasite from the skin to the oral, nasal and pharyngeal cavities, resulting in their severe mutilation and destruction [2,4,6]. This form of the disease seldom heals spontaneously and is mainly caused by L.braziliensis, L.guyanensis and L.panamensis, present in the New World. Most MCL cases are reported in South America [4]. In the Old World, MCL is rare but can be caused by L.infantum, L.donovani, L.major and L.tropica [4,6].

VL is a systemic infection, nearly always fatal if left untreated. The disease may be asymptomatic, oligosymptomatic or fully established. Active VL comprises a wide range of clinical symptoms characterized by undulating fever, diarrhoea, severe cachexia, hepatosplenomegaly, lymphadenopathy, hypergammaglobulinaemia, pancytopenia and hypoalbuminaemia [2,4,13]. Moreover, a dermal manifestation termed post kala-azar dermal leishmaniasis (PKDL) may develop after treatment of VL. Patients with PKDL may constitute reservoirs for VL because their skin lesions contain numerous parasites [2,6,13]. In the Old World, VL is caused by L.donovani and L.infantum, whereas in the New World it is caused by L.infantum (synonymous to L.chagasi [14,15]) [4]. L.infantum affects mostly children (< 5 years) and immunosuppressed adult individuals, whereas L.donovani affects all age groups (median, 13-23 years) [4]. The majority of VL cases occur in the Indian Subcontinent, East Africa, Mediterranean Basin, Middle East, West Asia and South America [4,6,13].

Canine leishmaniasis is a systemic disease manifested by non-specific clinical
signs, including loss of weight, cutaneous and mucosal lesions, lymphadenopathy, splenomegaly, fever, vomiting, polyuria, chronic renal failure, hyperglobulinemia, pancytopenia, hypoalbuminemia and ocular, vascular and neurological disorders. It is usually caused by *L. infantum* and can be present in the Mediterranean Basin, Africa, Asia and the American continent [16].

In Portugal, the dog is considered a host and a major reservoir of *L. infantum* [1], the prevalence of canine leishmaniasis being estimated at up to 20% in endemic regions [17,18]. In this country, the number of reported cases of human leishmaniasis is estimated at 15 cases per year [18,19,20]. Though human VL in Portugal has been considered predominantly an infantile disease, since the advent of HIV, a tendency to decrease children cases and increase infection in adults has been observed [17].

### 1.3. Diagnosis

The classical diagnosis method of leishmaniasis is microscopic visualization of *Leishmania* amastigotes in Giemsa stained samples (spleen, bone marrow and lymph node aspirates or skin). The more sensitive parasite culture from these samples is time-consuming and expensive, with contamination risks associated. In addition, the collection and management of biological samples demands well-equipped facilities and technical expertise and may be painful or life threatening for the patients [12,21,22]. Thus, non-invasive methods are more suited for routine diagnosis.

Serological diagnosis can be performed by indirect fluorescence antibody test, enzyme linked immunosorbent assay and western blot. These tests detect antibodies present in blood or serum, which can recognize a variety of *Leishmania* antigens with high sensitivity and specificity; yet, their application requires laboratory settings [13,21]. To overcome such drawback, freeze-dried or fast agglutination tests and the rK39 immunochromatographic strip test have been adapted to easily and rapidly diagnose VL in field conditions [23]. Nonetheless, serological tests are unreliable in CL patients due to their low or undetectable antibody levels [12,24]. Moreover, these tests do not readily discriminate between active, asymptomatic or past VL infection, since anti-leishmanial antibodies are easily detected in these three conditions [13,25,26]. This limitation can be theoretically overcome by antigen detection tests, which directly correlate antigen levels with parasite load or disease progression [21]. The latex agglutination test employs latex beads coated with antibodies to detect a leishmanial glycoconjugate antigen in the urine of VL patients with good specificity, but this test requires sensitivity improvement [23,27].
The intradermal or Montenegro skin test consists in the injection of Leishmania antigen or killed intact parasites into the patients’ forearm and measurement of the local induration of the skin 48 or 72 hours later [12,28]. This test is negative in acute cases of VL due to impaired T cell mediated immunity, but is positive after cure, allowing the identification of people previously exposed to the parasites [28]. Such distinction is problematic in CL patients because T cell mediated immunity usually also develops during active infection [12,24].

Polymerase chain reaction based techniques, which amplify Leishmania DNA, can be used in the diagnosis of all forms of leishmaniasis. They are sensitive, specific and allow rapid parasite load quantification and species identification in diverse samples [12,21,22]. High cost and technical complexity restrict its use to laboratories, but field application is being developed [21,22].

Combination or improvement of available diagnostic tests should permit an accurate diagnosis of leishmaniasis and prompt initiation of therapy. New tests are needed and they ought to be rapid, simple, affordable, field applicable, sensitive and specific, allowing discrimination between current, subclinical and past infections [13,21,22].

1.4. Treatment

1.4.1. Current therapeutic modalities

Priority has been given to the development and clinical implementation of drugs to treat VL, due to the associated high mortality rates of this form of leishmaniasis [21]. Although not fatal, CL is treated to hasten cure, decrease scar advance and to prevent parasite spreading or relapse [12].

The pentavalent antimonials (SbV) have been used in the treatment of VL, CL and MCL for more than 60 years [12,21]. They are affordable and highly effective in many endemic areas, but their use was discontinued in the Indian Bihar State due to escalating L. donovani resistance [29], a problem attributed to drug misuse. Besides, SbV present several disadvantages such as painful intramuscular or intravenous administration, prolonged treatment and toxicity (cardiac arrhythmias and pancreatitis) [21]. It is accepted that SbV are pro-drugs that require reduction to trivalent antimonials (SbIII) to become active. They act, in part, by interfering with the antioxidant defense system of both stages of L. donovani: SbIII induce efflux of reduced trypanothione (T(SH)2, the major thiol in Leishmania) and inhibit trypanothione reductase (TR), thus favouring intracellular accumulation of oxidized trypanothione (TS2) [30,31]. Consequently, uncontrolled oxidative metabolism leads to an apoptotic-like cell death of the parasite [32]. Therefore, it
is not surprising that resistance to Sb$\text{III}$ correlates with increased levels of T(SH)$_2$ [33], TR [34] and enzymes involved in the T(SH)$_2$ biosynthetic pathway [35,36] and ROS detoxification [37]. Finally, other mechanisms such as decreased drug uptake or increased efflux/sequestration of the drug may be involved in Sb$\text{III}$ resistance [38,39].

Pentamidine, an aromatic diamidine, is used only as a second line treatment for VL, CL and DCL [39] due to its serious adverse effects (insulin-dependent diabetes mellitus, shock, hypoglycaemia and death), high cost and declined efficacy over the years [21]. This drug induces alkalization of acidocalcisomes [40] and accumulates in mitochondria [41], causing collapse of membrane potential [42] and fragmentation of kinetoplastid DNA [43]. Resistance correlates with reduced pentamidine uptake and accumulation in the mitochondria, as well as with increased drug efflux from L.mexicana promastigotes [41].

Amphotericin B (AmB), a polyene macrolide antibiotic, is used as a first-line drug in areas of high unresponsiveness to Sb$\text{V}$. Although AmB is affordable and highly effective, it requires lengthy intravenous administration, hospitalization and causes infusion-related side effects (fever, chills, nephrotoxicity and hypokalemia) [21]. The safer lipid formulations of AmB are the drugs of choice for VL treatment in developed countries, though elevated cost precludes their use in poor countries [21]. Infusion of these formulations delivers AmB to tissue macrophages [21,44]. There, AmB binds to ergosterol present in Leishmania surface membrane and forms transmembrane channels, which affect membrane-bound enzymes and allow permeability to cations, water and glucose [45]. Although resistance to AmB is rarely reported [38], L.donovani promastigotes are able to resist its action by substituting membrane ergosterol by one of its precursors [46].

The aminoglycoside antibiotic paromomycin is effective against CL and VL [39], well tolerated and inexpensive, but requires intramuscular administration [47]. This molecule induces mitochondrial membrane depolarization and inhibits cytoplasmic and mitochondrial protein synthesis in Leishmania promastigotes [48], by binding to and interfering with the ribosome function [49]. Such effects together with drug uptake are less pronounced in L.donovani resistant promastigotes [48].

Miltefosine (hexadecylphosphocholine) is the only highly effective oral drug for the treatment of VL and CL [50], including cases unresponsive to Sb$\text{V}$. Although miltefosine is relatively safe, it exhibits teratogenic potential [21,51]. This molecule causes mitochondrial membrane depolarization, decreases oxygen consumption and ATP cytosolic levels and inhibits mitochondrial cytochrome c oxidase in L.donovani promastigotes [52]. Besides, it alters the phospholipid and sterol content of promastigote membranes [53]. Despite miltefosine’s high efficacy, major concerns on the development of resistance have been
raised because of drug misuse, its long half-life and easiness to experimentally induce resistance [21]. The decreased drug internalization by resistant *L. donovani* parasites [54,55] is due to defects in the function of the miltefosine translocation machinery, which is normally present at the plasma membrane [56,57]. In addition, resistant promastigotes can change the lipid composition of the surface membrane to impede miltefosine binding [58] or they can efflux the drug through the action of ABC-like transporters [59,60].

The treatment of CL and MCL can also be achieved, with variable efficacy, by the use of azoles, azithromycin, allopurinol, dapsone, rifampicin or imiquimod, either by parenteral or topical administration [12].

1.4.2. Developing new chemotherapeutics for VL: primaquine derivatives

The abovementioned drugs present several drawbacks, such as parasite resistance, specific toxicities, elevated costs, prolonged treatment regimens and painful administration routes that lead to treatment rejection by the patients. Hence, alternative drugs with improved therapeutic effectiveness and strategies to preserve the efficacy of the available ones are urgently required [21,38,61]. Many classes of compounds are being recently developed for the treatment of VL, including 2-quinolines, buparvaquone, alternative AmB formulations and 8-aminoquinolines (8-AQs) [21,50]. The latter are a promising class of drugs for the oral treatment of malaria, *Pneumocystis jirovecii* pneumonia, trypanosomiasis and leishmaniasis [62].

One of the 8-AQ members, sitamaquine, is currently being tested for the oral treatment of VL (completed phase IIb clinical trials). However, this drug presents variable efficacy and serious side effects (dyspepsia, vomiting, abdominal pain, headache, nephrotoxicity, cyanosis and methemoglobinemia) [63,64,65,66,67]. Sitamaquine crosses the *L. donovani* promastigote plasma membrane via electrostatic and hydrophobic interactions with phospholipids [68,69]. Then, it rapidly accumulates in and alkalizes the acidocalcisomes [40], although this process appears to be unrelated to its lethal action [70]. Moreover, sitamaquine inhibits mitochondrial succinate dehydrogenase and causes respiratory dysfunctions, which consequently trigger oxidative stress that leads to apoptosis-like death of *L. donovani* promastigotes [42,71]. Resistance to sitamaquine can be experimentally induced in both parasite stages [72] and is possibly owed to drug efflux by an ABCG-like transporter [59].

Primaquine (PQ, A in Figure 1), a well known anti-malarial 8-AQ, also displays activity against visceral *Leishmania* species in culture medium [73], macrophages [74,75,76] or animal infection models [76,77,78]. However, it is not used in the VL clinical
context since it exhibits lower efficacy than VL reference drugs [73], low oral bioavailability and considerable toxicity (methemoglobinemia and hemolytic anemia) [79]. Such disadvantages can probably be overcome by PQ encapsulation or direct modification of its structure. In fact, encapsulation in liposomes or nanoparticles enhances PQ activity against *L. donovani* in macrophages [74,75,76] and animal models of infection [76,77]. On the other hand, the modification of PQ structure has already led to the finding of other 8-AQs with excellent activity against experimental VL: tafenoquine [80], NPC1161B [81] and sitamaquine [78,81], which is already in clinical trials as mentioned earlier.

The modification of PQ structure can be accomplished by introduction of substituents on the quinoline moiety and/or modification of the terminal aliphatic amino group [79]. The latter approach intends to reduce PQ conversion into inactive carboxyprimaquine (carboxyPQ, B in Figure 1), the major metabolic process underlying PQ’s low oral bioavailability [79]. In particular, the terminal aliphatic amino group of PQ can be blocked by *N*-acylation with amino acids or peptides (C in Figure 1). However, this produces structures susceptible to premature proteolytic cleavage that yield PQ (and consequently allow its conversion into carboxyPQ) before it can reach target cells. Hence, these structures require extra protection of the peptide moiety [79]. This can be achieved by condensing the *N*-aminoacyl derivatives of PQ with carbonyl compounds, leading to the formation of an imidazolidin-4-one ring (D in Figure 1) [82], a classical strategy to protect peptide drugs from early degradation by amino- or endo-peptidases [83,84,85,86]. The imidazolidin-4-one approach has been applied to amino acid derivatives of PQ, yielding novel compounds which displayed superior stability in physiological buffer and human plasma [82,87], while retaining PQ’s activity against *Plasmodium* and *Pneumocystis* [87,88]. Furthermore, higher stability and moderate activity could also be obtained by further *N*'-acylation of the imidazolidin-4-one ring with an additional amino acid residue (E in Figure 1) [89,90]. Such procedure aimed at (i) completely supressing the hydrolysis of the imidazolidin-4-one ring, (ii) increasing the aqueous solubility of the entire compound and finally, (iii) increasing the therapeutic activity due to the addition of a basic amino group, a feature indispensable for anti-malarial activity [79,89]. These strategies may also prove useful to increase the anti-leishmanial activity of PQ.

To further improve efficacy against *Plasmodium* [91] and *Pneumocystis* [92], PQ and its amino acid derivatives were also coupled to a ferrocene (Fc) moiety by reaction with ferrocenecarboxylic acid (F in Figure 1). It is known that the Fc structure [93] and PQ induce the generation of reactive oxygen species (ROS) toxic to several pathogens [79]. Hence, their junction might have anti-leishmanial activity as well, since *Leishmania* are sensitive to ROS action [94]. Accordingly, the PQ analogues sitamaquine [71] and
tafenoquine [95] inhibit the activity of the mitochondrial succinate dehydrogenase and cytochrome c reductase, respectively, a process that leads to the production of ROS and culminates in *Leishmania* cell death.

Apart from the modifications referred to above, many others can be made to the PQ structure to try to improve its anti-leishmanial activity, but only the ones described are the focus of this thesis.

![Chemical structures of primaquine and some derivatives modified at the terminal aliphatic amino group.](image)

**Figure 1** | Chemical structures of primaquine and some derivatives modified at the terminal aliphatic amino group. The low oral bioavailability of primaquine (PQ, A) is a consequence of its metabolic conversion to carboxyprimaquine (carboxyPQ, B), a process that can be reduced by blocking PQ’s terminal aliphatic amino group with amino acids or peptides (C), an imidazolidin-4-one ring (D) $N^1$-acylated with an additional amino acid residue (E) or a ferrocene moiety (Fc, F). Adapted from [89,91,92].

### 1.5. Control and prevention

All forms of leishmaniasis should be ideally controlled and prevented, but more attention has been given to VL on this matter due to its associated mortality [2]. Prompt diagnosis and treatment of human cases is extremely important to avoid or decrease the transmission of anthropornotic VL [13,96]. In addition, it has been advised that the control of canine leishmaniasis ought to involve removal and humane destruction of seropositive and/or infected canines [13,96]. Chemotherapy represents an alternative to this ethically questionable procedure, though relapses are very frequent [13,96]. Hence, it has been suggested that vector control and vaccination should constitute the proper measures to prevent leishmaniasis [96]. The former approach encompasses the protection of human residences and animal shelters by indoor insecticide spraying and inclusion of insecticide treated nets [97]. Moreover, canine protection can be achieved by treatment with topical repellents and use of insecticide-impregnated collars [98]. Despite many efforts to develop a vaccine against human leishmaniasis, no formulation has been registered so far [99]. Conversely, the canine vaccines Leishmune® and Leish-Tec® (Brazil) or CaniLeish®
(Europe) have already been licenced [96]. In fact, canine vaccination with Leishmune®, which revealed protective [100] and transmission blocking [101] effects, has been reported to decrease VL incidence in both dogs and humans [102].

2. *Leishmania*-host interactions

2.1. Life cycle of *Leishmania*

The life cycle of *Leishmania* (Figure 2) can be initiated when a female sandfly ingests free amastigotes or infected macrophages during a bloodmeal on the skin of a vertebrate host. In the insect posterior midgut, macrophages disintegrate and released amastigotes experience a decrease in temperature and increase in pH that triggers their transformation into flagellated procyclic promastigotes (weakly motile and highly replicative stage). Then, they undergo a series of replication and differentiation events that culminate in their migration to the anterior midgut/foregut as infective metacyclic promastigotes (highly motile, non-dividing forms) [3,103]. When the sandfly takes another bloodmeal, around 1000 metacyclic promastigotes are regurgitated into the skin of a mammalian host together with sandfly saliva [104] and promastigote secretory gel [105], both potent enhancers of promastigote infectivity in mammalian hosts.

In the host dermis, metacyclic promastigotes are internalized by resident dendritic cells (DCs) [106], macrophages or rapidly infiltrating neutrophils [107]. Additional monocytes and neutrophils can be attracted to the bite site by the chemokines CCL2 and CXCL1, respectively, which are secreted by infected macrophages [108]. Although, *Leishmania* can infect diverse host cells, there is only evidence for replication and long-term survival within mononuclear phagocytes [5]. Promastigote attachment to the receptors of complement (CR1 and CR3), fibronectin and mannose [109,110] triggers actin-dependent phagocytosis that leads to *Leishmania* enclosure within a phagosome. This vesicle matures over time through fusion/fission events with early and late endosomes and lysosomes to form a phagolysosome-like compartment, known as the parasitophorous vacuole (PV) [111]. This acidic compartment is highly enriched in lysosomal proteases (cathepsins B, D, H and L) and its membrane is characterized by the presence of lysosomal-associated membrane proteins (LAMP) 1 and 2, the small GTPase Rab7p, macrosialin (CD68) and major histocompatibility complex (MHC) class II molecules [111,112]. However, promastigotes are able to delay phagosomal maturation [113,114] and acidification [115], possibly to allow time for differentiation into the non-flagellated amastigote, a process predominantly driven by the increased temperature of the host [116]. Once established, amastigotes are capable of surviving and proliferating
either in small PVs containing single amastigotes or in large PVs that can contain numerous amastigotes (*L. mexicana* complex species) [117]. Excessive amastigote replication may cause rupture of the macrophage and this allows re-infection of other local phagocytes [111]. Alternatively, the replicated amastigotes may be released from the macrophage by an exocytosis-like process [118]. Amastigotes may enter macrophages through the Fcγ, fibronectin, complement (CR3) or phosphatidylserine receptors [109,110] and do not delay phagosomal maturation [119]. In the case of visceral species of *Leishmania*, proliferation is paralleled by dissemination from the bite site in the skin to visceral organs (liver, spleen and bone marrow) [120,121]. Once dissemination or establishment of infection has been achieved, parasites must maintain a delicate balance between increasing the chance of transmission to the vector through proliferation and avoiding detection by the host immune system [111]. The transmission cycle of *Leishmania* is completed when infected phagocytes or free amastigotes are taken up by a sandfly during a blood meal from an infected host [3,103].

**Figure 2 | Life cycle of *Leishmania* parasites.** Inside the sandfly midgut, *Leishmania* amastigotes (1) transform into flagellated procyclic promastigotes (2). In turn, these undergo differentiation and replication events (3-4), ultimately converting to infective flagellated metacyclic promastigotes (5), which can be encountered at the foregut ready for transmission. During blood feeding, the sandfly introduces metacyclic promastigotes into the dermis of a mammalian host (5). Then, metacyclic promastigotes are phagocytosed by diverse cell types found in the local environment (6). However, only macrophages constitute the definitive host cell. After establishing intracellularly, metacyclic promastigotes differentiate into non-flagellated amastigotes (7). Amastigote replication (8) eventually leads to rupture of the host cell, which allows reinfection of local phagocytes (9). Alternatively, amastigotes may exit the macrophage by an exocytosis-like process (9). The transmission cycle completes when another sandfly ingests infected phagocytes or free amastigotes during a blood meal (10).
2.2. Host immune response to Leishmania

The type of immune response developed against Leishmania in mouse models is influenced by the host genetic background, the infecting parasite species and strain, the mode and site of parasite inoculation, as well as the inoculum size [122,123].

Neutrophils are one of the first cells to be recruited to sites of parasite inoculation [107] and they are involved in the resolution or aggravation of L.major lesions in resistant or susceptible mice, respectively [124,125,126]. In the former, resolution of L.major infection is linked to the secretion of neutrophil elastase [124] and IL-12 [127]. In addition, neutrophils are clearly involved in the early control of L.infantum and L.donovani growth [128,129,130]. Apart from the capacity to kill Leishmania through the release of ROS or neutrophil extracellular traps [131], neutrophils can also promote the development of protective immunity [128] and assist macrophages in parasite killing [124,132].

Monocytes also accumulate at the L.major inoculation site and may differentiate into dendritic cells (DCs) [133], which can capture amastigotes and activate CD4+ and CD8+ T cells [133,134]. These cells may also be activated by non-infected (bystander) DCs following exposure to L.braziliensis [135] and L.donovani [136], respectively.

Furthermore, the secretion of IL-12 by DCs is required for the early activation of natural killer (NK) cells in mice infected with L.infantum [137] and L.major [138]. Activated NK cells release IFNγ in order to (i) induce nitric oxide (NO•)-mediated killing of L.infantum by macrophages [139] and (ii) drive the differentiation of Th1 cells in the draining lymph nodes (dLNs) of L.major resistant mice [140]. In turn, CD4+ T cells secrete IL-2 that synergizes with DC-derived IL-12 to activate NK cells early after L.major infection [138,140].

All the cell types described above are involved in the early immune response to Leishmania and subsequent development of T cell responses. It is consensual that the development of T helper (Th) 1 immunity correlates with murine resistance to several cutaneous and visceral Leishmania species. In this case, IL-12 derived from antigen presenting cells drives the differentiation and proliferation of CD4+ Th1 cells capable of secreting IFNγ, which induces parasite killing by macrophages via NO• production [122,123,141,142]. By contrast, in mouse models of CL caused by L.major, susceptibility is generally associated with an IL-4-driven expansion of CD4+ Th2 cells and secretion of IL-4, IL-13 and IL-10 [122,123]. However, the association of Th2 responses with susceptibility to visceralizing Leishmania is not so clear. Although IL-10 overproduction is also related to susceptibility in mouse models of VL [122,143], IL-4 [144] and IL-13 [145] seem to promote the hepatic control of L.donovani growth.
Apart from Th1 and Th2 cells, other CD4+ T cell subsets also intervene in the immune response to *Leishmania*. The accumulation of CD4+CD25+Foxp3+ T regulatory (T<sub>reg</sub>) cells in skin lesions of resistant mice induces long-term persistence of *L.major*. These cells are able to suppress Th1 responses, in part, by secreting IFNγ [146]. Conversely, T<sub>reg</sub> cells protect susceptible mice against *L.major* infection by restraining the early production of IL-4 and the ensuing development of Th2 responses [147]. Non-healing *L.major* infection appears, instead, to correlate with IL-10 release by IFNγ-secreting CD4+ T cells and not by T<sub>reg</sub> cells [148]. Similarly, VL progression is linked to IL-10 production by regulatory DCs [149], NK cells [150] and CD4+ T cells that co-express or not IFNγ [151].

Th17 is another CD4+ T cell subset that produces cytokines of the IL-17 family [123]. Neutrophil recruitment mediated by IL-17 contributes to CL progression in susceptible mice [152] and likely promotes tissue inflammation and injury in MCL patients [153]. By contrast, IL-17 production correlates with resistance to human VL. The secretion of IL-17 by Th17 cells can be induced by exposure of peripheral blood mononuclear cells from healthy donors to *L. donovani*. Key cytokines required for the induction and maintenance of Th17 responses, namely IL-1β, IL-6 and IL-23, are also detected in such cultures [154].

The control of infection caused by *L.major* in skin lesions [155,156] and by *L. donovani* in the liver [157] also depends on the presence of IFNγ-secreting CD8+ T cells. Still, *L. donovani* is able to evade CD8+ T cell responses in the spleen by suppressing their proliferation and inducing their cell death [158]. Intriguingly, cytolytic CD8+ T cells have also been associated with lesion progression in *L. braziliensis*-infected humans [159].

Finally, the role of B cells in regulating immunity to CL is controversial. B cell-derived immunoglobulin Gs (IgGs) appear to mediate *L.major* uptake by DCs, resulting in activation of the latter and stimulation of T cells to produce IFNγ that leads to parasite killing by macrophages [160]. However, contradictory findings indicate that B cells are not involved in the development of either Th1 or Th2 responses during *L.major* infection [161]. In experimental VL, early B cell expansion (persistent throughout infection) and secretion of IgG and IgM have been implicated in the exacerbation of *L. infantum* infection [162]. This is supported by the fact that resolution of *L. donovani* infection is accelerated in the absence of B cells [129].

### 2.2.1. Organ-specific immunity in VL

The mouse liver is a site for initial multiplication of visceralizing *Leishmania* that eventually controls infection by forming cellular infiltrates around parasitized Kupffer cells (KCs), known as granulomas [120,121,163]. These structures start developing when
infected KCs secrete chemokines - like CCL3, CCL2 or CXCL10 - that recruit monocytes and neutrophils to encircle them (1 – 7 days) [164]. At this early stage, some infected KCs fuse to form multinucleated cells [165]. Such steps are then followed by the recruitment of CD4⁺ and CD8⁺ T cells to form a mature granuloma (7 – 30 days), a process dependent on the presence of IL-12, IFNγ, TNF, IL-2 [163], IL-4 [144] and IL-13 [145]. In this milieu, IFNγ activates infected KCs to eliminate intracellular amastigotes (30 days onward) [166]. Although several mature granulomas devoid of parasites - sterile granulomas - can be found in the hepatic parenchyma, sterile cure is never achieved [163]. It is likely that low parasite persistence in the liver, induced by IL-10, accounts for its resistance to re-infection [146]. Moreover, IL-10 secretion in developing granulomas may also counteract excessive pro-inflammatory responses and tissue injury [143].

Contrary to the liver, the spleen becomes chronically infected and a site of parasite persistency [120,121]. *Leishmania* are removed from the circulation in the spleen by macrophages located in the marginal zone (MZ) and red pulp. In the MZ, DCs acquire the parasite or its antigens, mature and migrate into the periarteriolar lymphoid sheaths (PALS). This recruitment occurs in response to CCL19 and CCL21, both chemokines produced by gp38⁺ stromal cells of the PALS. Once in the PALS, DCs secrete IL-12 and present *Leishmania* antigens to CD4⁺ T cells, leading to the generation of Th1 responses [167]. Hence, protective immunity occurs in the spleen during the acute phase of *L. donovani* infection. Still, infection progresses and causes disruption of the splenic architecture that is characterized by (i) loss of follicular DCs, (ii) remodelling of the MZ and (iii) changes in chemokine expression and cell migration [163]. Though the mechanism accounting for loss of follicular DCs is unknown, the other effects are primarily mediated by TNF and IL-10. Excessive TNF production causes the loss of MZ macrophages [168] and gp38⁺ stromal cells [169], with concomitant reduction of CCL19 and CCL21 expression. In addition, DCs become unresponsive to these chemokines due to down-regulation of CCR7 expression induced by IL-10. Consequently, DCs fail to migrate and interact with T cells in the PALS [169].

2.3. Host antimicrobial oxidative mechanisms

As previously mentioned, macrophages can be activated to eliminate intracellular *Leishmania*. Two of the most important antimicrobial systems of macrophages (and other phagocytes) are the NADPH oxidase and the nitric oxide synthase 2 (NOS2), which are the enzyme complexes responsible for the generation of superoxide (O₂⁻) and nitric oxide (NO⁻), respectively. These radicals may give rise to other more toxic intermediates
collectively known as reactive oxygen species (ROS) and reactive nitrogen species (RNS) that have the potential to kill intracellular pathogens [170,171].

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [171] catalyses the transfer of one electron from NADPH to oxygen (O\(_2\)) to yield O\(_2\)•* (Equation 1) [172]. NADPH oxidase activation can be elicited by microbial products (LPS), cytokines (IFN\(_{\gamma}\) and IL-8) or by binding to phagocytosis receptors (Fc\(_{\gamma}\) and complement receptors) [171,173]. Following activation by the mentioned stimuli, the cytosolic subunits (p40\(_{phox}\), p47\(_{phox}\), p67\(_{phox}\) and Rac1 or 2) migrate towards the membrane-bound subunits (p22\(_{phox}\) and gp91\(_{phox}\) and Rap1A) in order to assemble a functional oxidase [171]. The membrane subunits form the catalytic core of the oxidase, containing binding sites for flavin adenine dinucleotide (FAD), NADPH and two heme groups [170]. Hence, when a microbe is phagocytosed, the active NADPH oxidase present in the phagosomal membrane pours high amounts of O\(_2\)•* into the interior of the phagosome [171]. Then, O\(_2\)•* is rapidly converted to other oxidant successors that may cause oxidative damage to the target. The moderately reactive O\(_2\)•* is rapidly reduced to hydrogen peroxide (H\(_2\)O\(_2\)), either spontaneously or enzymatically by superoxide dismutase enzymes (SOD), particularly at acidic pH (Equation 2) [172,174,175]. In turn, H\(_2\)O\(_2\) may give rise to the highly reactive and short-lived hydroxyl radicals (HO•) in the Fenton reaction, by reacting with ferrous iron (Fe\(^{2+}\), Equation 3). The latter is regenerated when ferric iron (Fe\(^{3+}\)) reacts with O\(_2\)•* (Equation 4) [172,175]. Moreover, the formation of HO• can also be achieved by the reaction of O\(_2\)•* with hypochlorous acid (HOCl) (Equation 5). The latter molecule is generated by myeloperoxidase, an enzyme that catalyses the oxidation of halide ions to hypohalous acids (e.g. chloride to HOCl) at the expense of H\(_2\)O\(_2\) (Equation 6) [172].

\[
\begin{align*}
(1) & \quad 2 \text{O}_2 + \text{NADPH} \rightarrow 2 \text{O}_2\cdot* + \text{NADP}^+ + \text{H}^+ \\
(2) & \quad 2 \text{O}_2\cdot* + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
(3) & \quad \text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{HO}• + \text{HO}^- + \text{Fe}^{3+} \\
(4) & \quad \text{O}_2\cdot* + \text{Fe}^{3+} \rightarrow \text{Fe}^{2+} + \text{O}_2 \\
(5) & \quad \text{O}_2\cdot* + \text{HOCl} \rightarrow \text{O}_2 + \text{HO}• + \text{Cl}^- \\
(6) & \quad \text{H}_2\text{O}_2 + \text{Cl}^- \rightarrow \text{HOCl} + \text{HO}^- 
\end{align*}
\]

The NOS family of enzymes catalyse the oxidation of L-arginine to L-citrulline and NO•, in the presence of NADPH and O\(_2\) (Equation 7). They have an amino-terminal oxidase domain that contains a heme centre and binding sites for tetrahydrobiopterin and
L-arginine, linked by a calmodulin-binding domain to a carboxy-terminal reductase domain with binding sites for NADPH, FAD and flavin mononucleotide. Importantly, the production of NO• only occurs when NOS form a homodimeric structure [170,176,177,178]. The neuronal NOS (nNOS or NOS1) and endothelial NOS (eNOS or NOS3) are constitutively expressed and produce low levels of NO•, being their activity highly dependent on high intracellular calcium levels [176,177,178]. On the contrary, the inducible isoform (iNOS or NOS2) is the most widely distributed among cell types and its activity is not controlled by intracellular calcium concentrations [170,176,177,178]. Macrophages express the NOS2 enzyme [179], that can be found either in the cytosol or associated with intracellular vesicles [180]. Unlike other isoforms, NOS2 is capable of producing large quantities of NO• when suitably stimulated [170,177,178]. Its transcription can be induced by LPS, IFNγ and TNF [170,177,179] or suppressed by IL-4 [181]. However, the expression of NOS2 can also be regulated at the post-transcriptional and post-translational levels. TGFβ can decrease the stability and translation of NOS2 transcripts and enhance NOS2 protein degradation [182]. Moreover, NO• may regulate its own production by directly inactivating NOS2 [183]. Notwithstanding, when NOS2 is expressed and localized to the phagosome membrane [184] it likely favours the diffusion of NO• into the interior of the vesicle where its derivatives will cause deleterious effects on the phagocytosed target. Here, NO• undergoes successive oxidative degradation to form nitrite (NO2•), nitrogen dioxide (NO2•*) and nitrate (NO3•) [185]. Furthermore, NO• has a propensity to react with other free radicals, particularly with O2•* resulting in the formation of the strong oxidant peroxynitrite (ONOO•–) (Equation 8), which can further react with CO2 to yield NO2• and carbonate radical (CO3•–) [172,175].

(7) \[ \text{L-arginine} + 1.5\text{NADPH} + 2\text{O}_2 \rightarrow \text{L-citrulline} + 1.5\text{NADP}^+ + 2\text{H}_2\text{O} + \text{NO}\bullet \]  

(8) \[ \text{NO}\bullet + \text{O}_2\bullet^* \rightarrow \text{ONOO}^– \]

Several of the mentioned ROS and RNS are capable of inducing oxidative stress to microbes. This can be defined as a disturbance in the pro-oxidant and anti-oxidant equilibrium in favour of the former. Such state may lead to oxidative damage, that is, to molecular damage caused by the action of reactive species (RS) on DNA, lipids and proteins [172]. Damage to DNA affects its replication, gene expression and cell division. Several RS such as HO•, CO3•–, HOCl, ONOO•– can cause DNA strand breakage and/or chemical modifications to DNA bases or the deoxyribose sugar [172]. In addition, oxidative damage to polyunsaturated fatty acids tends to decrease the fluidity and functioning of biological membranes. Lipid peroxidation initiated by a RS (HO• and NO2•*) leads to the formation of a carbon radical (R•). Then, R• reacts with O2 to give a peroxyl
radical (RO$_2$•), which can abstract hydrogen from an adjacent fatty acid side chain. This reaction yields a lipid hydroperoxide and a new R• that can react again with O$_2$ and consequently propagate lipid peroxidation [172]. Finally, proteins may also suffer oxidative damage at both the protein backbone and on the amino acid side-chains, due to the attack of RS (RO$_2$•, HO•, HOCl, ONOO$^-$) or end products of lipid peroxidation. Protein damage may be reversible or irreversible and impair the function of receptors, signal transduction and transport proteins and enzymes [172].

### 2.3.1. *Leishmania* susceptibility to host derived oxidants

ROS and RNS generated in the absence of a host cell have the potential to kill *Leishmania*. In this context, both parasite stages are sensitive to the action of O$_2$•$^-$ [186,187] and H$_2$O$_2$ [187,188,189,190]. Amastigotes are notably more resistant than promastigotes to the effects of H$_2$O$_2$ [191,192], while infective promastigotes are more resistant than non-infective ones [193]. Presumably, H$_2$O$_2$ toxicity towards promastigotes is mediated by HO• and involves cessation of protein and RNA synthesis [193], which culminates in an apoptosis-like cell death [194,195]. Similarly, NO• or its derivatives decrease the viability of both *Leishmania* life stages [186,196,197,198,199,200,201], being amastigotes generally less sensitive to their action [198,199,202]. RNS interfere with the differentiation of amastigotes to promastigotes [199], inhibit the activity of cysteine proteinase [200], glyceraldehyde 3-phosphate dehydrogenase and aconitase [198,199] and eventually also induce an apoptotic-like cell death in amastigotes [203]. It has been suggested that ONOO$^-$ mediates the toxicity of NO• towards *L. donovani* promastigotes [190] and *L. amazonensis* amastigotes [204], but not *L. major* promastigotes [197].

During internalization and establishment of infection in the host cell, *Leishmania* face a deleterious mixture of ROS and RNS. Infection of macrophages with promastigotes elicits the release of O$_2$•$^-$ [186] and ensuing formation of H$_2$O$_2$ [189,205]. Promastigotes trigger the oxidative burst [195,206,207,208] more easily than amastigotes [188,191,205,209], explaining the former increased susceptibility to elimination by a mechanism reminiscent of apoptosis [195]. Importantly, the leishmanicidal activity of the host cell is compromised in the presence of ROS scavengers [186,189,210] or due to a genetic deficiency in the NADPH oxidase [191,205]. However, the fact that monocytes and macrophages deficient in ROS production are able to restrain the growth of *L. donovani* after IFN$\gamma$ activation [205], indicates that other mechanisms intervene in the control of the parasite. Indeed, macrophage activation (IFN$\gamma$ and LPS) enhances the production of RNS and leads to elimination of both parasite stages [196,197,211,212,213].
whereas inhibition of NOS2 activity [186,196,211,212] or a genetic deficiency in this enzyme [213,214] has the reverse effect. Moreover, activated macrophages from resistant mouse strains have higher capacity than those from susceptible strains to generate RNS and eliminate *L. major* [215] by an apoptotic-like process [203,216].

Several lines of evidence also support the notion that ROS and RNS are relevant to control *Leishmania* infection in animal models [217]. The healing of cutaneous lesions and life-long containment of persisting parasites correlate intimately with NOS2 expression in the *L. major* [196,213,214,218,219,220,221], *L. amazonensis* [204] and *L. braziliensis* [222] mouse models. However, unlike NOS2, NADPH oxidase is not required to control *L. amazonensis* [217] or *L. braziliensis* [222] infections. Still, its activity is required to clear *L. major* from the spleen and to prevent late parasite visceralization [214,223]. In contrast to what is observed in most mouse models of CL, resolution of lesions caused by *L. guyanensis* depends solely on the induction of NADPH oxidase activity [195,217]. This seemingly clear scenario is, however, not observed in animal models of VL. A first report showed that both NADPH oxidase and NOS2 acted in concert to control early *L. donovani* replication in the mouse liver, whereas NOS2 alone was required and sufficient to resolve late infection [166]. Contradictorily, a subsequent study demonstrated that neither of these antimicrobial mechanisms influenced the outcome of *L. donovani* infection in the mouse liver, though NOS2 exerted moderate protective effects in the spleen at late phases of infection [223]. To further complicate this picture, NOS2 deficiency appears to be the cause of unrestrained growth of *L. donovani* in the hamster’s liver, spleen and bone marrow. These animals have an intrinsic defect in the NOS2 promoter, whose responsiveness to activating stimuli (IFNγ and LPS) is reduced. As a consequence, NOS2 transcription and NO• production are impaired in the hamster’s macrophages [224,225].

2.3.2. *Leishmania* resistance to host derived oxidants

Despite the importance of ROS and RNS generated by the macrophage as leishmanicidal molecules, complete elimination of the parasite is seldom attained [219]. Naturally acquired [226,227] or experimentally induced [187,201] resistance to oxidative stress increases *Leishmania* infectivity and capacity to multiply or persist in the host. This highlights the parasite’s capacity to subvert the production of oxidants or avoid their toxicity through its antioxidant defense and repair systems [94].

Concerning the first situation, both *L. donovani* parasite stages interfere with the assembly of a functional NADPH oxidase by preventing the recruitment of p47phox and p67phox subunits to the phagosomal membrane [228,229]. Such effect is attributed to
lipophosphoglycan (LPG) in the case of promastigotes [228]. Other surface membrane molecules of *Leishmania* can also inhibit the oxidative burst of macrophages (metalloprotease Gp63) [230] or decrease NOS2 expression and activity (glycoinositolphospholipids [231] and LPG [232]). In particular, *L.amazonensis* promastigotes and *L.mexicana* amastigotes can decrease NO• synthesis by LPS-stimulated macrophages [233] and DCs [234], respectively. Besides, amastigotes can impair NO• synthesis by inducing TGFβ release from the macrophage [235] or by attenuating the IFNγ-induced activation of the Jak1/2-Stat1 pathway (Janus kinases 1/2 – signal transducer and activator of transcription 1) [236].

In addition to modulating the capacity of the host cell to generate oxidants, *Leishmania* also possess an antioxidant defense system that allows them to withstand oxidative stress. *Leishmania* surface LPG and related phosphoglycans [237,238,239], reduced pterins [240] and the reduced thiols ovothiol A, glutathione and trypanothione [241] are non-enzymatic scavengers of ROS/RNS.

In addition, the parasite actively detoxifies ROS and RNS through complex enzymatic machineries. *Leishmania* possess iron-containing SODs involved in O2•− detoxification mainly in the mitochondria and glycosomes [242,243,244]. However, this enzyme converts O2•− to the more toxic H2O2, which can be reduced by a variety of peroxidases that use electrons derived from reduced trypanothione, by the intermediate of tryparedoxin or ascorbate [94]. The *L.infantum* cytosolic 2-cysteine peroxiredoxin (2-cys PRX) is capable of detoxifying H2O2, t-butyl hydroperoxide (t-bOOH), ONOO− or NO• and its overexpression enhances parasite survival in macrophages [245,246]. In contrast, the mitochondrial 2-cys PRX appears to protect *L.infantum* from exposure to t-bOOH, but not H2O2 [245]. Another class of peroxidases are the non-selenium glutathione peroxidase-like enzymes (nsGPX). The *L.major* nsGPX1 displays slightly less affinity for H2O2 and is less abundant than 2-cys PRX [247]. Nevertheless, *L.major* also possesses a mitochondrial ascorbate peroxidase that confers resistance to H2O2 [248]. In addition, the *L.major* eukaryotic elongation factor 1B (eEF1B) exhibits trypanothione S-transferase and peroxidase activity [249]. This enzyme localizes to the endoplasmic reticulum and displays activity against hydrophobic hydroperoxides, but not H2O2, suggesting its participation in resistance to lipid peroxidation [249]. Finally, the presence of a NOS enzyme in *L.donovani* promastigotes, suggests that this parasite might have mechanisms to detoxify endogenously produced NO• that could offer resistance against exogenous NO• [250].
3. Iron and Leishmania-host interplay

3.1. Iron properties and functions

Iron is the fourth most abundant element of the earth’s crust and the most abundant transition metal in living organisms. Cells of virtually all forms of life are dependent on adequate quantities of iron for survival and proliferation [251,252]. The dynamic capacity to alternate between oxidation states (ferric iron, Fe$^{3+}$ or ferrous iron, Fe$^{2+}$) and to form coordination complexes with organic ligands, enables iron to participate in critical biological functions like oxygen binding and transport, electron transfer, NO• sensing, transcriptional regulation, structural stabilization or catalysis [252,253]. Whereas Fe$^{3+}$ is virtually insoluble under aerobic conditions at physiological pH, Fe$^{2+}$ is soluble and may readily catalyse the formation of pernicious radicals [172,252]. In fact, excessive accumulation of iron in tissues is strongly associated with DNA damage, lipid peroxidation, protein tyrosine nitration and oxidation [254,255,256]. In addition, iron-induced oxidative damage has been proposed to contribute to the pathology of several human disorders [257]. To reduce the threat of oxidative stress or damage in biological systems, iron is found associated to proteins in a variety of complexes [252,253].

3.2. Iron and infection

Both the host and invading pathogens require iron for survival and must have refined mechanisms for its acquisition and management. The vertebrates’ innate immune response to infection includes numerous mechanisms of iron restriction, which the pathogen attempts to circumvent to gain access to iron [258,259,260]. Data from humans and animal models indicate that the host iron status greatly influences susceptibility to infection. In most instances, iron availability favors the replication of pathogens, whereas iron deprivation hinders their growth [258,261,262].

3.2.1. Host iron homeostasis

The organism of an adult individual normally possesses 3000 to 4000 mg of iron, of which approximately 1-2 mg is lost per day, due to desquamation of skin and urinary cells, sloughing of intestinal epithelial cells, bleeding or sweat. In mammals, there is no regulated excretion of iron, hence the replenishment of such losses and consequent maintenance of iron balance occurs at the level of intestinal absorption. Dietary iron, inorganic or bound to heme, is absorbed at the brush border of enterocytes lining the proximal portion of the duodenum [263,264,265,266].
In order to be absorbed, Fe$^{3+}$ must be previously reduced to Fe$^{2+}$ by the ferrireductase duodenal cytochrome b (DCYTB or CYBD1) present in the apical membrane of enterocytes [267] or alternatively by STEAP ferrireductases [268,269]. Subsequently, Fe$^{2+}$ is transported across the membrane by the divalent metal transporter 1 (DMT1, also known as DCT1, NRAMP2 or SLC11A2) [270,271,272], a process requiring proton co-transport [271]. Heme iron absorption is poorly understood, as the nature of its importer remains uncertain [273,274]. Notwithstanding, internalized heme is likely degraded by heme-oxygenase to yield Fe$^{3+}$ [264]. Then, cytosolic Fe$^{3+}$ is transported to circulation by the basolateral membrane exporter ferroportin (FPN, also known as IREG1, MTP1 or SLC40A1) [275,276,277] and is oxidized to Fe$^{3+}$ by the multicopper ferroxidase hephaestin, the intestinal homologue of the abundant plasma protein ceruloplasmin [278].

Soon after release from the enterocyte, Fe$^{3+}$ is captured by transferrin (TF). This abundant high-affinity plasma iron transport protein distributes iron to cells containing the transferrin receptor 1 (TFR1), which is ubiquitously expressed [265]. Upon binding, the TF-TFR1 complex is internalized by clathrin-dependent endocytosis. Subsequent acidification (pH 5.5) of early endosomes through active proton influx causes dissociation of iron from the complex [279]. Free Fe$^{3+}$ is then reduced by the ferrireductase STEAP3 [280] and transported across the endosomal membrane by DMT1 into the cytosol [270,271,272,281]. Finally, empty TF and TFR1 are recycled to the cell surface to initiate further cycles of iron sequestration and internalization [279]. In particular, erythroid precursors, early lymphoid and neuroepithelial cells are strictly dependent on iron uptake by the TF-TFR1 pathway for normal development [282,283].

Erythropoiesis requires approximately 25 mg of iron per day to produce 200 billion new erythrocytes [284]. Such demands cannot be met by diet and iron must therefore come from erythropagocytosis. This process is most active in the spleen, where senescent or damaged erythrocytes are phagocytosed by macrophages of the reticuloendothelial system [263]. Hydrolytic enzymes of the macrophage phagolysosome lyse engulfed erythrocytes and freed hemoglobin (HB) then suffers proteolytic digestion to liberate heme [263]. Moreover, macrophages can take up HB and heme present in circulation due to intravascular hemolysis. HB-haptoglobin and heme-hemopexin complexes formed in the plasma are internalized, respectively, by CD163- [285] and CD91- [286] mediated endocytosis. Intracellular heme is either exported intact by the feline leukemia virus subgroup C cellular receptor (FLVCR) [287] or catabolized by heme-oxygenase 1 to biliverdin, carbon monoxide and Fe$^{2+}$ [288]. The latter can be exported by FPN [275,276,289] and oxidized to Fe$^{3+}$ by the multicopper ferroxidase ceruloplasmin in order to facilitate incorporation in TF [290]. Iron that is either not exported or used for
metabolic reactions is stored within the core of ferritin (FT). Degradation of the cytosolic (and ubiquitous) FT and concomitant iron release helps mobilize iron for cellular utilization [264,265]. Also, it is presumed that mitochondrial FT is involved in the protection of this organelle against iron-mediated toxicity [291].

Systemic iron homeostasis encompasses regulation of intestinal iron absorption, erythropoiesis, erythrophagocytosis and iron storage by hepatocytes and macrophages [264]. The regulation of these processes is the responsibility of hepcidin, a small peptide primarily synthesized by hepatocytes and secreted into circulation [292,293,294,295], that can also be produced by macrophages, monocytes and neutrophils [296,297,298]. Hepcidin production is subjected to regulation by systemic iron availability, among other factors. Its transcription is elevated in response to iron overload [295] and reduced in conditions of iron deficiency [299,300]. It acts by targeting cell surface FPN and eliciting its internalization and lysosomal degradation, thus leading to reduced intestinal iron absorption and macrophage iron export [301].

Intracellular iron balance is controlled by the interaction of cytosolic iron regulatory proteins (IRPs) with iron responsive elements (IREs). The latter are sequences present in the 5’ or 3’ untranslated regions (UTR) of mRNAs encoding proteins of iron metabolism. The formation of IRE/IRP complexes on the 5’UTR of FT mRNA [302,303] represses translation initiation [304], whereas such interactions in the 3’UTR of TFR1 mRNA [305] preclude its endonucleolytic cleavage and subsequent degradation [306]. IRPs bind IREs with high affinity in iron-depleted cells and with low affinity in iron-replete cells [307,308,309,310,311]. Hence, when cellular iron levels are low, more TFR1, but not FT, protein can be produced. The opposite is observed when cells are iron replete [264].

3.2.2. Host mechanisms of iron withholding

Several mechanisms of innate immunity are involved in the restriction of iron availability to microbes. These can function constitutively or be induced in response to infection [258,259,260].

Lactoferrin (LF) is a high affinity Fe$^{3+}$ chelator (at acidic pH) present in milk, saliva, tears, semen and secondary granules of neutrophils, being involved in the withholding of iron from invading pathogens at extracellular compartments [312,313]. Still, numerous microbes also secrete Fe$^{3+}$ chelators, known as siderophores, to effectively scavenge iron from their niches within the host. The host counteracts this microbial iron uptake system through the release of lipocalin-2 (also designated siderocalin, neutrophil gelatinase-associated lipocalin or 24p3) from neutrophils, macrophages and epithelial cells.
Lipocalin-2 acts by sequestering diverse Fe\(^{3+}\)-containing siderophores, hence preventing their acquisition by microbes [260,314]. Its importance for host defense is clearly shown by the enhanced susceptibility of lipocalin-2 deficient mice to infection by *Escherichia coli* [315] and *Mycobacterium tuberculosis* [316].

Other iron withholding strategies include the reduction of iron delivery to the infected host cell or the induction of iron efflux from the cellular compartments where microbes replicate [258,259]. The internalization of TF by monocytes can be blocked by decreasing the surface expression of TFR1 upon IFN\(_{\gamma}\) activation, thus limiting the availability of iron for the growth of bacteria such as *Legionella pneumophila* [317,318]. Moreover, IFN\(_{\gamma}\) can also induce the transcription of natural resistance associated macrophage protein 1 (NRAMP1 or SLC11A1) [319], which functions as a divalent metal-proton symporter [320] in phagosomal membranes of professional phagocytes [321]. This protein acts by depleting iron from phagosomal compartments [322], in this way starving pathogens like *M.bovis*, *Salmonella typhimurium* and *L.donovani* of this essential nutrient [323]. Similarly, FPN over-expression stimulates iron export from macrophages and consequently limits the growth of *S.typhimurium* [324,325], *Chlamydia* and *L.pneumophila* [326]. By contrast, FPN degradation mediated by hepcidin binding, blocks iron efflux from macrophages and results in increased availability of iron for these pathogens [324,326].

Although the host’s iron deprivation mechanisms are important to contain microbial growth, many pathogens are capable of counteracting their action [260]. *Leishmania* parasites are not only capable of subverting the host’s iron uptake systems, as they are also proficient in the acquisition of iron by their own mechanisms [327,328].

### 3.2.3. *Leishmania* iron acquisition

Since both *Leishmania* life stages lack critical enzymes of the heme biosynthetic pathway [329], they have to be cultured in medium supplemented with inorganic salts of iron, heme-containing compounds or heme alone [330]. This molecule is acquired through the *Leishmania* heme response 1 (LHR1) importer present in the plasma membrane and lysosomes of *L.amazonensis* promastigotes [331,332]. Other physiological iron sources such as HB [333,334], TF and LF [335], but not FT, may also promote the growth of this parasite stage. However, their uptake occurs by quite distinct mechanisms. The HB protein is acquired by endocytosis after its attachment to a high affinity receptor, a hexokinase present in the flagellar pocket of *L.donovani* promastigotes [333,334]. Subsequently, the degradation of internalized HB in the late endosomes/lysosomes releases heme that is possibly trafficked to the mitochondria by *Leishmania* ATP-binding...
cassette, sub-family G, member 5 (LABCG5). The latter is an intracellular ATP-binding cassette (ABC) protein present in the membranes of a vesicular network of *L. donovani* promastigotes [336]. Instead, TF and LF bind to a common surface receptor in *L. infantum* promastigotes, after which Fe\(^{3+}\) is presumably released from them and converted to Fe\(^{2+}\) by the action of a Fe\(^{3+}\) reductase before internalization by an yet unidentified transporter [337,338,339]. Accordingly, this Fe\(^{3+}\) reductase activity is detectable on the promastigote surface of different *Leishmania* species [340]. Consistent with promastigote studies, the growth of axenic amastigotes of *L.infantum* or *L.amazonensis* can also be supported by iron derived from HB and hemin [341] or TF [342], respectively. The uptake of heme by intramacrophagic *L.amazonensis* amastigotes is again mediated by LHR1 [331]. Furthermore, intracellular *L.amazonensis* also possesses a Fe\(^{3+}\) reductase, the LFR1, [340] which provides soluble Fe\(^{2+}\) for transport across the parasite plasma membrane by the Fe\(^{2+}\) iron transporter LIT1 [343,344]. Lastly, apart from the mechanisms of direct iron internalization, *Leishmania* parasites can also subvert the host's iron uptake systems for their own profit. In fact, *L.amazonensis* amastigotes can obtain TF by forcing the fusion of TF-containing endosomes with the PVs [342]. Alternatively, *L.donovani* is capable of decreasing the macrophage labile iron pool, a process that triggers an increased surface expression of TFR1 and internalization of TF, thus permitting a continuous provision of iron to the parasite [345].

In sum, the abovementioned variety of iron sources and acquisition mechanisms used by *Leishmania* parasites reflects the plasticity of their growth requirements and ability to survive in the diverse environments of the hosts encountered during their life cycle [327,328].

### 3.2.4. Host iron status and *Leishmania* infection

Most clinical and experimental evidence indicates that excessive iron levels in the host, owing to nutritional, hereditary or therapeutic reasons, favours the multiplication of pathogens and worsens the outcome of infections. Conversely, iron deprivation tends to hinder the multiplication of pathogens and improve the outcome of infections [258,261]. Experimental studies on the relationship between the host iron status and infection by *Leishmania* indicate that these parasites might be an exception to such tenet. So far, studies in this regard have never been conducted in humans.

The fact that *Leishmania* are equipped with diverse iron acquisition mechanisms and are capable of utilizing various iron sources, has led to the suggestion that iron deprivation could be an effective strategy to control leishmanial infections [327,328]. Such
hypothesis is supported by the finding that the iron chelators desferrioxamine (DFO) and hydroxypyridin-4-ones moderately inhibit the multiplication of *L. major* and *L. infantum* promastigotes in culture medium [346]. However, DFO has shown either no effect [347] or an inhibitory effect on the intramacrophagic growth of *L. donovani* [345,348] and *L. amazonensis* [342]. Moreover, treatment of mice with DFO reduces the hepatic and splenic growth of *L. infantum* [349], but does not affect the development of skin lesions caused by *L. major* [350]. Overall, *Leishmania* parasites do not seem to be particularly sensitive to iron chelation.

On the other hand, iron supplementation appears to mediate *in vivo* resistance to *Leishmania*, though such effect has not been reproduced *in vitro*. Iron treatment either favours the intramacrophagic growth of *L. donovani* [345] and *L. amazonensis* [342] and reverses the capacity of activated macrophages to eliminate *L. enriettii* [212] or has no influence on both effects [347]. By contrast, iron administration to susceptible mice leads to early containment of *L. major* in the skin and delays its dissemination to the dLNs, liver and spleen [350,351,352]. The control of this infection correlates with an early and sustained oxidative burst mediated primarily by neutrophils [351] and with the development of a Th1-type immune response [350]. The protective immune response to *L. major* displayed by iron overloaded mice is characterized by (i) an increased ability of splenic cells to present *L. major*-derived peptides, (ii) increased levels of IFNγ and NOS2 and decreased levels of IL-4 and IL-10 transcripts at the lesion site and (iii) reduced levels of serum IgE and IgG1 and increased levels of IgG2a [350]. Finally, iron overloaded mice are resistant to re-infection with *L. major*, which is linked to an enhanced proliferation of IFNγ-secreting CD4+ T cells in the dLNs [352]. The development of these cells and concomitant resistance to *L. major* is regulated by the transcription factor NF-κB [353]. In fact, the cellular activation and nuclear translocation of NF-κB is detectable in the dLNs of iron overloaded mice, during primary and secondary infections with *L. major* [352]. The abovementioned reports highlight the role of iron in mediating host protection against *L. major* infection. Clearly, more studies are needed to ascertain the role of iron during the course of infection with other *Leishmania* species.
Chapter II

Definition Of Objectives
Chapter II | Definition Of Objectives

Iron is an essential nutrient for the growth and survival of the host and the pathogen. It is generally acknowledged that iron chelation decreases and iron excess increases the growth of most pathogens [258,261]. However, this principle does not seem to apply to *Leishmania* for two main reasons. First, iron chelation does not consistently reduce the growth of *Leishmania* in macrophages [342,345,347,348] and in mouse models [349,350]. Second, the proliferation of *L.major* in the mouse is impaired by the host’s iron overload, an effect correlated to an enhanced oxidative burst [350,351,352]. In fact, it is well established that ROS and RNS can inhibit the replication of *Leishmania* in culture medium and inside macrophages [94]. However, their relevance to the control of these parasites in the mouse model has not been well defined [166,217,223]. Moreover, several lines of evidence indicate that visceralizing *Leishmania* are vulnerable to chemotherapeutics that act on the parasites by inducing the generation of ROS, as is the case of analogues of PQ [71,95]. Although PQ displays activity against visceral *Leishmania*, its aliphatic amine is prone to undergo oxidative deamination *in vivo*, which consequently causes PQ inactivation [79]. This drawback may be overcome by blocking PQ’s aliphatic amine with a peptidomimetic (imidazolidin-4-one) or organometallic (ferrocene) moiety. Indeed, optimization of PQ structure has already led to the development of potent anti-leishmanial compounds [78,80,81].

Given these facts, the key objectives of this PhD thesis were to:

1 | Clarify the effect of modulating the host iron status on the growth of *L.infantum* in the mouse model (chapter III);

2 | Define the relative contribution of ROS and RNS to the control of *L.infantum* infection in the mouse model (chapter IV);

3 | Explore the anti-leishmanial potential of peptidomimetic and organometallic derivatives of PQ, with higher resistance to proteolytic degradation and oxidative deamination (chapter V).
Chapter III

Iron Overload Favors The Elimination Of Leishmania infantum
From Mouse Tissues Through Interaction With Reactive Oxygen And Nitrogen Species

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Chapter III | Iron Overload Favors The Elimination Of
Leishmania infantum From Mouse Tissues Through
Interaction With Reactive Oxygen and Nitrogen Species

1. Abstract

Iron plays a central role in host-parasite interactions, since both intervenients need iron for survival and growth, but are sensitive to iron-mediated toxicity. Host's iron overload is often associated with susceptibility to infection. However, it has been previously reported that iron overload prevented the growth of Leishmania major, an agent of cutaneous leishmaniasis, in BALB/c mice.

In order to further clarify what is the impact of iron modulation on the growth of Leishmania in vivo, we studied the effects of iron supplementation or deprivation on the growth of L. infantum, the causative agent of Mediterranean visceral leishmaniasis, in the mouse model. We found that dietary iron deficiency did not affect the protozoan growth, whereas iron overload decreased its replication in the liver and spleen of a susceptible mouse strain. The fact that the iron-induced inhibitory effect could not be seen in mice deficient in NADPH dependent oxidase or nitric oxide synthase 2 suggests that iron eliminates L. infantum in vivo through the interaction with reactive oxygen and nitrogen species. Iron overload did not significantly alter the mouse adaptive immune response against L. infantum. Furthermore, the inhibitory action of iron towards L. infantum was also observed, in a dose dependent manner, in axenic cultures of promastigotes and amastigotes.

In conclusion, externally added iron synergizes with the host’s oxidative mechanisms of defense in eliminating L. infantum from mouse tissues. Additionally, the direct toxicity of iron against Leishmania suggests a potential use of this metal as a therapeutic tool or the further exploration of iron anti-parasitic mechanisms for the design of new drugs.

2. Introduction

Leishmania are trypanosomatid protozoans that alternate between two forms: the extracellular motile promastigote in the gut of phlebotomine insects and the intracellular non-motile amastigote inside the macrophages of mammalian hosts. These parasites cause leishmaniasis, a spectrum of human diseases that range from self-healing
cutaneous ulcers to fatal visceralizing infection. Every year, approximately 2 million people develop symptomatic disease (0.5 million of them the visceral form) [4]. In Europe, visceral leishmaniasis (VL) is caused almost exclusively by *L.infantum*, which is transmitted as a zoonosis. The domestic dog is one of the main reservoirs of this parasite and canine leishmaniasis is an important veterinary problem in European Mediterranean countries [1].

There are currently no effective vaccines to prevent human leishmaniasis [99]. Therefore, management of the disease relies on chemotherapy. However, available drugs are highly toxic and the frequency of resistant parasite strains is increasing worldwide [21,38]. The improvement of our knowledge on the mechanisms of host resistance to *Leishmania* is important to contribute to the development of new therapeutic strategies.

The important role played by iron metabolism in the interaction between host and pathogens is being increasingly highlighted by recent research [259,260]. Both the host and the pathogens absolutely need iron for survival and must have efficient mechanisms for its acquisition together with adequate mechanisms of cell defense to avoid iron toxicity. Data obtained in human patients, as well as in different animal infection models indicate that in most cases iron availability favors the multiplication of pathogens, whereas iron deprivation impairs their growth [258,261]. Interestingly, the vertebrates innate immune response to infection includes several mechanisms of iron with-holding such as lactoferrin, hepcidin, Nramp1 or lipocalin2 [258,259]. Still, adequate concentrations of iron are required to support macrophage killing mechanisms during infection [354,355]. Contrary to what happens with most pathogens, the growth of *Brucella abortus* inside macrophages [356] and that of *L.major* in the mouse [350,351,352] are decreased by host’s iron overload. In both cases, killing was correlated to oxidative burst [351,356]. These examples highlight that iron can be exploited, in some cases, by the host to strengthen its antimicrobial defense mechanisms.

Our group has previously shown that the infection by *Mycobacterium avium*, an intramacrophagic pathogen, is clearly exacerbated by host’s iron overload, either genetically determined [357] or caused by iron-dextran injection [358,359]. Furthermore, we have also demonstrated that iron chelation can be used to inhibit the growth of *M.avium* [360]. In the present work, we evaluated the effect of iron on the growth of *L.infantum*, the agent of European VL. We found that iron consistently inhibited the replication of *L.infantum* both by a direct effect and through the activity of the host’s macrophage.
3. Materials & Methods

3.1. Animals and ethics statement | BALB/c and C57BL/6 mice were purchased from Charles River (Madrid, Spain). Mice deficient on the p47 subunit of the NADPH oxidase complex, on a C57BL/6 background (p47phox−/−), were bred at IBMC from a breeding pair purchased from Taconic (Lille Skensved, Denmark). The p47phox+/− mice were administered trimethoprim-sulfamethoxazole (Bactrim; 600 mg l−1) in the drinking water, as prophylactic treatment against bacterial infection. This treatment was ceased when infection experiments began. Mice deficient in the nitric oxide synthase 2, on a C57BL/6 background (NOS2−/−), were bred at IBMC from a breeding pair kindly provided by Drs. J. Mudgett, J. D. MacMicking and C. Nathan (Cornell University, New York, USA). Mice deficient in HFE, on a C57BL/6 background (Hfe−/−) were bred at IBMC from a breeding pair obtained from Centre Nationale de la Recherche Scientifique. All animals were housed at IBMC facilities under specific pathogen free conditions and fed ad libitum, except for the diet experiments indicated below. All animals were used at 8 to 16 weeks of age. Only female mice (average 20 – 25 g) were used for in vivo experiments. Mice were euthanized by isofluorane anesthesia followed by cervical dislocation and tissues were collected in aseptic conditions. The experimental animal procedures were approved by the Local Animal Ethics Committee of IBMC and licensed by the Portuguese General Directory of Veterinary (DGV, Ministry of Agriculture, Rural Development and Fishing), in May 18, 2006 with reference 520/000/000/2006. All animals were handled in strict accordance with good animal practice as defined by national authorities (DGV, Law nu1005/92 from 23nd October) and European legislation EEC/86/609.

3.2. Parasites | All experiments were performed with L. infantum strain MHOM/MA/67/ITMAP-263 (zymodeme MON-1). For each experiment, parasites were obtained from the spleens of infected mice. Promastigotes were differentiated from spleen amastigotes by culturing at 25°C in complete Schneider’s medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (all from Gibco, Life Technologies, Carlsbad, CA, USA), 2% human urine, 5 µg/ml phenol red (Sigma) and 5 mM HEPES sodium salt (Sigma) pH 7.4. Promastigote cultures were expanded at 25°C, for a maximum of 5 passages, in RPMI 1640 GlutaMAX™-I medium (Gibco, Life Technologies), containing 20% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 25 mM HEPES sodium salt pH 7.4. Promastigote differentiation from the exponential to the stationary phase was promoted by culture at 25°C, without medium renovation, for 4 to 5 days.

Axenic amastigotes were derived from the above-mentioned L. infantum strain, by culture at 37°C with 7% CO₂ atmosphere, in a medium for axenic amastigotes
supplemented with 2 mM L-glutamine (GlutaMAX™, Gibco, Life Technologies) and 20% FBS (MAA20 medium, adapted from [361]).

3.3. Direct effect of iron on *Leishmania* promastigote and amastigote cultures | Promastigotes (2 × 10⁶ / well) and amastigotes (4 × 10⁵ / well) of *L. infantum* were cultured in complete RPMI (25°C) or MAA20 (37°C) medium, respectively, supplemented with iron-dextran (Fe³⁺ hydroxide-dextran complex, Sigma), iron citrate (Fe³⁺, Sigma) and iron sulphate (Fe²⁺, Merck) in the concentrations of 0.018, 0.035, 0.070, 0.14, 0.28, 0.56, 1.1, 2.2, 4.5, 9 and 18 mM (96 well plates). Equivalent concentrations of dextran (Sigma), tri-sodium citrate (Merck) and magnesium sulphate (Merck) were used as controls. After 24h of culture, 20 µl of a 2.5 mM resazurin solution (freshly prepared and filtered in phosphate buffered saline, pH 7.4, Sigma) was added to each well. The fluorescence intensity (excitation at 560 nm and emission at 590 nm) was determined 24h (for amastigotes) or 48h (for promastigotes) after resazurin addition to allow conversion to fluorescent resorufin, with a fluorometer SpectraMAX GeminiXS (Molecular Devices LLC, Sunnyvale, CA). In order to exclude a possible interference of iron with resazurin conversion to resorufin, appropriate controls were included without cells. No resazurin conversion was detected in the absence of parasites. Complete RPMI and MAA20 medium contains approximately 6 and 7.7 µM of iron, respectively, according to supplier’s information.

3.4. Animal experimental infection and parasite burden quantification | Mice were injected in the lateral vein of the tail with 2 × 10⁷ *L. infantum* stationary promastigotes in 200 µl of phosphate buffered saline (PBS) pH 7.4. At defined time points, the animals were euthanized and total livers and spleens were removed and homogenized, respectively, in 3.5 ml and 3 ml of complete Schneider’s medium. These suspensions were further diluted 1:100 (liver) or 1:10 (spleen). Four-fold serial dilutions of the homogenized tissue suspensions were performed in quadruplicate (96 well plates). After 7 to 14 days at 25°C, the wells were examined for viable promastigotes. The reciprocal of the highest dilution that was positive for parasites was considered to be the number of parasites per ml of suspension and was used to calculate the number of parasites per organ (parasite burden).

3.5. Animal experimental iron overload | In the kinetics experiments, 1 mg of iron, as iron-dextran (Sigma), was administered every other day intraperitoneally (i.p.) to each mouse, from day -20 to day -2 of infection (a total of 10 mg of iron per mouse). Control mice received equivalent amounts of dextran (Sigma) by the same route. Parallel studies allowed us to verify that the administration of 10 mg of iron in a single injection produced the same effect in terms of outcome of *Leishmania* infection and also that dextran alone had no effect on the course of infection. Consequently, in subsequent experiments, iron
overload was achieved by one single i.p. injection with 10 mg of iron per mouse. Control animals were injected with saline solution pH 6.0. None of the iron-dextran doses tested were toxic to the mice, as treated but uninfected mice remained healthy.

3.6. Animal experimental iron deprivation | Mice were fed iron-free chow (Mucedola, Milan, Italy), from weaning until the end of the experiment (24 weeks). Control mice were fed a chow that differed only at its iron content (180 mgKg\(^{-1}\), Mucedola, Milan, Italy). Chow was administered in plastic recipients to avoid metal contamination. Animals were allowed to drink deionized water.

3.7. Tissue iron determination | Non-heme iron was measured in tissues by the bathophenanthroline method [362]. Briefly, tissue samples (30 – 100 mg) were weighted, placed in iron-free Teflon vessels (ACV-Advanced Composite Vessel, CEM Corporation, Matthews NC, USA) and dried in a microwave oven (MDS 2000, CEM Corporation). Subsequently, dry tissue weights were determined and samples digested in an acid mixture (30% hydrochloric acid and 10% trichloroacetic acid) for 20 h at 65ºC. After digestion, a chromogen reagent (5 volumes of deionised water, 5 volumes of saturated sodium acetate and 1 volume of 0.1% bathophenanthroline sulfonate/1% thioglycollic acid) was added to the samples in order to react with iron and obtain a colored product that was measured spectrophotometrically at 535 nm. The extinction coefficient for bathophenanthroline is 22.14 mM\(^{-1}\)cm\(^{-1}\). Iron content in tissues was expressed as µg non-heme iron/ organ.

3.8. Cytokine quantification | Liver (100 mg) and spleen (50 mg) samples were lysed in Bio-plex cell lysis buffer containing 2mM phenylmethanesulfonyl fluoride (PMSF, Bio-Rad Laboratories Inc., CA, USA), sonicated for 5 min in a refrigerated bath and centrifuged at 4500g for 4 min at 4ºC to remove debris. Total protein was quantified in supernatants with the MicroBCA kit (Pierce, Thermo Fisher Scientific). Clear homogenates were diluted to 1.5 mgml\(^{-1}\) in PBS pH 7.4 containing 0.5% bovine serum albumin (BSA) and again centrifuged at 16000g for 10 min at 4ºC. Cytokine quantification in liver and spleen homogenates was performed following Bio-plex assay (Bio-Rad) instructions.

3.9. Flow cytometry analysis | Spleen cells were obtained by teasing these organs gently with forceps and incubating them in ammonium chloride hemolytic buffer to lyse any remaining erythrocytes. Cell suspensions were then washed with HBSS and resuspended in DMEM / 10% FBS. For immunofluorescence staining, \(10^5\) splenic cells were incubated for 15 min at 4ºC, in a 96 well plate, with fluorescein isothiocyanate (FITC)-conjugated anti-DX5 (1:200), anti-Gr1 (1:800) or anti-CD19 (1:200) antibodies,
phycoerythrin (PE)-conjugated anti-CD3 (1:200), anti-CD11c (1:200) or anti-CD11b (1:400) antibodies and allophycocyanin (APC)-conjugated anti-mouse F4/80 (1:200) antibodies (BD Pharmingen, San Diego, CA, USA), in PBS / 1% FBS in order to analyze spleen cell populations during infection. The cells were washed twice with PBS / 1% FBS. The analysis of the cell populations was based on the acquisition of 10 000 events in a Becton Dickinson (BD, Franklin Lakes, NJ, USA) FACSCalibur equipped with BD CELLQuest and FlowJo (Tree Star Inc., Ashland, OR, USA) softwares.

3.10. Histological analysis | Liver samples (50 mg) were fixated in 4% buffered paraformaldehyde pH 7.4 and embedded in paraffin. Tissue sections (5 µm) were stained with Perls' blue stain for iron (Fe$^{3+}$) detection. Representative pictures were obtained with an Olympus CX31 light microscope equipped with a DP-25 camera (Imaging Software Cell^B, Olympus, Center Valley, PA, USA).

3.11. Statistical analysis | Statistical analysis was carried out using GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). Student’s t-test was used to estimate the statistical significance of the differences between groups. Multiple comparisons were performed with One-way ANOVA followed by Dunnett or Student Newman-Keuls post hoc test. Differences between groups were considered statistically significant when p value was less than 0.05 (*p<0.05; **p<0.01; ***p<0.001).

4. Results

4.1. Iron deprivation does not affect L.infantum growth in mouse tissues

Iron withdrawal has been suggested as a means of controlling the growth of several unrelated pathogens [261,262]. To evaluate the effect of iron deprivation on the growth of L.infantum, BALB/c mice were fed normal or iron-deficient chow for the first 120 days of life. They were subsequently infected and kept in the respective diets for the next 60 days, before being euthanized. Non-heme iron quantification in the liver and spleen confirmed that mice kept on an iron-deficient diet had less than half the amount of iron found in controls (Figure 1A). However, no differences in parasite load were observed between groups fed control or iron-deficient diets (Figure 1B), indicating that a mild iron deficiency has no impact on L.infantum replication in mouse tissues.
Figure 1 | Effect of iron deprivation or overload on the growth of *L. infantum* in the mouse. A. B. BALB/c mice were kept on a control or iron deficient diet for 120 days prior to their intravenous (i.v.) infection with \(2 \times 10^7\) stationary promastigotes of *L. infantum*. Mice were sacrificed 60 days after infection. A. Non-heme iron content of liver and spleen was quantified in mice fed the control (white bars) or iron deficient diet (black bars). The graph shows the average ± standard deviation of the non-heme iron content, expressed as µg/organ (\(n=6\)). B. The parasite burden in the liver and spleen of groups fed the control (white bars) or iron deficient (black bars) diet was quantified by limiting dilution. The graph shows the average ± standard deviation of the \(\log_{10}\) number of parasites per organ (\(n=6\)). C. BALB/c mice were i.p. injected with 1 mg of iron (given as dextran), in 10 alternate days, from day -20 to day -2 of infection (a total of 10 mg of iron per animal). Control mice received equivalent amounts of dextran by the same route. Mice were infected i.v. with \(2 \times 10^7\) stationary...
promastigotes of *L. infantum* and were sacrificed at 7, 15, 30 and 60 days of infection. The parasite burden in the liver and spleen of control (white circles) or iron overloaded (black circles) groups was quantified by limiting dilution (n=5). D. Non-heme iron content in the liver and spleen was quantified in control (white bars) and iron overloaded (black bars) groups at 60 days after infection (n=4-5). Student’s *t*-test was performed to determine the statistical significance of the differences between groups (**p<0.01; ***p<0.001). E. F. G. H. Perl’s blue staining was performed in liver sections of uninfected (E), uninfected iron overloaded (F), 60 days-infected (G) and 60 days-infected iron overloaded (H) mice. Black bar corresponds to 50 μm. The results of one representative experiment are shown. Two experiments were performed with similar results.

4.2. Iron overload decreases the growth of *L. infantum* in mouse tissues

Iron overload correlates with increased susceptibility to a great variety of pathogens [261,262]. In order to determine the effect of host’s iron overload on the infection by *L. infantum*, BALB/c mice were injected with 10 mg of iron-dextran or an equivalent amount of dextran before infection. Parasite burdens were determined in the livers and spleens at 7, 15, 30 and 60 days after infection. The growth of *L. infantum* in the liver and spleen of non-treated mice followed kinetics similar to that previously reported [120,121]. During the first 30 days of infection, the parasite numbers increased significantly in both organs, returning to baseline levels in the liver thereafter, while continuing to grow in the spleen (Figure 1C). Conversely, parasite load was significantly lower in both organs of iron-overloaded mice throughout the experiment period (Figure 1C).

Non-heme iron quantification confirmed that iron-overloaded animals had 8 and 3 times more iron, respectively, in the liver and spleen than control mice at 60 days after infection (Figure 1D). Iron distribution was analyzed in the liver, by Perl’s staining. In those animals that were not injected with iron-dextran, iron deposition was very rarely seen (Figure 1E, G), the exception being the faint staining of some cell infiltrates (Figure 1G). The administration of iron to non-infected mice led to its accumulation predominantly in Kupffer cells (Figure 1F, black arrows), as expected from previous reports [253,363]. Infection with *L. infantum*, lead to the appearance of heavily iron-loaded macrophages inside cell infiltrates, the areas presumed to correspond to parasite containment (Figure 1H) [163,165].

In order to investigate whether the *in vivo* anti-leishmanial effect of iron could be attributed to a generalized host tissue oxidative damage, we measured DNA cleavage and lipid peroxidation in the hepatic parenchyma through the immunofluorescence staining for TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and 4-hydroxynonenal, respectively. Moreover, we assessed the formation of protein carbonyl groups in liver protein lysates by western blotting. However, we could not find any differences
between control and iron-treated mice (data not shown), indicating that iron supplementation in our model did not cause a generalized oxidative damage to the tissue.

These experiments revealed that the accumulation of iron inside macrophages at *L. infantum* infection foci correlates with reduced parasite’s multiplication, but not to generalized tissue damage.

4.3. High concentrations of iron inhibit the axenic growth of *L. infantum*

In order to clarify the mechanisms by which iron exerts its anti-leishmanial effect in our model, we first asked whether iron could be exerting a toxic effect directly on the parasites. Axenic promastigotes (Figure 2A–C) or amastigotes (Figure 2D–F) of *L. infantum* were grown in the presence of increasing concentrations of iron (0.018 – 18 mM) in the form of either dextran (A,D) or citrate (B,E) complexes or sulphate (C,F) salt. *L. infantum* viability was measured, based on the parasite’s capacity to metabolize the dye resazurin.

![Figure 2](image)

**Figure 2** | Effect of different forms of iron on the axenic growth of *L. infantum* pro- and amastigotes: Promastigotes in the exponential phase of growth (2 × 10^6 / well; A, B, C) or amastigotes (4 × 10^5 / well; D, E, F).
F) were incubated in RPMI (25°C) or MAA20 (37°C) medium, respectively, with iron-dextran (A, D), iron citrate (B, E) and iron sulphate (C, F) (black circles) in the concentrations of 0.018, 0.035, 0.070, 0.14, 0.28, 0.56, 1.1, 2.2, 4.5, 9 and 18 mM. Equivalent concentrations of dextran (A, D), tri-sodium citrate (B, E) and magnesium sulphate (C, F) were used as controls (white circles). Resazurin dye was added after 24h of culture. The fluorescence was measured 24h (for amastigotes) or 48h (for promastigotes) after resazurin addition. The results show the average ± standard deviation of the percentage viability in relation to the non-treated control (n=3). One-way ANOVA, followed by a Newman-Keuls multiple comparison post-hoc test, was performed to determine the statistical significance of the differences between control (white circles) and iron treated (black circles) groups (*p<0.05, **p<0.01; ***p<0.001). The results of one representative experiment are shown. Three experiments were performed with similar results.

Iron concentrations below 0.56 mM had no effect on the multiplication of the parasites (Figure 2). Amastigotes seemed to be more susceptible to iron toxicity, as iron decreased the viability of these parasites in a dose dependent manner from 0.56 to 18 mM irrespective of its molecular form (Figure 2D-F). Promastigotes were inhibited by iron-dextran (Fe³⁺) at 0.56 mM or above (Figure 2A), while iron citrate (Fe³⁺) and iron sulphate (Fe²⁺) were active against promastigotes only above 4.5 mM (Figure 2B, C). Promastigotes exposed to 9 – 18 mM of iron in any form displayed oval shape, atrophied cell body and reduced motility (not shown), changes which are characteristic of stress situations [364]. The results obtained by resazurin reduction were confirmed by the visual microscopic quantification of the parasites in a Neubauer chamber, on selected samples (not shown). Overall, these results indicate that iron can inhibit the growth or even kill *L. infantum* promastigotes and amastigotes, although the concentrations needed to achieve that effect are relatively high.

4.4. Iron overload does not affect the capacity of the *L. infantum* infected mice to induce expansion of splenic cell populations or expression of key cytokines

Since previous studies had suggested that host’s iron-overload interfered with the development of a protective immune response [350], we evaluated the impact of iron-overload on the induction of protective cytokines and specific splenic cell populations in our VL model.

BALB/c mice were treated with 10 mg of iron (given as iron-dextran) or saline solution 15 days prior to infection. They were infected with *L. infantum* and sacrificed 60 days later. Groups of non-infected mice were kept as controls. We performed a cytometric analysis of the number of splenic CD3⁺ (T cells), CD3⁺DX5⁺ (NK cells), CD19⁺ (B cells), CD11c⁺ (DCs), CD11b⁺F4/80⁺ (Macrophages) and CD11b⁺Gr1⁺⁺ (Neutrophils) cells. The
infection with *L. infantum* resulted in a significant increase in the numbers of CD19+ and CD11c+ cells, while other splenic cell sub-sets remained unaltered (Figure 3). More importantly, the number of cells belonging to each of the abovementioned populations was the same in control and iron overloaded groups (Figure 3).

Additionally, the in situ production of a number of cytokines was measured, using a multiplex assay. The results of this screening revealed that infection with *L. infantum* did not have a dramatic impact on cytokine production. Only IL-1β, IL-6, TNF and IL-4 were significantly induced by infection in the spleen (the latter also in the liver, Figure 4), while the production of IL-12p70 and IL-13 decreased with infection, in the liver (Figure 4). No significant differences were found between iron overloaded and control infected animals in any of the cytokines tested (Figure 4). The determination of cytokine mRNA expression in the tissues at earlier time-points did not reveal any differences between iron-overloaded and control infected mice (not shown).

Overall, these experiments indicate that the inhibitory effect of iron on the growth of *L. infantum* in the mouse does not result from an improvement of the activation of protective cells or increased production of protective cytokines.

Figure 3 | Effect of iron overload on the capacity of the mouse to induce the expansion of splenic cell populations. BALB/c mice were i.p. injected with saline solution or 10 mg of iron (-dextran, in a single dose) and were infected 15 days later by the i.v. route, with $2 \times 10^7$ *L. infantum* stationary promastigotes (inf) or were
left uninfected (non-inf). Mice were sacrificed 60 days later and the spleen was collected. The levels of CD3⁺, CD3⁺DX5⁺, CD19⁺, CD11c⁺, CD11b⁺F4/80⁺ and CD11b⁺GR1⁺⁺ cells were quantified in the spleen of mice without (white bars) or with (black bars) iron overload. The results express the average ± standard deviation of the total number of cells in the spleen (n=3-5). One-way ANOVA, followed by a Newman-Keuls multiple comparison post-hoc test, was performed to determine the statistical significance of the differences between all groups (*p<0.05). The results of one representative experiment are shown. Two experiments were performed with similar results.

Figure 4 | Effect of iron overload on the capacity of the mouse to induce the production of key cytokines. BALB/c mice were i.p. injected with saline solution or 10 mg of iron (dextran, in a single dose) and
were infected 15 days later by the i.v. route, with $2 \times 10^7 L.\infantum$ stationary promastigotes (inf) or were left uninfected (non-inf). Mice were sacrificed 60 days later and liver (100 mg) and spleen samples (50 mg) were collected and homogenised in protein lysis buffer with 2mM PMSF (Bio-plex Cell Lysis Kit, Biorad). The levels of IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL-12p70, IFNγ, TNF and IL-17 were quantified in liver and spleen homogenates of mice without (white bars) or with (black bars) iron overload, accordingly to 10-Plex assay instructions (Biorad). The results express the average + standard deviation of the cytokine levels, expressed in pg / mg of protein ($n=3$-5). Student’s t-test was performed to determine the statistical significance of the differences between the different groups (*$p<0.05$; **$p<0.01$). The results of one representative experiment are shown.

4.5. High iron doses and administration prior to infection are necessary for the inhibitory effect of iron

In order to further understand the mechanisms of the iron inhibitory effect on $L.\infantum$, we tested the importance of different experimental parameters. First, we decided to assess if the same protective effect could be obtained with lower iron doses. BALB/c mice were injected with different amounts of iron (given as iron-dextran complex) prior to infection with $L.\infantum$. Parasite loads were determined 30 days after infection. A significant inhibitory effect on the growth of $L.\infantum$ in the liver and spleen was seen only in mice that received 10 mg of iron (Figure 5A). The administration of lower iron doses (0.4, 1 and 4 mg) tended to decrease the parasite growth in the liver, but not to a statistically significant level (Figure 5A).

Next, we asked whether iron would still have an inhibitory effect on $L.\infantum$ growth when given after infection. We administered 10 mg of iron (-dextran) or saline solution to BALB/c mice, 1 or 15 days after infection. Parasite loads were determined 60 days post-infection. In the liver, similar levels of growth inhibition were seen when iron was given either before, 1 day after or 15 days after infection (Figure 5B). However, in the spleen and in contrast with iron pre-loading, no significant reduction on the growth of $L.\infantum$ was detected when the administration of iron was done after infection (Figure 5B).

These results suggested that high amounts of iron present in the host prior to infection favor the decrease of the multiplication of $L.\infantum$. In the model used, iron accumulation is observed in macrophages. To assess the relevance of the cellular location of iron deposition at the time of infection, we used HFE-deficient mice which are used as a model of human hemochromatosis and accumulate iron in parenchymal cells rather than inside macrophages [365,366]. We infected Hfe$^{-/-}$ mice with $L.\infantum$ and evaluated the parasite load and iron content in the livers and spleens at 60 days after
infection. HFE-deficient mice had around 3 times more iron in the liver than controls and normal amounts of iron in the spleen (Figure 6A). As expected, in non-infected Hfe\textsuperscript{-/-} mice, iron was found predominantly in the hepatic parenchyma (Figure 6D). However, in \textit{L. infantum}-infected mice, strong iron staining was found inside cell infiltrates both in wild-type mice and (more intensely) in Hfe\textsuperscript{-/-} mice (Figure 6E,F) showing that infection rerouted iron between the two cell types. Interestingly, the parasite loads in wild-type and Hfe\textsuperscript{-/-} mice were the same (Figure 6B).

Overall, these results indicate that a decrease in the growth of \textit{L. infantum} is observed when high amounts of iron are found inside the host’s macrophages, with a stronger effect when this occurs before the infection.

\textbf{Figure 5 | Effect of different iron doses and periods of administration on the growth of \textit{L. infantum}.} \textbf{A.} BALB/c mice were injected i.p. with 0.4, 1, 4 or 10 mg of iron (-dextran), in a single dose, 12 days before the infection. \textbf{B.} BALB/c mice were injected i.p. with 10 mg of iron (-dextran), in a single dose, 15 days before or 1 and 15 days after the infection (-15, +1 and +15 days, respectively). \textbf{A.} \textbf{B.} Control mice received saline.
solution by the same route. Mice were sacrificed 30 (A) or 60 (B) days after the i.v. infection with $2 \times 10^7$ stationary promastigotes of *L. infantum*. The parasite burden in the liver and spleen of mice without (white bar) and with (gray - black bars) iron administration was determined by limiting dilution. The data shows the average + standard deviation of the log_{10} number of parasites per organ ($n=4$-$5$). One-way ANOVA, followed by a Dunnett’s multiple comparison post-hoc test, was performed to determine the statistical significance of the differences between each of the iron treated groups and control group (*p<0.05; **p<0.01; ***p<0.001). The results of one representative experiment are shown. Two experiments were performed with similar results.

Figure 6 | Effect of genetically determined iron overload on the growth of *L. infantum* in mice. A. B. Wild-type (WT) and Hfe$^{-/-}$ mice were i.v. infected with $2 \times 10^7$ stationary promastigotes of *L. infantum* and were sacrificed 60 days after infection. A. Non-heme iron content in both organs was quantified in WT (white bars) and Hfe$^{-/-}$ (black bars) groups. The data shows the average + standard deviation of the non-heme iron content, expressed in $\mu g / organ$ ($n=4$-$6$). Student’s $t$-test was performed to determine the statistical significance of the differences between WT and Hfe$^{-/-}$ groups (***p<0.001). B. The parasite burden in the liver and spleen of WT (white bars) and Hfe$^{-/-}$ (black bars) mice was determined by limiting dilution ($n=5$-$6$). C. D. E. F. Perl’s blue staining was performed in liver sections of uninfected (C) and infected (E) WT and uninfected (D) and infected (F) Hfe$^{-/-}$ mice. Black bar corresponds to 50 $\mu m$. The results of one representative experiment are shown. Two experiments were performed with similar results.
4.6. Host production of reactive oxygen and nitrogen species is necessary for the inhibitory effect of iron overload on *L.infantum* growth

Iron concentrations necessary to inhibit *L.infantum* growth in axenic conditions are relatively high. So, we hypothesised that iron synergizes with antimicrobial mechanisms of macrophages, such as the production of reactive oxygen species (ROS) by the NADPH oxidase (respiratory burst) and reactive nitrogen species (RNS) by the nitric oxide synthase 2 (NOS2) to decrease *Leishmania* viability in the mouse tissues.

**Figure 7 | Effect of iron overload on the growth of *L.infantum* in mice: role of NADPH oxidase and NOS2.** Wild-type (WT) and p47phox⁻/⁻ (A, C) or NOS2⁻/⁻ (B, D) mice were i.p. injected with saline solution or 10 mg of iron (-dextran, in a single dose) 15 days before i.v. infection with $2 \times 10^7$ stationary promastigotes of *L.infantum* and were sacrificed 15 days later. The liver (A, B) and spleen (C, D) were removed and the parasite burden in mice without (white bars) or with (black bars) iron overload was quantified by limiting dilution. The results express the average ± standard deviation of the log$_{10}$ number of parasites per organ ($n=3$-$8$). One-way ANOVA, followed by a Newman-Keuls multiple comparison post-hoc test, was performed to determine the statistical significance of the differences between all groups (*$p<0.05$; **$p<0.01$; ***$p<0.001$). The results of one representative experiment are shown. At least two experiments were performed with similar results.
Mice genetically deficient in the p47phox subunit of NADPH oxidase (p47phox−) and in the NOS2 enzyme (NOS2−) were used to test this hypothesis. Animals were treated with 10 mg of iron (-dextran) or saline solution and 15 days later were infected with *L. infantum*. Mice were sacrificed 15 days after infection and the parasite load was determined in the liver and spleen. Iron overload decreased *L. infantum* growth in wild-type but not in knock-out mice in the liver (Figure 7A, B) and spleen (Figure 7C, D), indicating that the mechanism through which iron exerts its inhibitory effect is dependent on the production of ROS and RNS by the host. A similar experiment in which animals were sacrificed 30 days after infection gave identical results (data not shown).

5. Discussion

Iron is a central element in host-parasite interaction and several iron-depriving mechanisms are used by the host to inhibit pathogen proliferation [259,260]. In contrast, previous work has shown that host’s iron overload prevented the growth of *L. major* in BALB/c mice [350,351,352]. In those studies, it is shown that iron overload correlates with increased production of ROS upon *L. major* infection [351]. In the present work, we treated mice with iron-dextran and infected them with *L. infantum*. We observed iron-loaded macrophages inside hepatic infiltrates, the areas of parasite containment, concomitantly with the decrease of tissue parasite loads. Such iron-associated decrease did not occur in p47phox- or NOS2-deficient mice, suggesting that iron exerts its effects through the combination with ROS and/or RNS produced by the macrophage. In fact, superoxide (O$_2$•−) and nitric oxide (NO•), synthesized by the phagocytic NADPH oxidase and the NOS2, respectively, have been implicated in the elimination of *Leishmania* by the host’s macrophages [166,195,204,214,217,218,220,222,225]. Moreover, both macrophagic NADPH oxidase and NOS2 require iron for proper function [170].

The fact that the anti-parasitic effect of iron is lost in mice deficient in only one of the two enzymes, either NOS2 or NADPH oxidase, suggests that both ROS and RNS are simultaneously required for iron to exert its anti-leishmanial effect. Iron can possibly favour the formation of peroxynitrite (ONOO−), a strong oxidizing species formed by the reaction of NO• with O$_2$•− [172]. Since we could not find evidences of tissue oxidative damage (DNA damage, lipid peroxidation and protein oxidation) in iron-treated mice, we suggest that this formation of ROS and RNS in combination with iron has a highly localized activity, inside the macrophage.

It was somewhat surprising that Hfe−/− mice, which have spontaneous iron overload, predominantly in the liver, had tissue parasite loads similar to those of wild-type mice,
when infected with *L. infantum*. This could be justified by the fact that Hfe<sup>−/−</sup> mice develop spontaneous iron overload predominantly in hepatocytes, keeping macrophages relatively iron depleted [365,366,367]. When Hfe<sup>−/−</sup> mice were infected with *L. infantum*, we could see iron accumulation inside macrophages at the infection foci. However, as suggested by the experiments in which we treated mice with iron-dextran after infection, the inhibitory effect of iron is best accomplished when the macrophages are iron-loaded prior to infection. Another hypothesis to explain the lack of an impact of Hfe<sup>−/−</sup> iron overload on the growth of *L. infantum* is the level of iron overload in the tissues. Indeed, Hfe<sup>−/−</sup> mice had tissue iron levels that were significantly lower than those found in iron-dextran-injected mice. The fact that even with iron-dextran injection, we needed high iron doses to decrease the parasite burden in tissues, argues for this hypothesis.

In the mouse model of CL, iron-induced respiratory burst at the onset of *L. major* infection is coupled to later activation of the nuclear transcription factor NF-κB [352] and to the display of a protective immune response [350]. Iron and ROS can modulate the activation of NF-κB signaling pathways [368], known to regulate several genes involved in immune and inflammatory responses [369]. In the case of *L. major* infection, mouse resistance is clearly related to an IL-12-driven, IFN-γ-dominated Th1 immune response, whereas susceptibility correlates with an IL-4-driven Th2 response [122,123,141,370]. Experimentally iron overloaded BALB/c mice, infected with *L. major*, exhibited a Th1-type immune response, with increased levels of IFN-γ and NOS2 and decreased levels of IL-4 and IL-10 transcripts compared to untreated mice [350]. In accordance, supplementation of rats with iron-dextran [371] or saccharated colloidal iron [372] potentiated the induction of hepatic NOS2 and the production of NO• by LPS. However, the decrease of NO• production has also been observed in mice [373] and macrophages [374] treated with different iron sources. Additionally, delayed Th1 immune responses and Th2 phenotypes have been observed in response to iron supplementation in mice infected with *Cryptococcus neoformans* [375] and *Candida albicans* [376], indicating that each particular host-pathogen interaction responds differently to iron overload.

In the case of VL, an efficient control of infection is also dependent on Th1 responses, although a mixed Th1/Th2 cytokine profile is detected during the course of infection [120,121,141,377]. So, iron supplementation in our model, besides exerting a direct toxic effect on parasites in conjunction with ROS and RNS, could be improving the host’s capacity to control the infection, by modulating the adaptive immune response. When we evaluated the cytokine response to *L. infantum* infection, we saw a discrete induction of IL-4 both in the liver and the spleen of infected mice, together with increases in the splenic expression of the pro-inflammatory cytokines, IL-1β, IL-6 and TNF.
However, iron overload did not significantly alter the immune response profile induced by infection, leading us to conclude that the modulation of the adaptive immune response does not contribute significantly to the protective effect of iron.

Most studies indicate that iron deficiency impairs the growth of pathogens [258,261,262]. In our model, feeding mice with an iron deficient diet did not affect *L. infantum* growth. Observations regarding the effects of iron chelators on *Leishmania* growth are contradictory. Treatment of mice with desferrioxamine (DFO) led to the decrease of *L. infantum* proliferation [349] but not that of *L. major* [350]. Also, in *in vitro* models of macrophage infection, DFO has shown either no effect [347] or an inhibitory effect [342,345] on *Leishmania* growth. Finally, when tested on *Leishmania* promastigotes growing in culture medium, hydroxypiridinone-derived chelators showed some inhibitory effect, which was higher than that of DFO [346]. Thus, overall, *Leishmania* do not seem to be particularly sensitive to iron deprivation. In our model of nutritional iron deficiency, the low levels of iron in tissue stores were probably sufficient to maintain the growth of *Leishmania* and not low enough to impact on the host’s capacity to control the infection.

In addition to the results obtained *in vivo* and discussed above, we found axenic cultures of *L. infantum* to be sensitive to the direct toxicity of iron. In this regard, *L. infantum* promastigotes have been shown to accumulate iron in catalytically active forms, which contribute to their sensitivity to killing by hydrogen peroxide (H₂O₂) [193,335], possibly through the Fenton reaction. Upon exposure to high doses of this metal, *L. infantum* promastigotes exhibited impaired motility and morphological changes identical to those reported to occur after exposure to antimony (III) [364] (not shown). This metalloid, used for a long time as a first line treatment against leishmanial infections, was recently found to act through the induction of oxidative damage in *L. donovani* [31,364]. Interestingly, increased intracellular iron levels directly correlated to the parasite sensitivity to this drug [364].

In conclusion, the present work discloses that iron overload decreases *Leishmania* proliferation and induces parasite death, probably by promoting oxidative reactions pernicious to the parasite. The further investigation of the molecular mechanisms of these effects will be fundamental to explore a potential utilization of iron itself as a therapeutic tool and also to understand and improve the mechanisms of action of other anti-leishmanial drugs.
Chapter IV

Phagocyte NADPH Oxidase, But Not Nitric Oxide Synthase 2, Is Essential For The Control Of Leishmania infantum In The Mouse Tissues

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Unpublished results.
Chapter IV | Phagocyte NADPH Oxidase, But Not Nitric Oxide Synthase 2, Is Essential For The Control Of *Leishmania infantum* In The Mouse Tissues

1. Abstract

Reactive oxygen and nitrogen species generated by the phagocyte NADPH oxidase and the nitric oxide synthase 2 (NOS2) respectively, are of crucial importance for host resistance to numerous microbial pathogens. However, their contribution to host defense against visceralizing species of *Leishmania* in the mouse model has not been clearly defined. To help elucidate this subject, we investigated the capacity of mice genetically deficient in the p47phox subunit of NADPH oxidase (p47phox<sup>−/−</sup>) and nitric oxide synthase 2 (NOS2<sup>−/−</sup>) to combat an infection with *L.infantum*, a causative agent of visceral leishmaniasis. We found that NADPH oxidase is essential to restrain the initial establishment of *L.infantum* in the spleen and to inhibit late infection in the liver. In contrast, NOS2 does not confer protection against the parasite in none of the organs nor at any stage of the infection. Relevantly, NADPH oxidase deficiency did not impair the expression of NOS2.

In sum, our results demonstrate that NADPH oxidase, not NOS2, is the critical host effector mechanism involved in the control of *L.infantum* in the mouse model.

2. Introduction

*Leishmania* parasites maintain their life cycle by alternating between flagellated promastigotes in the gut of phlebotomine insects and non-flagellated amastigotes inside macrophages of mammalian hosts [3,5]. These protozoa cause leishmaniasis, a complex of mammalian diseases whose clinical symptoms range from self-healing cutaneous lesions to the more severe visceralizing infection [2]. Human visceral leishmaniasis (VL) results from infection with *L.donovani* and *L.infantum* and is usually fatal if left untreated, accounting for more than 50 000 deaths per year [4,13].

In the macrophage, *Leishmania* parasites have to overcome several microbicidal mechanisms in order to survive and replicate. Two of the most important antimicrobial systems of these cells, as well as of other phagocytes, are based on the activity of NADPH oxidase and nitric oxide synthase 2 (NOS2), which are the enzyme complexes
responsible for the generation of superoxide (O\textsubscript{2}•) and nitric oxide (NO•), respectively. These radicals can give rise to other more toxic intermediates collectively known as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [170,171].

There is plenty of evidence indicating that ROS and RNS inhibit the multiplication of visceral species of *Leishmania* in axenic cultures as well as inside macrophages [186,187,188,189,190,191,192,193,194,199,201,205,206,207,209,210]. However, their relevance to the control of infections caused by visceralizing *Leishmania* in mouse models has not been thoroughly characterized. The two available studies have been performed exclusively with *L. donovani* and have yielded conflicting results [166,223]. The first report demonstrated that both NADPH oxidase and NOS2 acted in concert to control early *L. donovani* replication in the liver, whereas NOS2 on its own was sufficient to resolve late infection [166]. Contrasting with these data, a subsequent study showed that none of these antimicrobial mechanisms influenced the growth of *L. donovani* either in the early or late stages of hepatic infection, although NOS2 exerted moderate protective effects in the spleen at late phases of infection [223].

In this work, we sought to explore the contribution of ROS and RNS to the control of *L. infantum* infections *in vivo*. We found that NADPH oxidase and ROS, but not NOS2 or RNS, are critical to restrain *L. infantum* infection in the mouse liver and spleen.

3. Materials and Methods

3.1. Animals and ethics statement | C57BL/6 mice were purchased from Charles River (Madrid, Spain). Mice deficient in the p47 subunit of the NADPH oxidase complex, on a C57BL/6 background (p47phox\textsuperscript{−/−}), were bred at IBMC from a breeding pair purchased from Taconic (Lille Skensved, Denmark). The p47phox\textsuperscript{−/−} mice were administered trimethoprim-sulfamethoxazole (Bactrim; 600 mg/l\textsuperscript{−1}) in the drinking water, as prophylactic treatment against bacterial infection. This treatment was ceased when infection experiments began. Mice deficient in the nitric oxide synthase 2, on a C57BL/6 background (NOS2\textsuperscript{−/−}), were bred at IBMC from a breeding pair kindly provided by Drs. J. Mudgett, J. D. MacMicking and C. Nathan (Cornell University, New York, USA). All animals were housed at IBMC facilities under specific pathogen free conditions and fed ad libitum. Only female mice (average 20 – 25 g) were used at the age of 8 to 16 weeks. Mice were euthanized by isoflurane anesthesia followed by cervical dislocation and tissues were collected in aseptic conditions. The experimental animal procedures were approved by the Local Animal Ethics Committee of IBMC and licensed by the Portuguese General Directory of Veterinary (DGV, Ministry of Agriculture, Rural Development and
Fishing), in May 18, 2006 with reference 520/000/000/2006. All animals were handled in strict accordance with good animal practice as defined by national authorities (DGV, Law nu1005/92 from 23rd October) and European legislation EEC/86/609.

3.2. Parasites | All experiments were performed with *L. infantum* strain MHOM/MA/67/ITMAP-263 (zymodeme MON-1). For each experiment, parasites were obtained from the spleens of infected mice. Promastigotes were differentiated from spleen amastigotes by culturing these at 25°C in complete Schneider’s medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (all from Gibco, Life Technologies, Carlsbad, CA, USA), 2% human urine, 5 µg/ml phenol red (Sigma) and 5 mM HEPES sodium salt (Sigma) pH 7.4. Promastigote cultures were expanded at 25°C, for a maximum of 5 passages, in RPMI 1640 GlutaMAX™-I medium (Gibco, Life Technologies), containing 20% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 25 mM HEPES sodium salt pH 7.4. Promastigote differentiation from the exponential to the stationary phase was promoted by maintaining the cultures without medium renovation for 4 to 5 days at 25°C.

3.3. Animal experimental infection and parasite burden quantification | Mice were injected in the lateral vein of the tail with $2 \times 10^7$ *L. infantum* stationary promastigotes in 200 µl of phosphate buffered saline (PBS) pH 7.4. At defined time points, the animals were euthanized and total livers and spleens were removed and homogenized, respectively, in 3.5 ml and 3 ml of complete Schneider’s medium. These suspensions were further diluted 10- to 100-fold accordingly to the time point being assessed. Four-fold serial dilutions of the homogenized tissue suspensions were performed in quadruplicate. After 7 to 14 days at 25°C, the wells were examined for viable promastigotes. The reciprocal of the highest dilution that was positive for parasites was considered to be the number of parasites per ml of suspension and was used to calculate the number of parasites per organ (parasite burden).

3.4. Indirect immunofluorescence assay | Liver and spleen samples were fixated in 4% buffered paraformaldehyde pH 7.4 and embedded in paraffin. Tissue sections (5 µm) were adhered to poly-L-lysine treated slides, deparaffinised in xylol and re-hydrated. Tissues were permeabilized for 5 min with the working solution PBS-TritonX-100 0.1%--Tween20 0.1% and antigen retrieval was performed in 10 mM citrate buffer pH 6.0 for 30 min at 96°C. After blocking with a rat purified anti-mouse CD16/CD32 (2.4G2, BD Pharmingen, San Diego, CA, USA) diluted 1:100 in working solution-BSA 5% for 30 min at room temperature (RT), the tissues were incubated overnight (ON) at RT with a rabbit
polyclonal anti-mouse NOS2 antibody (M-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in working solution. Then, tissues were washed in PBS and subsequently incubated for 3h at RT with Alexa Fluor 568 goat anti-rabbit IgG (A-11036, Molecular Probes, Eugene, OR, USA) diluted 1:500 in working solution. After washing in PBS for 3h at 4°C, the tissues were incubated for 15 min in a solution of 0.2 µg/mL DAPI (Sigma-Aldrich Co., St Louis, MO, USA) and were mounted with VectaShield (Vector Laboratories Ltd., UK). The images were obtained with a Zeiss AxioImager Z1 epi-fluorescence microscope and analysed with the Zeiss AxioVision Rel. 4.8.2 software (Carl Zeiss Microscopy GmbH, Germany). Background was subtracted with Photoshop CS5 (Adobe Systems Inc., San Jose, CA, USA). No significant signal was observed in the negative controls (no antibodies; no primary antibody; both antibodies in NOS2−/− mice).

3.5. Statistical analysis | Differences between groups were estimated with Student’s t-test and considered significant when p value was less than 0.05 (*p<0.05; ***p<0.001).

4. Results

The role of ROS and RNS in the control of infection caused by visceralizing *Leishmania* species in mouse models is not clearly defined [166,223]. In this study, we proposed to dissect how the production of ROS and RNS affects the outcome of *L.infantum* infection in the mouse. To address this issue, we followed the kinetics of the infection of mice genetically deficient in the p47phox subunit of the NADPH oxidase (p47phox−/−) and in the NOS2 enzyme (NOS2−/−). Both knock-out and wild-type mice were infected with *L.infantum* stationary promastigotes and the parasite load in the liver and spleen was estimated at 3 hours or 1, 7, 15, 30, 60 and 120 days after infection.

Our results show that the parasite burden in the liver of wild-type and p47phox−/− mice is identical during the first 30 days of infection, but is increased in the knock-out mice at 60 and 120 days after infection (Figure 1A, left). Hence, NADPH oxidase is required to control *L.infantum* but only at late phases of infection in the liver. A different picture is observed in the spleen, where p47phox−/− mice have parasite loads significantly higher than wild-type animals as soon as 3 and 24h after infection (Figure 1B, right). This 1-logarithmic difference in parasite burden is maintained at all other time points analyzed (Figure 1A, right) and, most likely, is a consequence of the early restrain of *L.infantum* infection by NADPH oxidase.
Figure 1 | Role of NADPH oxidase and NOS2 on the control of *L.infantum* infection in the mouse. Wild-type (WT) and p47phox<sup>-/-</sup> (A, B) or NOS2<sup>-/-</sup> (C) mice were i.v. infected with 2 × 10<sup>7</sup> *L.infantum* stationary promastigotes and sacrificed at 1, 7, 15, 30, 60 and 120 days after infection (A, C) or at 3 and 24 hours after infection (B). The parasite burden in the liver and spleen of WT (white bars) or knock-out (black bars) mice was quantified by limiting dilution. The data shows the average + standard deviation of the log<sub>10</sub> number of parasites per organ (n=4-9). Student’s *t*-test was performed to determine the statistical significance of the differences between groups (*p<0.05; **p<0.001).
In contrast to the results above, wild-type and NOS2<sup>−/−</sup> mice were equally susceptible to <i>L. infantum</i> infection at all time points assessed and in both organs (Figure 1C). Importantly, we confirmed that the NOS2 protein was expressed in the liver and spleen of wild-type and p47phox<sup>−/−</sup> mice at 30, 60 (not shown) and 120 days after infection (Figure 2A), but not in NOS2<sup>−/−</sup> mice. We also observed that while the number of hepatic infiltrates expressing NOS2 was identical in both groups (Figure 2B), the number of splenic foci staining for this protein in p47phox<sup>−/−</sup> was clearly higher than in the wild-type (Figure 2A). Together, these results indicate that NOS2 expression in the mouse tissues analysed does not correlate with protection against <i>L. infantum</i>.

In short, we conclude that the activity of NADPH oxidase, unlike that of NOS2, is critical for the control of <i>L. infantum in vivo</i>.

Figure 2 | Expression of NOS2 protein in the hepatic and splenic tissue of <i>L. infantum</i> infected mice. Liver and spleen sections from wild-type (WT), p47phox<sup>−/−</sup> or NOS2<sup>−/−</sup> mice infected with 2 × 10<sup>7</sup> stationary promastigotes of <i>L. infantum</i> for 30, 60 and 120 days were stained for NOS2 protein (red). Nuclei were counterstained with DAPI (blue). A. It is only shown the NOS2 staining detected at 120 days after infection, as it is similar to that of other time points. White bar represents 20 µm (liver) or 100 µm (spleen). B. The expression of NOS2 in the liver was determined as the percentage (%) of the number of NOS2-positive
infiltrates towards the total number of infiltrates, which were counted in 15 random microscopic fields per liver section \((n=4-6)\). A similar quantification was not performed in the spleen, since no infiltrates were observed. No statistically significant differences were found.

5. Discussion

The present study shows that NADPH oxidase is a key factor controlling the initial establishment of \(L.\)\textit{infantum} in the spleen and restricting late infection in the liver. In contrast, NOS2 does not confer protection against the parasite in none of these organs at any stage of the infection.

The generation of ROS by the NADPH oxidase of professional phagocytes is known to be triggered by phagocytosis of \textit{Leishmania} promastigotes \([170,171,186,188,189,191,195,205,206,207,209]\). Accordingly, we have observed that ROS restrain the onset of infection by \(L.\)\textit{infantum} promastigotes in the mouse spleen, since mice deficient in the p47phox subunit of NADPH oxidase (p47phox\(^{-}\)) have significantly higher parasite burdens than control mice at 3 and 24h after infection. Neutrophils \([130]\) and macrophages, which produce high levels of ROS, are likely to play a decisive role in the early containment of \(L.\)\textit{infantum} in the mouse spleen. Importantly, the same difference in splenic parasite burden between wild-type and p47phox\(^{-}\) mice was seen throughout the entire course of infection, reinforcing that ROS were important to regulate the initial extent of \(L.\)\textit{infantum} replication. Nevertheless, infection progression still occurs in both groups of mice, meaning that several parasites survive the early respiratory burst. Indeed, the spleen is a site of parasite persistency that becomes chronically infected in VL \([120,121,163]\).

In the liver, the role of NADPH oxidase appears to be different from the spleen as its activity is only apparent in the late phase of infection. The liver is a site of early multiplication for visceralizing \textit{Leishmania} that spontaneously controls infection by forming granulomas \([120,121,163]\). It is assumed that infected Kupffer cells, comprised inside granulomas, are activated to eliminate \textit{Leishmania} amastigotes through the production of ROS and RNS \([163]\). We hypothesize that Kupffer cells or recruited blood monocytes only produce ROS to eliminate \(L.\)\textit{infantum} amastigotes when the granuloma becomes fully matured and the adequate activating stimuli are present \([165]\). Although granuloma formation is an asynchronous process, these structures tend to become mature around 30 - 60 days upon infection with visceral \textit{Leishmania} \([163,165]\), which argues in favour of our hypothesis.
The fact that IFN-γ-activation of human macrophages genetically deficient in the NADPH oxidase leads to eventual restrain of *L. donovani* growth, indicates that other effector mechanisms like NOS2 might intervene in the control of visceralizing *Leishmania* [205]. Contradictorily, we found that NOS2 is not essential to restrain the growth of *L. infantum*, since mice deficient in the NOS2 enzyme (NOS2−/−) and wild-type mice display identical parasite loads in both the liver and spleen. Of relevance, control and p47phox−/− mice developed progressive infection in the spleen, despite the high NOS2 protein expression. Thus, NOS2 expression and RNS generation do not correlate with protection against *L. infantum* infection. The latter observations have also been made in the spleen of mice chronically infected with *L. major* [214], *L. infantum* [121] and *L. donovani* [378]. Together, these results suggest that the strain of *L. infantum* studied in this work may be resistant to the action of RNS. Remarkably, NO•-resistant strains of *L. infantum*, which exhibit increased capacity to survive and multiply inside macrophages, have been isolated from infected humans and dogs [227]. Otherwise, *L. infantum* may have the capacity to locally subvert the expression of NOS2. In fact, we have spotted some infected cells in the hepatic parenchyma that were not expressing NOS2 (data not shown). However, we cannot exclude the possibility that NOS2 had not yet been activated in those parasitized cells, since this observation was made only at 30 days after infection.

Finally, previous studies showed that NADPH oxidase and NOS2 had little or no influence on the control of *L. donovani* growth in the mouse liver and spleen [223] or that NOS2 alone was sufficient for the resolution of hepatic infection [166]. Our work clearly demonstrates that NADPH oxidase, not NOS2, is crucial for the control of *L. infantum*. The discrepancy between these experimental models of VL might be attributed to several factors. First, *L. donovani* and *L. infantum* might have distinct sensitivities to ROS and RNS in vivo. Second, we infected mice with *L. infantum* promastigotes, while the other studies used the *L. donovani* amastigote stage, which barely triggers the respiratory burst of macrophages [188,191,205,209]. Third, the genetic background of mice was different between these studies.

In conclusion, understanding the host mechanisms that govern resistance to *Leishmania* may open new avenues to develop adequate therapeutic strategies. Our work discloses that the control of *L. infantum* in vivo is mediated by ROS and not by RNS.
Chapter V

Peptidomimetic And Organometallic Derivatives Of Primaquine Active Against *Leishmania infantum*

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Chapter V | Peptidomimetic And Organometallic Derivatives Of Primaquine Active Against *Leishmania infantum*

1. Abstract

The current treatment of visceral leishmaniasis is made difficult by the low efficacy, elevated costs, low bioavailability and high toxicity of many of the available drugs. Primaquine, an anti-malarial 8-aminoquinoline, displays activity against *Leishmania* spp. and several of its derivatives have been developed as potential anti-leishmanial drugs. However, primaquine exhibits low oral bioavailability due to oxidative deamination of its aliphatic chain. We have previously developed peptidomimetic and organometallic derivatives of primaquine with higher resistance to proteolytic degradation and oxidative deamination, which presented significant activity against primaquine-sensitive pathogens like *Plasmodium* or *Pneumocystis*. In light of these relevant findings, we decided to evaluate these compounds against both the promastigote and the intramacrophagic amastigote forms of *Leishmania infantum*, the agent of Mediterranean visceral leishmaniasis. We found that several of these compounds had significant activity against *L. infantum*. One of the peptidomimetic (3c) and one of the organometallic (7a) derivatives of primaquine were active against the clinically relevant intramacrophagic amastigote form of the parasite, causing more than 96% reduction in the number of amastigotes per 100 macrophages at 60 and 40 µM respectively, while being less cytotoxic for host cells than reference drugs sitamaquine or miltefosine. Hence, 3c and 7a represent new entries towards the development of new anti-leishmanial leads.

2. Introduction

*Leishmania* are digenetic protozoa that alternate between motile promastigotes in the gut of the sand fly and non-motile amastigotes inside macrophage phagolysosomes of the mammalian host. These parasites are the causative agents of leishmaniasis, a disease with clinical symptoms that range in severity from self-healing cutaneous lesions to serious mucocutaneous disfigurement and fatal visceralizing infection. The World Health Organization estimates that more than 12 million people are currently infected, with 2 million new cases occurring every year and 350 million people, in 98 different countries, at risk of acquiring the infection [4].
Visceral leishmaniasis (VL) is the most severe form of the disease, fatal if left untreated, with an annual incidence estimated at 0.5 million cases, causing around 50,000 deaths annually (a rate exceeded only by malaria among protozoan diseases) [4]. VL is caused by Leishmania donovani in East Africa and Indian subcontinent and L. infantum in Europe, North Africa and Latin America [13]. Active VL is characterized by weight loss, fever, weakness and hepatosplenomegaly, among other symptoms [2]. The increasing co-infection HIV/Leishmania has raised the incidence of the disease, reduced the likelihood of a therapeutic response and greatly contributed to the probability of relapse [11].

Since there are currently no effective vaccines to prevent Leishmania infections, management of VL relies on chemotherapy with first line drugs - pentavalent antimonials (sodium stibogluconate and meglumine antimoniate) - and second line drugs - pentamidine, paromomycin, amphotericin B or its lipid formulations and miltefosine [13]. However, these drugs present several problems, such as specific toxicities, elevated costs, prolonged treatment regimens, low patient compliance and parasite resistance [38]. Therefore, alternative drugs and combination regimens with improved therapeutic effectiveness are urgently needed to treat VL [61].

8-Aminoquinolines (8-AQ) have been established as a promising class of drugs for the oral treatment of malaria, Pneumocystis jirovecii pneumonia, leishmaniasis and trypanosomiasis [62, 89]. Primaquine (PQ, 1 in Figure 1), an anti-malarial 8-AQ, is known to exhibit activity against visceral Leishmania [73, 74, 75, 76, 77, 78]. Since it leads to some adverse side effects and has a lower therapeutic index than VL reference drugs, PQ has currently no applicability in the VL clinical setting. The optimization of the PQ structure has already led to the discovery of three promising 8-AQ: NPC1161B [81], tafenoquine [80] and sitamaquine [78, 81], which demonstrated high activity against experimental VL. Sitamaquine has completed phase IIb clinical trials by GlaxoSmithKline, although with variable results and unexpected cases of toxicity [63, 64, 65, 66, 67].

Interestingly, earlier works suggested that PQ encapsulation in liposomes or nanoparticles enhanced its leishmanicidal activity, either in infected macrophages [74, 75, 76] or in animal models of infection [76, 77]. This enhanced effect may be due to the fact that encapsulation of PQ prevents it from undergoing metabolic inactivation to carboxyprimaquine (2, Figure 1) [79], thus increasing the drug’s bioavailability. Therefore, it is reasonable to expect that alternative strategies that prevent PQ conversion into its inactive metabolite, 2, will possibly contribute to the improvement of the drug’s activity against VL.
We have been working on the synthesis and biological evaluation of PQ derivatives where the aliphatic amine of the parent drug has been masked by acylation with a peptidomimetic (3, Figure 1) or organometallic moiety (5-10, Figure 2), as a strategy to (i) avoid premature oxidative deamination of PQ to 2 and (ii) confer resistance to proteolytic degradation, which affects PQ dipeptide derivatives such as 4 (Figure 1) [87,89,379,380]. Both compounds 3 and 5-10 have previously revealed remarkable activity against PQ-sensitive pathogens, namely *Plasmodium* (malaria) and *Pneumocystis* (pneumocystic pneumonia), in some cases with better performances than PQ at the activity and/or toxicity levels [88,89,91,92]. In view of this, and of the known anti-leishmanial properties of 8-AQ analogues of PQ, as sitamaquine, the present work aimed to explore the activity of the peptidomimetic and organometallic PQ derivatives against *L.infantum*, the causative agent of Mediterranean VL. As presented forward, some of these compounds exhibited anti-leishmanial activity, which was stronger than that of PQ and comparable to reference anti-leishmanial drugs sitamaquine or miltefosine, while having lower cytotoxicity than the latter. These findings emphasize the importance of scrutinizing the activity of PQ derivatives or analogues against protozoan pathogens other than *Plasmodia*.

*Figure 1* | Chemical structures of primaquine (1), its main metabolite carboxyprimaquine (2) and its peptidomimetic (3a-e) or dipeptidic (4a-i) derivatives. Cyt P_450_: cytochrome P_450_; MAO: monoamine oxidase.
Figure 2 | Chemical structures of primaquine’s organometallic derivatives 5-10.

Figure 3 | Chemical structures of sitamaquine (11) and miltefosine (12).
3. Materials & Methods

3.1. Chemical synthesis | Synthetic procedures and structural data have been reported elsewhere on compounds 3a-e [89] and 4b [380]. Other compounds of the PQ-Pro-Xaa series, 4a,c,i, were synthesized as previously described for 4b [380], and spectroscopic data as well as HPLC traces are given as Supporting Information (Supp. Info.). Synthetic procedures and chromatographic/spectroscopic data were reported elsewhere for all compounds 5-10 [91,92], except 7h, for which relevant procedures and data are available in Supp. Info.

3.2. Reagents | Sitamaquine was supplied by GlaxoSmithKline (Brentford, Middlesex, UK). Miltefosine was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). All compounds used in this study were dissolved in dimethyl sulfoxide (DMSO) and stored at -20ºC.

3.3. Parasites | Promastigotes of the *L.infantum* strain MHOM/MA/67/ITMAP-263 (zymodeme MON-1) were differentiated at 25ºC in complete Schneider’s medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin (all from Gibco, Life Technologies, Carlsbad, CA, USA), 2% human urine, 5 µg/ml phenol red (Sigma) and 5 mM HEPES sodium salt (Sigma) pH 7.4, from amastigotes present in the spleen of infected mice. Promastigote cultures were expanded at 25ºC, for a maximum of 5 passages, in RPMI 1640 GlutaMAX™-I medium (Gibco, Life Technologies), containing 20% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 25 mM HEPES sodium salt pH 7.4. Promastigote differentiation from the exponential to the stationary phase was promoted by culture at 25ºC, without medium renovation, for 4 to 5 days.

3.4. Drug screening assay on promastigotes | Promastigotes (1 × 10^6 /well) were cultured at 25ºC in complete RPMI medium, supplemented with the various compounds at concentrations between 2.5 and 160 µM (200 µL total volume). After 24h of culture, 20 µL of a 2.5 mM resazurin solution (freshly prepared and filtered in phosphate buffered saline, pH 7.4, Sigma) was added to each well. The fluorescence intensity, corresponding to resazurin conversion to the fluorescent compound resorufin was determined 48h after resazurin addition (excitation wavelength of 560 nm and emission wavelength of 590 nm, with a fluorometer SpectraMAX GeminiXS, Molecular Devices LLC, Sunnyvale, CA). All experimental conditions were carried out in triplicate. The IC₅₀ (µM) values were determined with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) from plots of percentages of parasite growth in relation to control versus inhibitor concentration.
3.5. Bone marrow derived macrophages | Macrophages were derived from the bone marrow of BALB/c mice as follows. Each femur was flushed with 5 ml of HBSS (Gibco). The resulting cell suspension was centrifuged and the cells re-suspended in complete DMEM (Gibco), containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 Uml\(^{-1}\) penicillin, 50 µgml\(^{-1}\) streptomycin, 10 mM HEPES pH 7.4 and 10% L929 cell conditioned medium (LCCM), as a source of macrophage-colony stimulating factor. The cells were distributed in 24 or 96-well plates containing or not, sterile and LPS-free microscopy slides and incubated at 37ºC in a 7% CO\(_2\) atmosphere. Three days after seeding, 100 µL of LCCM was added. On the 7\(^{th}\) day of culture, the medium was renewed.

3.6. Infection of bone marrow derived macrophages | On the 10\(^{th}\) day of culture, completely differentiated macrophages adhered to microscopy slides (4 × 10\(^5\) /well) were infected with stationary promastigotes of *L.infantum* at an approximate macrophage:parasite ratio of 1:10. After 4h at 37ºC in a 7% CO\(_2\) atmosphere, cells were washed with warm HBSS to remove non-internalized promastigotes and re-incubated in complete DMEM. Compounds were added after 24h of culture at the desired concentrations. Cells were fixed and stained with Hemacolor® (Merck KGaA, Darmstadt, Germany) 24 or 72h hours later. The slides were visually inspected under the light microscope to determine the number of parasites present inside macrophages. The “parasite index” was calculated as the number of amastigotes per 100 macrophages. Each experimental condition was run in triplicate wells. A minimum of 400 macrophages was counted per slide. Representative pictures of stained cells were obtained with an Olympus CX31 light microscope equipped with a DP-25 camera (Imaging Software Cell^B, Olympus, Center Valley, PA, USA).

3.7. Drug cytotoxicity assay on bone marrow derived macrophages | On the 10\(^{th}\) day of culture, macrophages (8 × 10\(^4\) /well) were treated with the various compounds at the desired concentrations (200 µL total volume). 24 or 72h later, 20 µL of a 1.25 mM resazurin solution was added to each well and the plates were re-incubated at 37ºC for another 3 h. The fluorescence intensity was determined as above-mentioned. All experimental conditions were carried out in triplicates. The CC\(_{50}\) (µM) values were determined with GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) from plots of percentages of cell viability in relation to control versus inhibitor concentration.

3.8. Statistical analysis | One-way ANOVA, followed by Bonferroni's multiple comparison *post hoc* test, was performed to determine statistical significance of the differences between vehicle and groups treated with the different compounds.
4. Results

4.1. In vitro activity against *L.infantum* promastigotes

Our first approach was to test PQ and its peptidomimetic, dipeptidic and organometallic derivatives on axenic cultures of *L.infantum* promastigotes. The promastigote stage of *Leishmania* is best suited to this purpose due to the simplicity of cultivation, allowing a fast and easy way to screen a large number of drugs.

Results obtained for compounds of the 3 and 4 series (Figure 1) are depicted in Table 1. Two of these compounds, 3c and 4c, were as active as PQ (1) against *L.infantum* promastigotes, whereas all other derivatives from these series displayed lower or no activity. Although 3c and 4c are amongst the PQ derivatives with highest lipophilicity (3c ranks 1st) – there is no direct correlation between clogP and IC\(_{50}\) values (Table 1). This is clearly shown by the significantly different IC\(_{50}\) values obtained with 4d-4f, whose calculated clogP values are identical. It is interesting to notice, though, that \(\beta\)-ramification of the aminoacid (Xaa) residue may have a role in antimicrobial activity, as all PQ derivatives which displayed IC\(_{50}\)<35 \(\mu\)M bear a \(\beta\)-ramified Xaa residue. Other structural features in Xaa, like presence/absence of ionizable (as in 4h) or of hydrogen-bonding (as in 4g or 4h) groups, as well as the bulkiness of the hydrocarbon aminoacid side chains in subsets 3a-c or 4a-f (expressed by the Charton’s steric factor \(\nu\) [381] (Table 1)), do not seem to have any particular effect on compound’s activity against *L.infantum*.

Since several reports indicate that iron potentiates the leishmanicidal activity of several drugs [364,382] and that we have previously shown that ferrocene (Fc) derivatives of PQ were active against *Plasmodium* [91] and *Pneumocystis* [92], we next tested a series of Fc derivatives of PQ for their activity against *L.infantum* promastigotes. The results obtained with compounds 5-10 (Figure 2) are shown in Table 2. The compounds of series 7, where PQ is linked to Fc through a variable amino acid spacer, were generally inactive or performed worse than parent PQ and reference drugs sitamaquine (11) or miltefosine (12) (Figure 3). Relevantly, removal of the amino acid spacer between PQ and Fc, to give compound 5, led to an increase in activity, which became higher than that of PQ, though lower than those of both reference drugs sitamaquine (11) and miltefosine (12). Compounds 9 and 10 were inactive, although the latter had shown the highest activity against *Plasmodium* [91]. Compound 10 was obtained by directly binding a hexylFc moiety to the PQ heteroaromatic core, 8-amino-6-methoxyquinoline. Interestingly, inserting the same hexylFc moiety of 10 on PQ itself led to compound 6, which exhibited a remarkably higher activity against promastigotes, being more potent than both reference drugs (11, 12). Finally, compound 8, whose structure encompasses an imidazolidin-4-one
and an Fc moiety, also showed a significant activity against *L.infantum* promastigotes, comparable to that of miltefosine (12). With the exception of compound 6, no other PQ derivative ranked better than sitamaquine (11). Nonetheless, toxicity issues with this drug (11), the fact that peptidomimetic derivatives of PQ are promising alternatives to PQ due to enhanced chemical and metabolic resistance [89,383] and the good activities demonstrated by the organometallic series of compounds, motivated us to further explore the potential of some of them in the amastigote form of *Leishmania* parasites.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sup&gt;1&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>R&lt;sup&gt;3&lt;/sup&gt;</th>
<th><em>L.infantum</em> promastigotes (IC&lt;sub&gt;50&lt;/sub&gt; ± SD / μM; 72h)</th>
<th>Mouse bone marrow-derived macrophages (CC&lt;sub&gt;50&lt;/sub&gt; ± SD / μM; 24h)</th>
<th>clogP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ν (R&lt;sup&gt;2&lt;/sup&gt; or R&lt;sup&gt;3&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>32.2 ± 1.0</td>
<td>&gt; 60</td>
<td>2.04</td>
<td>-</td>
</tr>
<tr>
<td>3a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>H</td>
<td>H</td>
<td>-</td>
<td>74.8 ± 1.7</td>
<td>-</td>
<td>1.82</td>
<td>0</td>
</tr>
<tr>
<td>3b</td>
<td>H</td>
<td>Me</td>
<td>-</td>
<td>64.4 ± 1.2</td>
<td>-</td>
<td>2.22</td>
<td>0.52</td>
</tr>
<tr>
<td>3c&lt;sup&gt;c&lt;/sup&gt;</td>
<td>H</td>
<td>Pr</td>
<td>-</td>
<td>31.8 ± 1.0</td>
<td>&gt; 60</td>
<td>3.03</td>
<td>0.76</td>
</tr>
<tr>
<td>3d</td>
<td>H</td>
<td>(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt; SMe</td>
<td>-</td>
<td>&gt; 80</td>
<td>-</td>
<td>2.40</td>
<td>-</td>
</tr>
<tr>
<td>3e</td>
<td>Me</td>
<td>H</td>
<td>-</td>
<td>&gt; 80</td>
<td>-</td>
<td>2.10</td>
<td>-</td>
</tr>
<tr>
<td>4a</td>
<td>-</td>
<td>-</td>
<td>H</td>
<td>&gt; 80</td>
<td>-</td>
<td>1.05</td>
<td>0</td>
</tr>
<tr>
<td>4b</td>
<td>-</td>
<td>-</td>
<td>Me</td>
<td>&gt; 80</td>
<td>-</td>
<td>1.45</td>
<td>0.52</td>
</tr>
<tr>
<td>4c&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>Pr</td>
<td>31.6 ± 1.0</td>
<td>&gt; 60</td>
<td>2.25</td>
<td>0.76</td>
</tr>
<tr>
<td>4d</td>
<td>-</td>
<td>-</td>
<td>SMe</td>
<td>37.8 ± 1.0</td>
<td>-</td>
<td>2.72</td>
<td>1.02</td>
</tr>
<tr>
<td>4e</td>
<td>-</td>
<td>-</td>
<td>iBu</td>
<td>&gt; 80</td>
<td>-</td>
<td>2.72</td>
<td>0.98</td>
</tr>
<tr>
<td>4f</td>
<td>-</td>
<td>-</td>
<td>BzI</td>
<td>52.0 ± 1.4</td>
<td>-</td>
<td>2.72</td>
<td>0.70</td>
</tr>
<tr>
<td>4g</td>
<td>-</td>
<td>-</td>
<td>p-(OH)BzI</td>
<td>&gt; 80</td>
<td>-</td>
<td>2.43</td>
<td>-</td>
</tr>
<tr>
<td>4h</td>
<td>-</td>
<td>-</td>
<td>(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>62.9 ± 1.5</td>
<td>-</td>
<td>1.35</td>
<td>-</td>
</tr>
<tr>
<td>4i</td>
<td>-</td>
<td>-</td>
<td>(CH&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;SMe</td>
<td>43.3 ± 1.0</td>
<td>-</td>
<td>1.62</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.4 ± 1.0</td>
<td>&lt; 60</td>
<td>5.84&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14.4 ± 1.1</td>
<td>&gt; 60</td>
<td>3.30</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculated using the OSIRIS Property Explorer (http://www.organic-chemistry.org/prog/peo/);  
<sup>b</sup>Taken from reference [381];  
<sup>c</sup>Experimental value taken from reference [384];  
<sup>d</sup>In 3a to 4i, R stands for amino acid side chain; H, hydrogen; Me, methyl; Pr, isopropyl; Bu, isobutyl; SMe, sec-butyl; BzI, benzyl.  
<sup>e</sup>[β]-ramified amino acids
Table 2 | Anti-parasitic activity and macrophage toxicity of primaquine (1), its organometallic derivatives 5-10 and reference drugs sitamaquine (11) and miltefosine (12).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>L. infantum promastigotes (IC50 ± SD / µM; 72h)</th>
<th>Mouse bone marrow-derived macrophages (CC50 ± SD / µM; 72h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–</td>
<td>26.5 ± 1.2</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>22.7 ± 1.1</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>6</td>
<td>–</td>
<td>4.9 ± 2.3</td>
<td>6.8 ± 1.2</td>
</tr>
<tr>
<td>7a*</td>
<td>H</td>
<td>&gt; 80</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>7b</td>
<td>Me</td>
<td>44.7 ± 1.1</td>
<td>–</td>
</tr>
<tr>
<td>7c*</td>
<td>iPr</td>
<td>&gt; 80</td>
<td>–</td>
</tr>
<tr>
<td>7d</td>
<td>iBu</td>
<td>&gt; 80</td>
<td>–</td>
</tr>
<tr>
<td>7e</td>
<td>Bzl</td>
<td>&gt; 80</td>
<td>–</td>
</tr>
<tr>
<td>7f</td>
<td>(CH2)3NH2</td>
<td>40.3 ± 2.5</td>
<td>–</td>
</tr>
<tr>
<td>7g</td>
<td>CH2NH2</td>
<td>39.2 ± 6.2</td>
<td>–</td>
</tr>
<tr>
<td>7h</td>
<td>CH(CH3)OH</td>
<td>63.3 ± 1.3</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>11.5 ± 1.0</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>&gt; 80</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>&gt; 80</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>–</td>
<td>7.4 ± 1.0</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>–</td>
<td>14.4 ± 1.1</td>
<td>24.7 ± 0.5</td>
</tr>
</tbody>
</table>

*In 7a to 7e, R stands for amino acid side chain; H, hydrogen; Me, methyl; iPr, isopropyl; iBu, isobutyl; Bzl, benzyl.

**4.2. In vitro activity against intramacrophagic L. infantum amastigotes**

In light of the observations made in the previous section, compounds 3c and 4c (Figure 1), as well as 5 and 8 (Figure 2) were selected for evaluation on intramacrophagic L. infantum amastigotes, the clinically relevant parasite stage. The high toxicity of compound 6 towards mouse bone marrow derived macrophages (Table 2) excluded it from further testing. Compound 7a was included in these studies for comparison with 8, of which it is the synthetic acyclic precursor. Moreover, PQ (1), sitamaquine (11) and miltefosine (12) were included as reference drugs.

The anti-leishmanial activity of the peptidomimetic PQ derivative 3c was confirmed in this assay, as this compound was found to be more effective than PQ at eliminating intramacrophagic L. infantum (Table 3). Conversely, the dipeptide derivative 4c had no effect on the elimination of intramacrophagic L. infantum amastigotes (Table 3).
As shown in Table 4, among the organometallic derivatives, compound 5 showed no activity against amastigotes, while the activity of compound 8 was confirmed on this intramacrophagic form of the parasite (Table 4). Surprisingly, compound 7a also showed potent activity against amastigotes, revealing an efficacy at 40 µM that was comparable to that of miltefosine (12) at 20 µM (Table 4). Due to its high toxicity towards macrophages (Table 2 and Figure 4C), miltefosine (12) could not be used in these assays at concentrations higher than 20 µM.

Table 3. Activity of PQ (1), 3c, 4c and sitamaquine (11) against intramacrophagic *L. infantum*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration / µM</th>
<th>Nr amastigotes / 100 macrophages ± SD (24h)</th>
<th>% reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>313.0 ± 18.3</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>60</td>
<td>221.4 ± 29.5</td>
<td>29.3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>235.0 ± 53.7</td>
<td>24.9</td>
</tr>
<tr>
<td>3c</td>
<td>60</td>
<td>7.3 ± 1.8</td>
<td>97.7a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>168.5 ± 35.9</td>
<td>46.2a</td>
</tr>
<tr>
<td>4c</td>
<td>60</td>
<td>322.3 ± 57.1</td>
<td>-3.0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>320.5 ± 54.4</td>
<td>-2.4</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>8.7 ± 6.9</td>
<td>97.2a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>84.1 ± 27.1</td>
<td>73.1a</td>
</tr>
</tbody>
</table>

a p<0.001

Table 4. Activity of PQ (1), 5, 7a, 8 and miltefosine (12) against intramacrophagic *L. infantum*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration / µM</th>
<th>Nr amastigotes / 100 macrophages ± SD (72h)</th>
<th>% reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>135.8 ± 27.5</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>80</td>
<td>10.6 ± 5.1</td>
<td>92.2b</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>66.8 ± 23.6</td>
<td>50.8a</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>127.8 ± 33.5</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>99.0 ± 32.2</td>
<td>27.1</td>
</tr>
<tr>
<td>7a</td>
<td>80</td>
<td>3.7 ± 1.1</td>
<td>97.3a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.3 ± 1.7</td>
<td>96.1b</td>
</tr>
<tr>
<td>8</td>
<td>80</td>
<td>29.0 ± 5.4</td>
<td>78.6a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>30.5 ± 3.3</td>
<td>77.6b</td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>5.0 ± 1.0</td>
<td>96.3a</td>
</tr>
</tbody>
</table>

a p<0.05; b p<0.001
Lastly, both 3c (Figure 4E and Table 1) and 7a (Figure 4F and Table 2) caused no significant toxicity towards macrophages in contrast with reference drugs 11 and 12 (Figure 4B and 4C, Table 1 and 2). Reference drugs 11 (60µM) and 12 (20µM) clearly decreased the confluency and altered the morphology of infected macrophages as compared to vehicle, whereas compounds 1 (60µM), 3c (60µM) and 7a (80µM) did not significantly affect any of these parameters at the highest concentrations tested (Figure 4).

Figure 4 | Micrographs of *L. infantum* infected bone marrow-derived macrophages exposed to: vehicle (A) and compounds 11 at 60 µM (B), 12 at 20 µM (C), 1 at 60 µM (D), 3c at 60 µM (E) and 7a at 80 µM (F). Black bar corresponds to 50 µm.

5. Discussion

Chemotherapy is currently the only option for the management of the life threatening VL. However, the limited number of available drugs and their serious drawbacks reinforce the urgent need for adequate therapies [13]. The ideal drug should be non-toxic, affordable, extremely effective and easily administered during a short period in the outpatient setting with no parasite resistance associated. To acquiesce with these criteria, the rational modification of known anti-parasitic molecules such as PQ is a valuable strategy.

Structural modifications that involve the inclusion of imidazolidin-4-one (peptidomimetics 3) and Fc (organometallics 5 – 10) moieties into PQ improve its activity against *Plasmodium* and *Pneumocystis* and/or its safety [88,89,91,92]. The results here in disclosed show that peptidomimetic 3c and organometallic 7a derivatives of PQ were...
more effective at eliminating intramacrophagic amastigotes of *L. infantum* than the parent drug and showed decreased toxicity when compared to sitamaquine and miltefosine.

We tested 5 peptidomimetic and 9 dipeptidic derivative of PQ for their activity against *L. infantum* promastigotes. Among these, 3c and 4c were the most active, with an IC$_{50}$ close to that of PQ (1). It is remarkable that compounds 3c and 4c are closely related, as 3c is the imidazolidin-4-one peptidomimetic surrogate of PQ’s dipeptide derivative 4c. This is hardly coincidental and shows that the valine residue, common to the two, is beneficial for in vitro anti-leishmanial activity against promastigotes. Interestingly, when tested for anti-leishmanial activity inside macrophages, 3c was much more active than 4c. This is not surprising since 4c is more susceptible than 3c to proteolytic degradation [383], which could be aggravated by the fact that protease activity is increased in *Leishmania*-infected macrophages [385]. Proteolytic degradation would cause cleavage of the dipeptide moiety in 4c, leading to PQ release. However, if this was the case, 4c would expectedly display an activity similar to that of the parent drug, which did not occur. Another possible explanation for the discrepant behavior of 1, 3c and 4c against either promastigotes or intracellular amastigotes could be the fact that the macrophage metabolizes 3c, but not 1 or 4c, to originate a compound which is more toxic to *L. infantum*. This lack of activity of 4c may also be due to the action of a macrophage efflux pump able to recognize dipeptide substrates as, e.g., multi-substrate transporters from the ABC super-family [386] or peptide/histidine transporters PHT1 and PHT2 [387]. It is likely that such transporters would not recognize the peptidomimetic imidazolidin-4-one moiety in 3c. The most remarkable finding on 3c, however, is the fact that this compound was as potent as sitamaquine (11) at the highest concentration tested, 60 µM (Table 3), with the further advantage that 3c is not cytotoxic at this concentration, whereas sitamaquine is (Table 1, Figure 4). Though sitamaquine (11) performed better than 3c at the lowest concentration tested, 30 µM, its high toxicity hampers its application in clinics, which urges the discovery of safer anti-leishmanial leads, like 3c.

Moreover, this study allowed the drawing of some conclusions regarding structure-activity relationships. β-ramification of the aminoacid (Xaa) residue may have a role in antimicrobial activity, as all PQ derivatives which displayed IC$_{50}$<35 µM bear a β-ramified Xaa residue (Table 1). Possibly, this feature favors the uptake by *L. infantum* parasites, mediated by specific amino acid permeases or transmembrane peptide transporters [388], parallel to, e.g., the arginine-specific transporter previously reported for *L. donovani* [389]. Curiously, enhanced activity and uptake when β-ramified amino acids were present has been previously described, although with other compounds and pathogens [390,391]. Although the most active compounds had high lipophilicity, we found no direct correlation
between this parameter and anti-parasitic activity. Other structural features in Xaa do not seem to have any relevant effect on compound’s activity against *L. infantum* (Table 1).

Concerning the Fc derivatives of PQ 5-10 (Figure 2), results obtained on promastigotes (Table 2) were very variable, reflecting the high structural diversity of these organometallic compounds. Series 7a-h, where PQ is linked to Fc through a variable amino acid spacer, was generally not very promising, while the removal of the amino acid spacer between PQ and Fc, to give 5, led to a substantial increase in activity. Also somewhat surprising was the fact that compound 10, the most promising as an anti-malarial [91], was inactive against *L. infantum* promastigotes. Interestingly, compound 6, which has the same hexylFc moiety as 10, but linked to the PQ’s aliphatic amine group than directly bound to the aryl-amine group in 8-amino-6-methoxyquinoline (the heteroaromatic core of PQ), appeared remarkably active against promastigotes, being more potent than both sitamaquine (11) and miltefosine (12). The second most active compound against *L. infantum* promastigotes was 8, with an activity comparable to that of miltefosine (12). This is an interesting finding, as 8 includes both structural modifications pursued by our group over the past few years in order to improve PQ’s therapeutic properties [87,89,91,92]: it encompasses an imidazolidin-4-one and an Fc moiety, i.e., it can be seen as an organometallic surrogate of peptidomimetic compounds 3 (Figure 1).

Since the intramacrophagic amastigote form of *Leishmania* is the most biologically relevant, the most promising organometallic PQ derivatives were also tested in this model. Unfortunately, compound 6, which had shown the highest activity against promastigotes, revealed a high toxicity towards bone marrow derived macrophages, which excluded it from further studies. The CC₅₀ on macrophages was of about 7 µM, in agreement with the cytotoxicity previously displayed on Huh-7 human hepatoma cells [91]. On the contrary, compounds 5 and 8 (similarly to the peptidomimetics of the 3 and 4 series tested previously) had very low cytotoxicity and were selected for subsequent analysis on intramacrophagic amastigotes. Compound 7a, though inactive against promastigotes, was also taken further for such studies, as representative of the non-cytotoxic (Table 2) acyclic precursor of 8. In fact, compounds 5, 7a and 8 can be seen as sequential analogues of each other, i.e., 7a is the analogue of 5 where the simplest amino acid (glycine) has been introduced as spacer between the PQ and the Fc moiety; in turn, 8 is the derivative of 7a where an additional cyclization step introduced an imidazolidin-4-one ring. While compound 5 lost its activity inside macrophages, 8 revealed a significant activity on this setting. Yet, the most striking observation was that 7a had potent activity against amastigotes, not far from that of miltefosine (12) (Table 4). So, contrary to what was observed in the peptidomimetic series, the presence of an imidazolidin-4-one ring was not
necessary for intramacrophagic activity in the case of the organometallic derivatives. Eventually, joining the constrained cyclic imidazolidin-4-one motif with the bicyclic Fc moiety in 8 represents a structure too large or too stiff to easily enter the macrophage and/or the amastigote. By removing the imidazolidin-4-one ring, as in 7a, such problem might be minimized or eliminated, explaining the higher activity of 7a as compared to 8. Another clear fact is the key role of the amino acid residue: 7a differs from 5 only in the fact that the latter misses the glycine residue present in the former, yet this difference was enough to remove anti-leishmanial activity in 5. Curiously, both 7a and 5 were previously found to be inactive either against blood-stage Plasmodia or as malaria transmission-blocking agents, while presenting very good activity against liver-stage malaria parasites [91,92]. This suggests stage-specific anti-parasitic activity, which might deserve further investigation in the future.

Factors underlying the different activities described in this study remain to be determined. However, it is clear that the screening of a variety of PQ derivatives for anti-leishmanial activity revealed two compounds with interesting (3c) or highly potent (7a) effects on the clinically relevant stage of L.infantum, with very low toxicity for host cells. This makes them worthy candidates as leads for the development of novel and safer PQ-based anti-leishmanials and emphasizes the relevance of exploring the potential of PQ-based structures against protozoan pathogens other than Plasmodia.
Chapter VI

General Discussion
Chapter VI | General Discussion

1. Relationship between the host iron status and *Leishmania* infection

Iron is an indispensable nutrient for the survival and proliferation of nearly every living organism. The biological utility of iron originates from its capacity to readily undergo electron transfer reactions and to interact with numerous coordinating ligands, features that enable its participation in the most basic cellular functions. Yet, when redox active iron is present in excess it can promote oxidative reactions pernicious to biological systems [172,175,253,392]. Hence, organisms must have mechanisms to supply their own iron needs and avoid iron’s toxicity [258,264,265,266,327,328].

Several studies show that vertebrates use an elaborate iron withholding system to restrict the access to iron by invading microbes [258,259,260]. So, when the host is iron overloaded by nutritional, therapeutic or hereditary reasons, the iron withholding system is overwhelmed and the growth of pathogens is favoured [258,261]. Intriguingly, *L.major* [350,351,352] and *L.infantum* (our work) parasites appear to be an exception to this tenet, as discussed below. It is currently unknown if the proliferation of other *Leishmania* species is also inhibited by the host’s iron overload, a subject worthy to explore in the future.

1.1. Iron mediates host resistance to *Leishmania*: a role for ROS and RNS

One of our most significant findings was that iron overload protected mice against *L.infantum* visceral infection (chapter III). By administering high iron doses via the parenteral route, we forced its accumulation in the mouse tissue macrophages, as previously reported [175,253,363,393]. Upon infection with *L.infantum*, we observed that iron-loaded macrophages were found inside hepatic infiltrates, which are structures of parasite containment [163,165]. This observation allied to the reduced tissue parasite proliferation led us to conclude that iron acted as a toxic insult to *L.infantum* and not as a growth factor. The fact that the anti-leishmanial effect of iron overload was abrogated in mice genetically deficient in the NADPH oxidase or NOS2, suggested that ROS and RNS generated by macrophages are the mediators of iron toxicity towards *L.infantum*. Likewise, treatment of mice with diphenyleneiodonium chloride, which is an inhibitor of both the NADPH oxidase and NOS2, reverted the protective effects of iron overload against *L.major* [351]. It is possible that the high iron levels inside macrophages increased the extent of the labile iron pool, which comprises iron in a form that can potentially engage in redox reactions and induce oxidative damage to biological structures [394].
Moreover, the macrophage PVs were probably enriched in redox-active iron, since the mouse strains used in our studies do not express a functional NRAMP1 protein, whose role is to deplete iron from phagosomal compartments [320,321,322,323]. In the future, we could directly ascertain whether iron is enhancing the macrophage oxidative defense mechanisms against *L. infantum*. This could be achieved by quantifying the level of ROS and RNS released in response to *L. infantum* infection by macrophages treated or not with iron [208]. Alternatively, the detection of ROS and RNS could be performed *ex vivo* in macrophages isolated from the liver and spleen of iron overloaded mice infected with *L. infantum* [204,351].

Notably, we verified that *L. infantum* hepatic parasite burden was significantly reduced in iron overloaded mice early after infection, meaning that iron might have interacted with ROS and RNS during phagocytosis (chapter III). In agreement, iron overload significantly enhanced the respiratory burst of rat Kupffer cells (liver macrophages) in response to phagocytosis of inert particles [392,395]. In addition, iron overloaded mice exhibited increased levels of NADPH oxidase-mediated oxidative burst [351] and NOS2 transcripts [350] early after *L. major* inoculation in the skin. The iron-induced effects on parasitemia were detected in the spleen only at later time-points (chapter III). This discrepancy between organs might be related to the initial iron distribution. A larger proportion of the injected iron is taken up by Kupffer cells in the liver. Although the spleen has a higher basal amount of iron per mg of tissue, this iron is probably distributed among cells that are not involved in the initial uptake of *Leishmania* parasites. The fact that iron overload does inhibit the growth of *L. infantum* in the spleen at later stages of infection, raises the hypothesis that iron may exert its effects during cell-to-cell spread of the parasites or once parasites are proliferating inside an iron-loaded macrophage, by pathways unexplored in the present work.

The ROS and RNS synthesized by NADPH oxidase and NOS2, respectively, have been implicated in the elimination of several other *Leishmania* species by the host’s macrophages [166,195,204,213,214,217,218,220,222,225]. However, we showed that ROS, but not RNS, are required to control *L. infantum* infection in the tissues of non-iron overloaded mice (chapter IV). Moreover, we hypothesize that *L. infantum* might be resistant to the action of RNS (chapter IV). So, it is conceivable that in an iron overloaded mouse, the generation or propagation of both ROS and RNS reaches a level that cannot be withstood by the parasite. On the other hand, iron might have promoted the formation of alternate reactive species to which the parasite displays no antioxidant defense capacity. For instance, the reaction of iron with peroxynitrite (ONOO’) yields nitronium ion (NO$_2^+$), a strong oxidizing species to which no detoxification system in *Leishmania* has
been reported [392,396]. Furthermore, *L.infantum* promastigotes were shown to contain iron in catalytically active forms, which contribute to their sensitivity to killing by ROS [193,335] and possibly also by RNS.

Relevantly, both macrophagic NADPH oxidase and NOS2 require iron (as part of the heme co-factor) for proper function [170]. In a few models of macrophage infections with different bacteria, macrophages were shown to need iron to exert their antimicrobial activity. Iron increased the capacity of macrophages to eliminate or prevent the multiplication of *B.abortus*, by catalyzing the production of HO• [356] and iron loading of *Staphylococcus aureus* prior to infection enhanced bacterial killing by monocytes, most likely by promoting oxidative damage [397]. Also in the case of *Listeria monocytogenes* and *S.typhimurium*, iron seems to be needed for intramacrophagic killing of these bacteria [354,355]. However, several studies performed with macrophage infection models failed to reproduce the *in vivo* anti-leishmanial effect of iron. Indeed, treatment of macrophages with different iron sources either did not alter [347] or increased the multiplication and survival of several *Leishmania* species [212,342,345]. In these cases, iron doses were probably not sufficient to promote oxidative reactions pernicious to *Leishmania*. Instead, the *in vivo* effects of iron may also be mediated by other cell types, such as neutrophils, as has been previously proposed for the *L.major* model [351].

The findings discussed above led us to question if genetically determined host iron overload would also inhibit *L.infantum* multiplication. To address this question, we used the HFE deficient mouse model, which mimics the human iron overload disorder hereditary hemochromatosis. This pathology is most often associated with mutations in HFE, a MHC class I-like molecule, and is characterized by increased serum TF saturation and iron deposition predominantly in the liver [264,265,365,366]. We observed that HFE-deficient mice had tissue parasite loads comparable to those of wild-type mice. Such result did not corroborate our finding that iron-dextran administration inhibited *L.infantum* growth (chapter III). A possible explanation might be the fact that HFE-deficient mice develop spontaneous iron overload mainly in hepatocytes, while macrophages are relatively depleted of this metal [365,366]. Still, upon infection with *L.infantum*, we clearly detected high accumulation of iron inside macrophages of the hepatic infiltrates in HFE-deficient mice. Notably, an identical observation has been previously reported in *M.avium* infection of this mouse model [357]. Redistribution of iron towards accumulation inside macrophages is likely a strategy of the host’s iron withholding system to decrease circulating iron levels in response to infection and/or inflammation [264]. Non-infected HFE-deficient mice have reduced hepatic hepcidin transcripts [264,398] and increased FPN expression in the liver and spleen [399,400], an expression pattern not consistent
with iron retention in macrophages. Although we ignore the mechanisms of macrophage iron retention in chronically infected HFE-deficient mice, it is possible that the hepcidin/FPN expression is altered to favor iron accumulation in this condition.

Other justifications may then account for the absence of effect of iron overload, caused by HFE deficiency, on the growth of *L. infantum*. Firstly, the inhibitory effect of iron is best accomplished when the macrophages are iron-loaded prior rather than after infection. To validate this hypothesis, we could infect the *flatiron* mouse model, which has spontaneous iron loading in Kupffer cells due to a loss-of-function mutation in FPN [401]. In this mouse model, iron would be present in tissue macrophages before infection. Secondly, the function of NADPH oxidase and NOS2 may be compromised in HFE-deficient mice. This is, however, unlikely since their expression is identical in the spleen of wild-type and HFE-deficient mice infected with *S.typhimurium* [399]. Thirdly, HFE-deficient mice had tissue iron levels that were considerably lower than those found in iron-dextran-injected mice and were probably not enough to promote oxidative reactions pernicious to *L.infantum*. Supporting this hypothesis is the fact that in iron-dextran-injected mice, high iron doses had to be administered to significantly reduce the tissue parasite burden (chapter III).

1.2. Influence of iron overload on the host immune response to *Leishmania*

Mouse resistance or susceptibility to *L.major* infection is dependent on the development of Th1 or Th2 immunity, respectively [122,123,141,142,370]. Iron supplementation, besides enhancing the early oxidative burst [351], also promoted the development of a Th1-like immune response to *L.major* in a susceptible mouse strain [350]. Such a protective immune response in iron overloaded mice was characterized by (i) reduced levels of serum IgE and IgG1 and increased levels of IgG2a, (ii) an increased ability of spleen cells to act as antigen presenting cells, (iii) increased number of IL-12+ cells and decreased number of IL-4+ and IL-10+ cells at the inoculation site, and (iv) increased levels of IFNγ and NOS2 and decreased levels of IL-4 and IL-10 transcripts at the lesion site [350,351]. However, we can argue that the iron-induced oxidative burst at the onset of *L.major* infection may have significantly reduced the parasite numbers at the lesion site, thus facilitating the establishment of a protective immune response. Indeed, it has been described that low parasite numbers lead to the mounting of a Th1-like immune response to *L.major* irrespective of the infection route, mouse or parasite strain [402].

In mouse models of VL, resistance to infection also correlates with the development of Th1 responses, but it is not clear that Th2 responses determine susceptibility. In fact, a
mixed Th1/Th2 cytokine profile is detected during the course of infection with visceral Leishmania [122,141,142,163,377]. Although IL-10 overproduction strongly correlates with susceptibility to VL [122,143], other Th2 cytokines such as IL-4 [144] and IL-13 [145] seem to promote the control of L. donovani growth. Since the L. major model indicated that iron supplementation favoured the development of a Th1-like immune response [350,351], we also addressed whether this fact was occurring in our model. Our results suggest that the host’s iron overload did not significantly affect the adaptive immune response to L. infantum infection (chapter III). However, we cannot ignore that the absence of differences in the immune response profile induced by L. infantum in iron overloaded and untreated mice might be due to the number of parasites in the tissues. That is, although iron might have increased activation of a protective response, the decreased parasite numbers in iron overloaded mice might have elicited a lower activation of protective cells or production of protective cytokines than in control mice, resulting overall in a similar level of response. Anyhow, this result reinforces that the inhibitory effect of iron on the growth of L. infantum is predominantly due to its interaction with ROS and RNS.

Earlier studies showed that iron overload adversely affected the performance of the host immune system, namely by (i) impairing macrophage phagocytosis, antigen presentation and cytokine production, (ii) depressing NK cell cytotoxic activity, (iii) suppressing the functions of the complement system and (iv) altering lymphocyte numbers, proliferation and cytokine secretion, as well as T-cell subset ratios [260,403,404,405]. The fact that iron overload either did not affect (chapter III) or favoured [350,351] the induction of a protective immune response to Leishmania infection differs from what is observed in other infection models. Generally, the host’s iron overload correlates with the induction of Th2 immunity and/or the suppression of Th1 responses [260]. For instance, iron overloaded mice exhibit Th2 or delayed Th1 immune responses to C. albicans [376] or C. neoformans [375] infection, respectively, which are characterized by increased production of IL-4 and IL-10 and decreased production of IFNγ and IL-12. In line with this, the generation of NO• and secretion of TNF in response to LPS [406] or S. typhimurium infection [325] are impaired in iron-loaded macrophages. Decreased NO• production has also been observed in mice [373] and macrophages [374] treated with different iron sources. It should be noted, however, that the latter effect has not been consistently observed in all studies of iron supplementation. The treatment of rats with iron-dextran [371] or saccharated colloidal iron [372] potentiated the LPS-induction of hepatic NOS2 expression and NO• production. These two studies agree with our hypothesis that iron supplementation favours the production or propagation of RNS that leads to inhibition of L. infantum growth. Lastly, a recent study showed that iron overloading of macrophages,
as occurs in mouse models and humans with chronic inflammatory diseases, can direct the polarization of these cells towards an unrestrained pro-inflammatory M1 phenotype [407,408]. This M1 macrophage population secretes high levels of TNF, HO• and ONOO• [407,408,409]. In turn, M1 macrophages retain iron due to increased expression of the iron storage protein FT and decreased expression of the iron exporter FPN [410]. So, it is left unexplored the possibility that iron accumulation in our model might have induced macrophage polarization into the M1 phenotype and consequently promoted oxidative defense mechanisms against *L.infantum*.

1.3. Host iron overload offers resistance to re-infection with *Leishmania*

Iron treatment strongly inhibited the replication of *L.major* and *L.infantum*, but did not lead to complete parasite elimination from the mouse tissues. In other words, sterile cure was not achieved by iron supplementation. It has been postulated that the long-term persistence of *Leishmania* in a host after cure is important to develop resistance in case of re-infection [411]. In fact, others have previously demonstrated that iron overloaded mice are resistant to re-infection with *L.major* [352]. The iron-induced oxidative burst elicited during both primary and secondary infections with *L.major* correlates with the activation of the transcription factor NF-κB and with an enhanced proliferation of IFNγ-secreting CD4+ T cells in the dLNs [352]. This is substantiated by the fact that iron and ROS/RNS can modulate the activation of macrophagic NF-κB signalling pathways [175,368,412], which are known to regulate numerous genes involved in immune and inflammatory responses [369]. Indeed, NF-κB regulates the development of IFNγ-secreting CD4+ T cells and concomitant resistance to *L.major* [353]. Hence, iron not only seems to synergize with the host’s oxidative mechanisms of defense, but also interacts with ROS and RNS in order to activate signalling cascades that regulate the development of protective immunity against *Leishmania*. Understanding the molecular mechanisms involved in iron-induced resistance to *Leishmania* infection and re-infection deserves further research in the future.

1.4. Host iron deficiency does not correlate with susceptibility to *Leishmania*

Malnutrition is associated with susceptibility to VL in humans [9,10] and mice [413]. Although iron deficiency is the most common micronutrient deficiency in the human population [414], the relationship between human iron deficiency alone and increased risk of acquiring VL has never been investigated. We showed that feeding mice with an iron deficient diet did not affect the course of infection with *L.infantum* (chapter III). Although these nutritionally iron-deprived mice had half the normal iron stores in the liver and the
spleen, they presented normal haematocrit and body weight (not shown). To possibly attain a more dramatic iron deprivation, we could eventually feed the mice with an iron-deficient diet while simultaneously treating them with an iron chelator, such as desferrioxamine (DFO), deferasirox [415] or hydroxypyridinones [346,416]. Such iron deprivation approach has led to complete protection of mice against *Trypanosoma cruzi*, a related trypanosomatid [417].

Prior studies concerning the impact of iron chelation on *Leishmania* growth have yielded conflicting results. First, treatment of mice with DFO significantly impaired the growth of *L.infantum* [349], but not that of *L.major* [350]. Second, DFO had either no effect [347] or an inhibitory effect on the intramacrophagic replication of *L.donovani* [345,348] and *L.amazonensis* [342]. Third, the multiplication of *L.donovani, L.major* and *L.infantum* promastigotes in culture medium was either not inhibited by DFO [348] or was modestly inhibited by hydroxypyridin-4-one-derived chelators [346]. Altogether, the present and previous studies indicate that *Leishmania* parasites do not seem to be particularly sensitive to iron deprivation. This is probably related to their high capacity to scavenge iron in starvation conditions [331,340,343], since *Leishmania* are endowed with multiple iron acquisition mechanisms [327,328]. Hence, we suppose that, in our model, the lowered levels of iron in tissue stores were enough to support the growth of *L.infantum*.

Though iron chelation has been suggested as an effective therapeutic strategy against several infections [258,261], we feel it may be inadequate for the treatment of leishmaniasis, especially in areas where both this disease and malnutrition co-exist. Effective iron chelation could worsen the anemia that is frequently observed in VL patients [418] or adversely affect the proper performance of the host immune system. In fact, iron deficiency has been associated with (i) impaired microbicidal action of phagocytes, (ii) supressed NK cell cytotoxicity and (iii) reduced T lymphocyte numbers, proliferation and altered cytokine expression profile [404,414,419]. Of relevance, the activity of NADPH oxidase [355] and NOS2 [420] in response to PMA and *M.tuberculosis*, respectively, is impaired in iron-depleted macrophages. Also, the expression of the pro-inflammatory cytokines IL-6 and TNF in response to *S.typhimurium* infection is considerably attenuated in macrophages with low intracellular iron levels [400]. Lastly, mice fed an iron deficient diet produce less IFNγ upon stimulation of splenic T cells with a mitogen stimulus [421] and have reduced serum levels of IFNγ and IL-12 [422] in comparison to control mice.
1.5. Iron exerts direct toxicity towards *Leishmania*

In this work, we showed that axenic cultures of *L.infantum* promastigotes and amastigotes are sensitive to the direct toxicity of iron (chapter III). In line with our results, the excessive production and subsequent internalization of Fe\(^{2+}\) by the LFR1 reductase and LIT1 importer, respectively, markedly reduced the viability of *L.amazonensis* amastigotes [340]. Although we have not approached how *L.infantum* acquired iron from the different added sources, some explanatory hypothesis can be suggested. Iron-dextran may have been internalized by *L.infantum* parasites through a receptor or transporter of carbohydrates present at the surface membrane. In fact, the uptake of several hexoses (glucose, mannose, fructose or galactose) by distinct carbohydrate transporters has been described in *L.mexicana* [423]. Alternatively, iron bound to dextran or citrate may have been released from these complexes upon reduction by a Fe\(^{3+}\) reductase and subsequently internalized by a Fe\(^{2+}\) importer. These iron acquisition pathways have been proposed to occur in *L.infantum* promastigotes [339,340] and have been well characterized in intramacrophagic amastigotes of *L.amazonensis* [340,343].

Upon exposure to high iron concentrations, promastigotes exhibited impaired motility and morphological changes identical to those reported to occur after exposure to antimony (III) and arsenic (III) ([364] and data not shown). These metalloids were found to act through the induction of oxidative injury to *L.donovani* [31,32,39,364]. By analogy, iron may also inhibit the growth or even kill both life stages of *L.infantum* by catalysing the endogenous production of ROS. To address this hypothesis, we could try to measure whether iron causes direct intracellular generation of ROS [240,364] or oxidative damage to the parasite and/or if iron impairs the parasite’s antioxidant defense system [190,424].

Relevantly, the concentrations of iron needed to impair the viability of both life forms of *L.infantum* were relatively high, in the millimolar range (chapter III). At lower iron concentrations, the parasite possibly avoided its toxicity by down-regulating the expression of some iron importers or by increasing the efflux of iron. In fact, decreased uptake and increased efflux of antimony, another heavy metal, have been proposed to occur in antimony-resistant *Leishmania* parasites [38,39]. Instead, iron may have been compartmentalized by the parasite in a relatively safe form. However, this hypothesis is unlikely, since it has been described that *L.infantum* promastigotes store iron in redox-active forms, which greatly increases their susceptibility to killing by H\(_2\)O\(_2\) [193,335]. Another hypothesis that could explain the relative resistance to iron’s toxicity is the up-regulation of the parasites’ antioxidant defense or repair systems [94]. We envisage that whatever parasitic mechanisms were used for protection against the noxious effects of iron, they were overwhelmed by high iron concentrations (chapter III).
1.6. Iron potential as a therapeutic tool to fight *Leishmania* infections

Iron not only enhanced or synergized with the host’s oxidative mechanisms of defense against *Leishmania*, but also exerted direct toxicity towards both life forms of the parasite (our results and [351]). This suggests that iron may be exploited as a therapeutic tool to fight infections caused by *Leishmania* parasites. This contention is substantiated by several pieces of evidence. First, an increase in intracellular iron levels directly correlates with the sensitivity of *L. donovani* promastigotes to antimony (III) [364], a drug that causes oxidative stress to *Leishmania* by interfering with the parasites’ antioxidant defense system [31,32,39]. Second, iron potentiates the leishmanicidal activity of artemisinin by inducing oxidative injury that culminates in cell death of *L. donovani* promastigotes [382]. Third, iron treatment can induce accumulation of pentamidine in the mitochondria of *L. enriettii* promastigotes, consequently increasing their sensitivity to the drug. This effect is probably due to the action of the multidrug resistance protein 1 (LeMDR1), a possible mitochondrial iron importer [425]. Fourth, the coupling of an iron-containing group (ferrocene) to PQ significantly enhanced its activity towards *L. infantum* promastigotes and intramacrophagic amastigotes (chapter V). Hence, the increase of intracellular iron levels in *Leishmania* overall increases their vulnerability to chemotherapy. Noteworthy, in most instances, such effect seems to be intertwined with the generation or propagation of ROS pernicious to the parasites.

In sum, the interaction between pathogens and their hosts are complex processes dependent not only on the genome of both, but also on nutritional factors. It is generally acknowledged that iron excess increases and iron chelation decreases susceptibility to infection [261,262]. However, murine models of infection by *Leishmania* indicate that these parasites represent an exception to such rule. Moreover, despite several reports that iron supplementation (to correct nutritional iron deficiency) can significantly increase the risk of several infections [261,414], no correlation between iron administration and susceptibility to human leishmaniasis has, to our knowledge, ever been described. Although iron chelation has been suggested as an effective therapeutic strategy against several infections, in the case of leishmaniasis and especially in areas where this disease and malnutrition co-exist, iron chelation may be inappropriate. Iron overload decreases *Leishmania* growth, likely by promoting oxidative reactions harmful to the parasites. Understanding the molecular mechanisms of these effects will aid a possible exploitation of iron as a therapeutic tool and may also help to unravel and improve the mode of action of other anti-leishmanial drugs.
2. Role of ROS and RNS in the control of *Leishmania* infection

The fact that we found that ROS and RNS seem to mediate the toxicity of iron towards *L. infantum* (chapter III), motivated us to fully characterize the relative contribution of these microbicidal molecules to the control of *L. infantum* infection in a non-iron overloaded mouse. Our results showed that NADPH oxidase and ROS were required to control the initial establishment of *L. infantum* in the spleen and to restrict late infection in the liver. In contrast, NOS2 and RNS did not afford protection against the parasite in both organs at any stage of the infection (chapter IV).

2.1. ROS, but not RNS, mediate the *in vivo* control of *L. infantum* infection

The oxidative burst of professional phagocytes [170,171] is prompted by phagocytosis of *Leishmania* promastigotes [186,188,189,191,195,205,206,207,209], leading to parasite killing by a mechanism reminiscent of apoptosis [195]. In agreement, we showed that ROS restrain the onset of infection by *L. infantum* promastigotes in the mouse spleen (chapter IV). We hypothesize that neutrophils and macrophages, host cells known to generate high amounts of toxic oxidants, are responsible for the early containment of *L. infantum*. In fact, it has been described that neutrophils contribute to the early restraint of *L. infantum* [130] and *L. donovani* [128,129] infection and to the establishment of a protective Th1 immune response [128] in the mouse spleen. Moreover, they can also assist infected macrophages to produce O$_2$•$^*$ and NO• and consequently kill *L. major* [124], *L. infantum* and *L. braziliensis* [132]. Although ROS were important to regulate the initial extent of *L. infantum* replication, the infection in the mouse spleen still progressed (chapter IV). This means that several parasites either survive the early respiratory burst or barely activate the NADPH oxidase of certain phagocyte populations in this organ. The latter situation is supported by the fact that the levels of ROS generated as well as the proportion of infected cells producing detectable oxidants are reduced in human monocytes phagocytosing non-opsonized as opposed to opsonized *L. infantum* promastigotes [208]. Indeed, the spleen is a site of parasite persistency that becomes chronically infected in VL [120,121,163].

In contrast to what was observed in the spleen, ROS seem to control *L. infantum* in the liver at late stages of infection (chapter IV). The mouse liver permits an early and rapid proliferation of visceral *Leishmania* but spontaneously controls infection by forming cellular infiltrates around parasitized resident macrophages (Kupffer cells) [120,121,163]. Within these structures, known as granulomas, infected Kupffer cells are presumably activated to kill amastigotes via generation of ROS and RNS [163]. We propose that
Kupffer cells - which are poor producers of ROS when cultured in vitro [426] - or recruited blood monocytes only release ROS to eliminate *L.infantum* amastigotes when the granuloma fully matures and the required activating stimuli are present [165]. Though the assembly of granulomas is not a synchronized process, they become functionally and structurally mature during the progression from the acute to the chronic stage of infection with visceralizing *Leishmania* [163,165], which agrees with our hypothesis. Alternatively, neutrophils could also be recruited to granulomas at late infection stages to aid in the control of infection. Notably, the late influx of neutrophils into the skin lesions of *L.amazonensis* [204] and *L.major* [152] susceptible mice has been described as a process governed by IL-17 [152]. However, neutrophils do not appear to be involved in the containment of *L.infantum* in the liver [130].

A pertinent finding was the fact that NOS2 was not critical to contain the growth of *L.infantum* in the liver and spleen. We also showed that the expression of NOS2 protein was not related to protection against *L.infantum* infection, since both wild-type and NADPH oxidase-deficient mice developed gradual splenic infection in the face of a prominent NOS2 expression (chapter IV). The latter observation has also been made in the spleen of mice chronically infected with *L.major* [214], *L.infantum* [121] and *L.donovani* [378] or in the lesions of mice parasitized by *L.amazonensis* [204]. Moreover, we detected higher NOS2 expression in the spleen of NADPH oxidase-deficient mice than in the wild-type mice, probably to compensate for the deficiency in NADPH oxidase (chapter IV). In fact, it has been previously reported that compensatory mechanisms can arise in other gene-disrupted mice infected with *Leishmania* [165]. Overall, these results raise the possibility that our strain of *L.infantum* may be resistant to the lethal effects of RNS. Several NO•-resistant strains of *L.infantum* [227], *L.amazonensis* and *L.braziliensis* [226] have been isolated from infected humans. Both naturally [227] and experimentally-induced [201] NO•-resistant *L.infantum* displayed enhanced infectivity and ability to survive or replicate inside macrophages. Although axenic amastigotes of *L.infantum*, *L.amazonensis* and *L.mexicana* are susceptible to the action of RNS [199,204], tissue amastigotes may exhibit enhanced antioxidant defense and resistance to RNS toxicity. In this respect, *L.infantum* possesses three cytosolic 2-cysteine peroxiredoxins, one of which was reported to be predominantly expressed in the amastigote stage [427], that is capable of detoxifying NO• and ONOO• [246]. Moreover, it has been proposed that amastigotes resist NO• toxicity by overexpressing metabolic enzymes that can act as NO• scavengers, such as aconitase and glyceraldehyde-3-phosphate dehydrogenase [428]. Interestingly, a NOS enzyme has also been isolated from *L.donovani* promastigotes, reinforcing that this parasite might have mechanisms to detoxify endogenously produced NO• that could also
offer resistance against NO• generated by the host cell [250]. Another possible explanation for our results may be the capacity of _L.infantum_ to locally suppress the expression of NOS2. Indeed, we observed a few infected cells in the liver tissue that were not expressing NOS2 (data not shown). Nevertheless, we cannot exclude the possibility that NOS2 had not yet been activated in those parasitized cells or that the infected cells were not macrophages and hence did not express NOS2. It is well documented that _Leishmania_ can also infect cell types devoid of direct antimicrobial capacity, which function as safe targets particularly during late stages of infection [411].

### 2.2. In vivo control of other _Leishmania_ species by ROS and RNS

The role of ROS and RNS in the restraint of infection caused by visceralizing _Leishmania_ species in mouse models is quite controversial. A prior report demonstrated that both NADPH oxidase and NOS2 acted in concert to control early _L.donovani_ replication in the liver, whereas NOS2 alone was sufficient to resolve late infection [166]. In contrast, a subsequent study showed that these antimicrobial mechanisms had no influence on the outcome of _L.donovani_ infection in the liver, though NOS2 exerted moderate protective effects in the spleen at late phases of infection [223]. Our work clearly establishes that NADPH oxidase, not NOS2, mediates the control of _L.infantum_ infection in the mouse tissues. The incongruities between these studies might be due to several reasons. First, _L.donovani_ and _L.infantum_ might have distinct intrinsic vulnerabilities to the levels and type of ROS and RNS generated by the mouse cells. Second, we infected mice with _L.infantum_ promastigotes, whereas the other works used the _L.donovani_ amastigote life stage, known to scarcely elicit the oxidative burst of macrophages [188,191,205,209]. Third, the genetic background of mice differed between these studies.

Noteworthy is the fact that resolution of cutaneous lesions caused by _L.guyanensis_ also depends solely on the induction of a strong respiratory burst by NADPH oxidase [195,217]. This is in contrast to what is observed in most other mouse models of CL. The healing of lesions and long-term containment of persisting parasites correlate intimately with the expression of NOS2 in the _L.major_ [196,213,214,218,219,220,221], _L.amazonensis_ [204] and _L.braziliensis_ [222] mouse models. Unlike NOS2, NADPH oxidase is not required to control _L.amazonensis_ [217] or _L.braziliensis_ [222] infections. However, its activity is required to clear _L.major_ from the spleen and to prevent late parasite visceralization [214]. Hence, the containment of _Leishmania_ by NOS2 and NADPH oxidase _in vivo_ may be organ- and infection stage-specific.
2.3. Factors influencing the in vivo control of *Leishmania* by ROS and RNS

Lastly, several parameters, apart from parasite species, may dictate the influence of NADPH oxidase and NOS2 on the control of *Leishmania* infections. These include the parasite strain, inoculum dose and administration route as well as the infected host. We are now planning to ascertain the sensitivities to ROS and RNS of distinct *L. infantum* strains in the mouse model. This seems a pertinent step in our research, given that *L. infantum* strains with different virulence phenotypes elicit different humoral and cellular immune responses in the mouse [429,430]. We cannot, however, avoid mentioning that the mouse model may not reflect the contribution of ROS and RNS to the control of *L. infantum* in naturally infected hosts. It has been described that neutrophils and monocytes derived from *L. infantum*-infected dogs exhibit impaired respiratory burst and leishmanicidal capacity in relation to non-infected dogs [431,432], effects that may be attributed to LPG [433] and reversed by IFNγ activation [434]. Moreover, some studies suggest that *L. infantum* may resist RNS toxicity or suppress NOS2 expression by dog macrophages. Both high [435] and negligible [436] NOS2 expression in the tissues of *L. infantum*-infected dogs appears to correlate with disease severity and high parasite loads. Furthermore, co-culture of lymphocytes and macrophages derived from infected dogs inhibits the macrophagic production of NO• in response to re-infection with *L. infantum* [437]. Nevertheless, the NOS2-mediated leishmanicidal activity of dog macrophages [438,439] can be re-established *in vivo* through dog vaccination [216] or chemotherapy [440].

To summarize, a better understanding of the host mechanisms of resistance to *L. infantum* may open novel therapeutic avenues for intervention in VL. We have found that ROS, but not RNS, are pivotal for the control of *L. infantum* in the mouse tissues. In this connection, improving drugs that act by inducing the production of pernicious ROS in target pathogens, such as PQ and other 8-AQs [79], may be a valuable strategy to develop new chemotherapeutics for the treatment of infections caused by *L. infantum*.

3. VL treatment: potential of PQ-derived compounds as therapeutic agents

The treatment of VL is particularly challenging because several of the currently available chemotherapeutics exhibit high toxicity, require prolonged and often painful treatment regiments, are expensive and their efficacy is variable and hindered by parasite resistance. For these reasons, alternative drugs devoid of such disadvantages are urgently required to treat VL [13,21,38,441]. To respond to such demands, recent efforts have been focused on the rational modification of pre-existing anti-parasitic drugs, such
as PQ. The modification of the PQ scaffold has already led to the discovery of new 8-AQs with potent anti-leishmanial activities, such as NPC1161B [81], tafenoquine [80] and sitamaquine [78,81]. However, the first two drugs have yet no applicability in the clinical scenario and the latter has been correlated with adverse side effects [63,64,65,66,67]. Moreover, the screening of several chemical libraries, using an ex vivo splenic explant culture system from hamsters infected with *L. donovani*, revealed that the most frequently identified leads with both anti-leishmanial activity and low toxicity were quinolinic compounds, including 2-, 4- and 8-AQs [442]. This highlights the importance for the continuous design of new PQ-derived compounds as anti-leishmanial agents.

3.1. Modification of PQ side chain as a strategy to improve its anti-leishmanial activity: imidazolidin-4-one and ferrocene moieties

By protecting PQ's terminal aliphatic amine with a dipeptide, a peptidomimetic (imidazolidin-4-one) or an organometallic (ferrocene, Fc) moiety, we intended to possibly avoid the oxidative deamination of PQ and increase its anti-leishmanial activity. Increased anti-leishmanial and decreased *in vitro* hematotoxicity, has actually been accomplished by attaching an amino acid residue to the PQ’s side chain amino group together with the placement of a bulky 2-tert-butyl group at the C-2 of the quinoline ring [73]. We found that the conjugation of an imidazolidin-4-one (*3c*) and a ferrocene (*7a*), but not a dipeptide, moiety to PQ improved its activity against the intramacrophagic amastigote stage of *L. infantum*. Notably, the most active compounds, *3c* and *7a*, exhibited lower cytotoxicity towards host cells than VL reference drugs sitamaquine and miltefosine (chapter V).

Our experimental approach to dissect the possible anti-*L. infantum* activity of PQ derivatives consisted in the selection of the most active compounds against promastigotes to next test them towards intramacrophagic amastigotes. Of all the dipeptide and peptidomimetic derivatives of PQ tested, only peptidomimetic *3c* and dipeptide *4c* revealed activity similar to the parent drug against promastigotes, whereas most other compounds were inactive (chapter V). Both *3c* and *4c* contain a valine residue at the end of the its side chain, which may be related to their anti-leishmanial activity against *L. infantum* promastigotes. All other amino acid residues did not seem to be beneficial for the activity of PQ against this stage of the parasite. Nevertheless, the behaviour of *3c* and *4c* was completely different in the intramacrophagic amastigote stage. The former displayed activity superior to PQ and comparable to sitamaquine, whereas the latter was completely inactive. Several reasons may account for this fact. Firstly, the internalization, metabolism or efflux of both compounds might differ between the promastigote and
amastigote stages of *L. infantum*. Secondly, 3c might have been internalized to a higher extent than 4c by the macrophage. Thirdly, even if the entry into the macrophage occurred to the same extent, they might have been differently metabolized by the macrophage. It is possible that 4c might have been more easily degraded by proteases present in the PV. In fact, it has been determined that PQ-derived imidazolidin-4-ones like 3c, but not dipeptides like 4c, display higher enzymatic stability *in vitro* [383]. This hypothesis is, however, unlikely because proteolytic digestion of 4c would cause cleavage of the dipeptide group to yield PQ and therefore, 4c would expectedly exhibit activity at least close to PQ, which was not verified (chapter V). Alternatively, 3c may have been transformed into a compound that is more toxic to *L. infantum* than PQ, and/or 4c might have been converted to an inactive compound. Fourthly, the absence of activity of 4c might be caused by its externalization by the action of macrophage efflux pumps with affinity for dipeptides, as is the case of peptide/histidine transporters 1 and 2 [387]. By contrast, 3c would be allowed to accumulate inside the macrophage because these efflux systems would not recognize its imidazolidin-4-one moiety. Finally, we cannot exclude the hypothesis that 4c is a slow-acting compound and that a longer incubation with infected macrophages would permit the expression of anti-leishmanial activity.

The fact that iron potentiates the leishmanicidal activity of several drugs [364, 382], exerts toxicity towards axenic cultures of *L. infantum* promastigotes and amastigotes and that the host’s iron overload protects mice against *L. infantum* visceral infection (chapter III), led us to investigate the activity of Fc derivatives of PQ against this parasite. Contrary to what was observed with the dipeptidic and peptidomimetics, we found several organometallics (5, 6 and 8) with better performance than PQ against promastigotes (chapter V). The activity of PQ was slightly improved by the attachment of a Fc group to its terminal aliphatic amine (5). However, the insertion of a distinct amino acid residue between PQ and Fc, as in 7a-h, led to loss of activity. Notably, the introduction of a 6-carbon chain between PQ and Fc, to give 6, caused a very pronounced increase in activity. In fact, compound 6 was the most effective of the organometallic derivatives, being more active than sitamaquine and miltefosine. We hypothesize that the 6-hydrocarbon chain might have increased the lipophilicity of 6, which in turn could have favored its transport across the promastigote membrane, though we have not proven this experimentally. It is known that the lipophilicity of sitamaquine, which possesses a 6-carbon side chain directly attached to the heteroaromatic core of PQ, is a pivotal feature for crossing promastigote membranes [68, 69, 384]. However, compound 10, which has the same hexylFc group as 6, but attached to the heteroaromatic core of PQ instead of being linked to the PQ’s terminal aliphatic amine group, does not display activity against
promastigotes. Possibly, the lack of the terminal aliphatic amino group in 10, which is present in PQ, 6 and in sitamaquine, led to complete loss of anti-leishmanial activity. Finally, the attachment of an imidazolidin-4-one and a Fc group (in this order) to the PQ’s terminal aliphatic amino group led to compound 8, the second most active organometallic against *L. infantum* promastigotes, reinforcing that both structural modifications have a beneficial anti-leishmanial effect.

The most active and non-toxic organometallic derivatives of PQ, 5 and 8, were further tested for activity against intramacrophagic amastigotes, except compound 6 that displayed high cytotoxicity towards macrophages. Compound 7a, though inactive against promastigotes, was also selected for such studies, since 7a represents the precursor of compound 8 where an additional cyclization step introduced an imidazolidin-4-one group. We found that the activity of 8, but not that of 5, against promastigotes was confirmed on the intramacrophagic amastigote stage. Moreover, we verified that 7a had significantly higher activity than PQ against amastigotes, contrary to what was observed in the promastigote stage. These results suggest that the activity of 5 and 7a is either parasite stage-specific or influenced by the macrophage metabolism. In addition, the fact that 7a was more active than 8 indicated that the imidazolidin-4-one moiety was not crucial for the intramacrophagic activity of the organometallic PQ derivatives. Perhaps, the presence of an imidazolidin-4-one together with a Fc group in 8, but not 7a, may yield an excessively bulky or rigid structure than does not easily enter the macrophage and/or the amastigote. On the other hand, the glycine residue present between PQ and the Fc moiety in 7a seems important for activity against amastigotes, as its absence in 5 led to a complete loss of activity. Hence, it is possible that other amino acid residues linking PQ to Fc, as in 7b-h, may have potential activity against the intramacrophagic stage of the parasite, a subject left to explore in the future.

### 3.2. Screening for anti-leishmanial drugs: intramacrophagic amastigote assay

We now recognize that selecting the most effective drugs on the promastigote stage may not be the most adequate procedure, since the activity of a drug candidate towards the promastigote stage may not be reflected in the intramacrophagic amastigote stage. This was clearly demonstrated by the distinct behaviour of 4c, 5 and 7a between both parasite stages (chapter V). In support of this notion is the fact that half of the compounds present in a large library of kinase and phosphatase inhibitors, which were active against intramacrophagic amastigotes, were not active against promastigotes of *L. donovani* [443]. We performed drug screening against promastigotes growing in culture medium as it is a
simple, rapid and inexpensive technique that requires minute amounts of test compound. However, this methodology has been gradually dismissed because the metabolic pathways may significantly differ between both parasite stages and because it does not assess drug internalization, metabolism and efflux by the host cell [444,445]. Conversely, drug screening against amastigotes growing inside macrophages (primary or cell lines) is more likely to reproduce the parasite environment in an infected host. Although the latter methodology allows the evaluation of drug toxicity towards the parasite and the host cell, it holds several limitations. Drug sensitivity may vary accordingly to the species/strain from which the host cell was isolated and the drug effect on parasite growth may be confounded by replication of the host cell line [445]. Furthermore, direct counting of stained intracellular amastigotes, as performed in our study (chapter V), is time consuming, labour intensive, incompatible with the screening of high numbers of compounds and requires technical expertise. Relevantly, the recent engineering of recombinant parasites carrying a reporter gene - either as an episomal copy or after its integration in a defined locus (green fluorescent protein, β-galactosidase, β-lactamase or luciferase) - has permitted the objective analysis of drugs in large-scale, with considerable sensitivity, reproducibility, rapidity and simplicity [442,444,445]. Some caution must, nevertheless, be taken when using these methods for several reasons. First, the antibiotic resistance marker may confer cross-resistance to the test drug. Second, the engineering procedure may disrupt the genomic architecture and interfere with ordinary biological processes. Third, the reporter may have variable sensitivity and be masked by the background activity of the host cell. Fourth, the expression of the reporter gene may depend on the copy number of the transfected plasmid and not on the activity of the drug itself [444,445]. Finally, we propose that the future evaluation of activity of PQ derivatives (and other classes of compounds) should be performed exclusively in the intramacrophagic amastigote stage, using the robust reporter gene technology described.

3.3. In vivo studies on the efficacy, stability and toxicity of PQ derivatives

Our goal for the future is to ascertain the in vivo efficacy, stability and toxicity of PQ-derivatives 3c and 7a. In fact, we have initiated the assessment of the efficacy of 3c in a mouse model of VL. A pilot experiment revealed that 3c was as effective as PQ at decreasing the hepatic parasite burden (51.6% versus 41.8%) when administered in low doses (116.5 nmol/mouse/day for 21 days), without indications of toxicity (data not shown). Notably, we have also obtained experimental evidence that peptidomimetic derivatives of PQ, such as 3c, have lower tendency than PQ to undergo metabolic transformation mediated by rat liver enzymes [383]. Hence, these preliminary data
indicate that at least PQ peptidomimetics exhibit in vivo anti-L.infantum activity, low toxicity and superior stability than the parent drug. We now intend to explore the effect of escalating doses of 3c and 7a in short courses by oral administration to the mouse, as these compounds were developed to potentially improve the oral bioavailability (and hence the activity) of PQ. The testing of promising leads in animal models is important as it enables the determination of compound activity in relation to absorption, distribution, metabolism and excretion and gives an early estimation of toxicity [445]. Of note, the identification of compounds that act as pro-drugs or through the host’s immune system probably will only be clearly identified in animal models that can reproduce the immunological responses and pathological features of human VL [442,445].

3.4. Mechanism of action of PQ and its analogues

The mode of action of most leishmanicidal drugs is scarcely known [38,39,50]. Certainly, the unravelling of the mechanism of anti-leishmanial activity for peptidomimetic (3c) and organometallic (7a) derivatives of PQ would contribute to the rational optimization of their structure in order to obtain new and more potent analogues. We speculate that they might act in a similar way to PQ and other structurally related 8-AQs. Sitamaquine crosses L.donovani promastigote plasma membrane via electrostatic and hydrophobic interactions with phospholipids [68,69]. It was initially postulated, but recently discredited [70], that sitamaquine’s lethal action was related to its accumulation in and alkalinization of the acidocalcisomes of L.donovani promastigotes [40]. More convincing is the fact that sitamaquine [42,71] and tafenoquine [95] inhibit the activity of mitochondrial succinate dehydrogenase and cytochrome c reductase, respectively. In turn, this triggers the generation of ROS that culminates in an apoptotic-like death of L.donovani promastigotes. Though the anti-leishmanial mode of action of PQ has never been reported, it is likely that PQ (and the derivatives tested in this work) also targets the mitochondrial electron transport chain and/or favours the formation of ROS, as occurs in Plasmodium, T.cruzi and P.jirovecii [79]. Overall, PQ and its 8-AQ analogues appear to act by inducing oxidative stress in Leishmania. Relevantly, we have shown that ROS are involved in the control of L.infantum growth in vivo (chapter IV), which reinforces that drugs that act by inducing oxidative stress may be valuable against L.infantum. Furthermore, the anti-leishmanial activity of PQ and its derivatives is probably enhanced by conjugation to a Fc moiety, given that the Fe²⁺ present in this structure catalyses the generation of HO• via a Fenton-like reaction in vitro [93]. In fact, iron can positively influence the anti-leishmanial activity of unrelated drugs. Iron potentiates the leishmanicidal activity of the anti-malarial drug artemisinin [382] or the metalloids arsenic
(III) and antimony (III) [364], by inducing oxidative injury that culminates in an apoptotic-like cell death of *L. donovani* promastigotes. Thus, we can infer that strategies intended to augment the intracellular iron levels of *Leishmania*, like the inclusion of a Fc moiety into a drug, might increase the sensitivity of the parasite to chemotherapy. Supporting this contention is the fact that mice with elevated iron status are more resistant to *L. major* [350,351,352] and *L. infantum* (chapter III), which is related to an enhanced generation of ROS pernicious to the parasite. Lastly, our compounds might also display immune-activating properties, as it has been described that macrophages secrete pro-inflammatory cytokines in response to stimulation with imidazoquinolines via the toll-like receptor 7-MyD88-dependent signalling cascade [446].

### 3.5. Improvement of PQ peptidomimetic and organometallic derivatives

In sum, we have found one peptidomimetic (3c) and one organometallic (7a) derivative of PQ to be active against the intramacrophagic amastigote form of *L. infantum* that did not display apparent toxicity to the host cell. Therefore, we propose that these derivatives might be good scaffolds for the development of efficacious and safer PQ-based anti-leishmanials. We envision that introduction of substituents at the quinoline ring of 3c or 7a may further improve their anti-leishmanial action, while keeping their safety. For instance, the insertion of a 2-tert-butyl group together with a 3-trifluoromethylphenoxy group at the C-2 and C-5 positions of the PQ quinoline ring, respectively, has greatly improved the activity of PQ against *L. donovani* promastigotes, while maintaining its safety [447]. It may also be worthy to explore the effect of incorporating the substituents 3-trifluoromethylphenoxy, 3,4-dichlorophenoxy or prop-2-en-1-ol into the C-5 or C-2 positions of the quinoline ring of our PQ derivatives. In fact, these groups are present in tafenoquine [80], NPC1161B [81] and 2-quinoline [448], respectively, which are oral agents with potent *in vivo* anti-leishmanial activity and limited toxicity.
Chapter VII

Concluding Remarks
Chapter VII | Concluding Remarks

The main conclusions resulting from the work disclosed in this thesis were the following:

1| While dietary iron deficiency did not affect *L.infantum* growth, iron overload inhibited its replication in the host tissues likely via interaction with ROS and RNS. Furthermore, iron exerted direct inhibitory action, in a dose dependent fashion, towards axenic cultures of *L.infantum* promastigotes and amastigotes (chapter III).

2| The phagocyte NADPH oxidase, not NOS2, seems to be the critical host effector mechanism involved in the control of *L.infantum in vivo* (chapter IV).

3| The screening of several derivatives of PQ, an 8-AQ drug with modest antileishmanial activity, revealed a peptidomimetic (imidazolidin-4-one) and an organometallic (ferrocene) derivative which were active against the clinically relevant intramacrophagic amastigote stage of *L.infantum*, while displaying less cytotoxicity towards macrophages than VL reference drugs (chapter V).

With this work, we intended to contribute to a better understanding of the host mechanisms that govern resistance to *Leishmania* and to the development of more efficacious and safer chemotherapeutics to treat infections caused by these protozoa.
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Appendix

Publications And Communications In Scientific Meetings
Appendix | Publications And Communications In Scientific Meetings

1. Publications

1.1. Thesis related


1.2. Collaboration related


2. Communications in scientific meetings


Vale-Costa S, Pereira SG, Teixeira CM, Tomás A, Appelberg R, Gomes MS (2008) “Iron overload favours the elimination of *Leishmania infantum* from mouse tissues by enhancing the production of reactive oxygen and nitrogen species”, (oral communication), Workshop “Immunoregulation in infectious diseases: from immunity to bugs and back”, School of Health Sciences, Life and Health Sciences Research Institute, University of Minho, Braga, Portugal.

Vale-Costa S, Pereira SG, Teixeira CM, Tomás A, Appelberg R, Gomes MS (2009) “Iron overload favours the elimination of *Leishmania infantum* from mouse tissues through the production of reactive oxygen and nitrogen species”, (poster), XXXV Annual Meeting of the Portuguese Society of Immunology, Instituto de Medicina Molecular, Lisbon, Portugal.

Vale-Costa S, Tomás A, Appelberg R, Gomes MS (2010) “Role of reactive oxygen and nitrogen species in the infection by *Leishmania infantum* in the mouse”, (poster), XXXVI Annual Meeting of the Portuguese Society of Immunology, School of Health Sciences, Life and Health Sciences Research Institute, University of Minho, Braga, Portugal.


3. Awards