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The cytosolic hydroperoxide detoxification system of *Leishmania infantum*

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Preface

The dissertation entitled *The cytosolic hydroperoxide detoxification system of Leishmania infantum* is organized into 5 chapters. Chapter 1 consists in a general introduction to the subject and presents the aims of the research project. Chapter 2, 3 and 4 present the original results obtained in this study, structured as scientific articles. Finally, chapter 5 concerns a general discussion and final considerations of the work. The publications that resulted from this research project, communications in international scientific meetings, as well as, other collaboration works are listed in Appendix 1. Having in mind the importance of communicating science to the general public, my participation in scientific promotion projects is also listed in Appendix 1. In addition, Appendix 2 presents a compilation of interviews with leishmaniasis researchers, physicians, veterinarians and patients, in a simple approach to the disease and scientific research.

Em memória de Josué, que me ensinou a resistir

Aos meus pais, a quem devo tudo o resto

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Summary

Leishmania infantum is a protozoan parasite that can infect macrophages from different mammalian species causing visceral leishmaniasis. One distinctive feature between *Leishmania* and their hosts is their thiol-based metabolism, which in the parasites largely depends on an unique dithiol conjugate known as trypanothione. The need to maintain redox homeostasis is a constant during the lifetime of any cell, although in the case of *Leishmania* this is perhaps even more relevant since they can be exposed to powerful macrophage-derived oxidants. Thus, antioxidant defense might be determinant for the successful establishment of *L. infantum* infection. In this matter, cytosolic antioxidant enzymes are probably very suitably located to deal with the oxidative attack derived from an exogenous origin. In this thesis we particularly focused on the cytosolic trypanothione-dependent system for hydroperoxide detoxification that includes the oxidoreductases tryparedoxin (TXN) and classical 2-cysteine peroxiredoxin (2-Cys PRX). We demonstrated that the cytosolic tryparedoxin (*Li*TXN1) is essential for the survival of *L. infantum* throughout their life cycle, i.e. both the promastigote and the disease-causing amastigote stages depend on *Li*TXN1 expression to survive. Furthermore, we showed that *L. infantum* can upregulate the expression of *Li*TXN1 from a single allele, suggesting that the parasites require more than 50% of wild-type content of the enzyme to sustain normal development. Besides the cytosolic *Li*TXN1, *L. infantum* possess a redox-active mitochondrial enzyme (*Li*TXN2) and also two other *TXN*-like sequences with the potential to encode functional proteins. Here we also described the characterization of these *L. infantum* TXN-like proteins (*Li*TXN3 and *Li*TXN5), evidencing that they cannot function as classical TXN enzymes. Moreover, we showed that *Li*TXN3 is associated to the mitochondrial membrane and *Li*TXN5 to the endoplasmic reticulum membrane. Together our findings clearly show that in *L. infantum* no other molecule can functionally substitute

for *Li*TXN1 and also that no other TXN-like protein is capable of substituting *Li*TXN2 in reduction of mitochondrial 2-Cys PRX.

In the trypanothione/TXN antioxidant pathway classical 2-Cys PRXs are the enzymes that directly react with hydroperoxides. In other organisms besides reducing hydrogen peroxide and small chain organic hydroperoxides, 2-Cys PRXs also display peroxynitrite reductase activity. This study supports that the same is true for both *L. infantum* cytosolic 2-Cys PRXs (*Li*TXNPx1 and *Li*TXNPx2). To investigate if increased infectivity is directly linked to the capacity of the parasites to cope with macrophage-derived reactive oxygen and nitrogen species, we used *L. infantum* lines overexpressing one of the cytosolic 2-Cys PRX (*Li*TXNPx2) in ex vivo infection assays. Our results provide evidence that upregulation of *Li*TXNPx2 enhanced the parasites capacity to survive inside two different types of host cells, murine and human macrophages. This suggests that indeed the antioxidant defense of these parasites might be a significant factor in the infectious process.

Resumo

Leishmania infantum é um protozoário que pode infectar macrófagos de diferentes espécies de mamíferos, causando leishmaniose visceral. Uma das características que distingue *Leishmania* das células dos seus hospedeiros são os mecanismos baseados nas reacções com tióis, que nos parasitas dependem principalmente de um ditiol designado por tripanotiono. A necessidade de manter a homeostase redox é uma constante durante a vida de qualquer célula, apesar de no caso de *Leishmania* isto ser porventura ainda mais relevante, uma vez que, estes parasitas podem ser expostos a oxidantes poderosos derivados dos macrófagos. Assim, os sistemas de defesa antioxidante de *L. infantum* poderão ser determinantes para o efectivo estabelecimento de uma infecção. Nesta matéria, as enzimas citosólicas antioxidantes estão favoravelmente localizadas para lidar com o ataque oxidativo originado a partir de uma fonte exógena. Nesta tese focámos particularmente o sistema citosólico para a eliminação de hidroperóxidos dependente do tripanotiono que inclui as oxidoreductases: triparredoxina (TXN) e peroxirredoxina clássica de 2-cisteínas (2-Cys PRX). Demonstrámos que a triparredoxina citosólica (*Li*TXN1) é essencial para a sobrevivência de *L. infantum* ao longo do seu ciclo de vida, i.e. tanto o promastigota como o agente causador da doença, o amastigota, dependem da expressão de *Li*TXN1 para sobreviver. Para além disso, mostrámos que *L. infantum* consegue sobre-expressar a proteína *Li*TXN1 a partir de um só alelo, sugerindo que os parasitas necessitam de mais de 50% do conteúdo wild-type desta enzima para garantir o seu normal desenvolvimento. Além da enzima citosólica *Li*TXN1, *L. infantum* apresenta uma enzima mitocondrial redox-activa (*Li*TXN2) e também duas outras sequências idênticas a *TXNs* com a potencialidade de codificar proteínas funcionais. Esta tese descreve também a caracterização destas proteínas semelhantes a TXNs (*Li*TXN3 e *Li*TXN5), evidenciando que estas moléculas não operam como TXNs clássicas. Mostrámos ainda que

*Li*TXN3 se encontra associada à membrana mitocondrial e que *Li*TXN5 está associada à membrana do retículo endoplasmático. Em conjunto os nossos resultados indicam que em *L. infantum* não existe outra molécula que possa substituir funcionalmente *Li*TXN1 e também que nenhuma outra TXN é capaz de substituir a enzima *Li*TXN2 na redução da 2-Cys PRX mitocondrial.

As 2-Cys PRXs clássicas são as enzimas que directamente reagem com os hidroperóxidos na via antioxidante dependente de tripanotiono e TXN. Noutros organismos as 2-Cys PRXs para além de reduzirem peróxido de hidrogénio e outros hidropéroxidos, apresentam igualmente actividade de reductase de peroxinitrito. Este estudo sustenta que o mesmo se aplica a ambas as 2-Cys PRXs citosólicas (*Li*TXNPx1 e *Li*TXNPx2). Para investigar se o aumento da infectividade está directamente relacionado com a capacidade dos parasitas lidarem com espécies reactivas de oxigénio e nitrogénio provenientes dos macrófagos, usámos linhas de *L. infantum* que sobre-expressavam uma 2-Cys PRX citosólica (*Li*TXNPx2) em ensaios de infecção ex vivo. Os nossos resultados evidenciam que a sobre-expressão de *Li*TXNPx2 aumenta a capacidade de sobrevivência dos parasitas em dois tipos de células hospedeiras diferentes, macrófagos murinos e humanos. Isto sugere que de facto a defesa antioxidante destes parasitas poderá ser um factor preponderante no processo de infecção.

Résumé

La *Leishmania infantum* est un protozoaire qui peut infecter les macrophages de différentes espèces de mammifères, provoquant la leishmaniose viscérale. Une des caractéristiques qui distingue les *Leishmania* des cellules de ses hôtes est un mécanisme basé sur les réactions par rapport aux thiols, qui dans les parasites dépendent surtout d'un dithiol nommé trypanotione. La nécessité de conserver l'homéostasie redox est une constante de la vie de toutes les cellules, dans le cas des *Leishmania* ceci est d'autant plus notable, étant donné son exposition aux oxydants dérivés des macrophages. Ainsi, les systèmes de défense antioxydant des *L. infantum* seraient déterminants dans établissement effectif d'une infection. Les enzymes cytosoliques antioxydants sont localisés favorablement pour faire face à l'attaque oxydative venant d'une source exogène. Dans cette thèse nous abordons particulièrement le système cytosolique pour l'élimination d'hydroperoxydes dépendant du trypanotione qui inclut les oxydoréductases: tryparedoxine (TXN) et peroxyredoxine classique de 2-cystéines (2-Cys PRX). De plus, nous avons démontré que la tryparedoxine cytosolique (*Li*TXN1) est essentiel pour la survie de la *L. infantum* tout au long de son cycle de vie, i.e. tant pour le promastigote que pour l'agent causant la maladie, l'amastigote, qui dépendent de l'expression de *Li*TXN1 pour survivre. De plus nous avons montré que *L. infantum* peuvent produire une sur-expression de la protéine *Li*TXN1 à partir d'un seul allèle, évoquant que les parasites ont besoin de plus que 50% du contenu wild-type de cette enzyme pour assurer leur développement normal. En plus de l'enzyme cytosolique *Li*TXN1, *L. infantum* présente une enzyme mitochondriale redox-active (*Li*TXN2) ainsi que deux autres séquences identiques aux *TXN*s avec le potentiel à codifier des protéines fonctionnelles. Cette thèse décrit la caractérisation de ces protéines similaires aux TXNs (*Li*TXN3 et *Li*TXN5), en mettant en évidence que ces molécules ne fonctionnent pas comme des TXNs classiques.

Nous avons aussi montré que *Li*TXN3 se trouve liée à la membrane mitochondriale et que *Li*TXN5 est associée à la membrane du réticulum endoplasmique. L'ensemble de nos résultats indique que, au sein de *L. infantum,* il n'y a pas une autre molécule qui puisse remplacer fonctionnellement *Li*TXN1, mais également qu'aucune autre TXN n'est capable de substituer l'enzyme *Li*TXN2 pour la réduction de la 2-Cys PRX mitochondriale.

Les 2-Cys PRXs classiques sont les enzymes qui réagissent directement avec les hydroperoxydes dans la voie antioxydante dépendante de trypanotione et TXN. Dans d'autres organismes les 2-Cys PRXs, en plus de réduire le peroxyde d'hydrogène et d'autres hydroperoxydes, présentent également une activité de réductase de peroxynitrite. Cette étude soutient que le même mécanisme s'applique aux deux 2-Cys PRXs cytosoliques (*Li*TXNPx1 et *Li*TXNPx2). Pour analyser si l'augmentation de l'infectiosité est directement liée à la capacité des parasites à faire face aux espèces réactives de l'oxygène et de l'azote provenant des macrophages, nous avons utilisé les lignes de *L. infantum* qui sur-expriment une 2-Cys PRX cytosolique (*Li*TXNPx2), ceci au cours d'essais d'infection ex vivo. Nos résultats mettent en évidence que la sur-expression de *Li*TXNPx2 augmente la capacité de survie des parasites dans deux types de cellules hôtes différentes, des macrophages murins et humains. Cela suggère que les défenses antioxydantes de ces parasites pourraient effectivement être un élément prépondérant dans le processus d'infection.

Chapter 1

General introduction

1. Leishmaniasis

1.1. Historical notes

The history of parasitology blends necessarily with the one of tropical medicine. This medical field, created by imperialist policies to protect the colonizers against unknown diseases in tropical and subtropical regions, was responsible for a huge scientific advance on tropical pathogen comprehension (Farley, 1991; Cox, 2002). Parasitic diseases are also linked to human evolution, meaning that much of the present facts of parasitology, such as parasite distribution in the world, were shaped by mankind.

After the establishment of Pasteur's germ theory of disease, at the end of the 19th century, many pathogens were identified and related to a specific disease. This was the case of *Leishmania* parasites that were first seen in 1901 by William B. Leishman, a British army medical officer. However, his discovery of oval bodies invading the spleen tissue of a patient who died from kala-azar, the Hindi word for black fever, did not immediately lead to the recognition of a new parasite species. In 1903, Charles Donovan who worked for the Indian Medical Service, made similar observations and defined the round bodies has being a novel sort of organism. In that same year, the physician Ronald Ross ultimately determined that the agent of kala-azar was a new parasitic genus, which he named *Leishmania* (www.who.int/leishmaniasis). Still, 40 more years were needed to understand the global mechanisms of parasite transmission (Cox, 2002).

Naturally, *Leishmania* infections existed long before the formal designation of the infectious agent as *Leishmania* spp. and the disease as leishmaniasis. Ancient documents, paintings and sculptures, in some cases dating back to 2500 B.C., illustrate the presence of what is now believed to be leishmaniasis lesions or symptoms in Old and New World civilizations (Elliott, 1863; Manson-Bahr, 1996; www.who.int/leishmaniasis). At present,

the World Health Organization reports on leishmaniasis estimate that 12 million people are infected with different *Leishmania* species, in approximately 88 countries (www.who.int/leishmaniasis). We can, therefore, conclude that *Leishmania* parasites and humans have been probably co-evolving for a very long period of time.

1.2. Clinical manifestations and treatment

The leishmaniasis are a complex of diseases caused by several *Leishmania* species with different clinical manifestations. Typically human leishmaniasis can be grouped into 3 major forms: cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL) (reviewed in Desjeux, 2004).

Cutaneous leishmaniasis is the most common form of the disease and causes non-lethal skin lesions or ulcers that might be localized or spread all over the body (diffuse CL). In many cases the lesions heal spontaneously, however, almost always leaving permanent scars. Mucocutaneous leishmaniasis is characterized by lesions at the mucosal regions, which might cause extensive facial disfiguring and severe disability. Contrary to CL lesions, mucosal ulcers do not self-heal and treatment success depends primarily on the severity of the infection. The first choice treatment for MCL are antimony salts, although other drugs such as amphotericin B, pentamidine and allopurinol might also be used. The clinical response is satisfactory, with approximately 70% of cure (Amato et al., 2007).

Visceral leishmaniasis, known as kala-azar in India, is the most severe manifestation of the disease and the main responsible for the 50.000 annual deaths by leishmaniasis (www.who.int/leishmaniasis). It is a systemic infection, in which the parasites invade vital organs (spleen, liver, bone marrow and lymph nodes) eventually causing their failure. The symptoms vary between individuals, which can present

intermittent fever, abdominal pain, diarrhea, anemia, cachexia, pancytopenia and hepato-splenomegaly (Chappuis et al., 2007). For over 80 years, first-line treatment for VL has been based on antimony compounds (VL treatments reviewed in Sundar and Chatterjee, 2006). Antimonals are, however, very toxic and in northeastern India and in some regions of Sudan, they are no longer effective due to parasite drug resistance. Alternative chemotherapeutics applied include pentamidine and amphotericin B. Nevertheless, pentamidine also revealed to be extremely toxic and its efficacy has been diminishing over the past years. On the contrary, the antifungal amphotericin B revealed high efficacy against *Leishmania* infection (>95% cure rates) and its lipid formulation is far less toxic than any of the above-mentioned leishmanicidal drugs. One of the limiting factors for a broader use of liposomal amphotericin B is the price, since it can be 30 times more expensive than the antimonial treatment. Since most of the leishmanicidal compounds are in the form of injectable drugs, the development and application of oral chemotherapeutic agents is a very important field in leishmaniasis therapy. So far, miltefosine is the only oral leishmanicidal compound approved. Its relatively low toxicity, high efficacy (>94% cure rates) together with an easier administration represents a substantial improvement in the combat against the disease. Even though no cases of resistance have been reported in Indian patients, miltefosine's prolonged half-life (Sundar and Chatterjee, 2006) and parasite resistance in culture (Perez-Victoria et al., 2003) raises some apprehension. Despite many attempts to develop a vaccine against human VL, these have up to now remained unfruitful (leishmaniasis vaccines reviewed in Palatnik-de-Sousa, 2008). However, promising results are currently coming out with Leishmune®, a vaccine for canine leishmaniasis licensed in 2003 in Brazil, with both protective and transmission blocking activities (Saraiva et al., 2006).

1.3. Epidemiology of the disease

The *Leishmania* genus comprises a number of species complexes with distinct features including world distribution, insect vector, reservoir, vertebrate host and pathology (Table 1). In the Old World the main species complexes are *L. tropica*, *L. major*, *L. aethiopica*, *L. donovani* and *L. infantum* and are transmitted by sandflies of the genus *Phlebotomus* (Bryceson, 1996). *Leishmania major* is the main responsible for CL in northern Africa, Middle East, Arabian Peninsula, Kenya, Sudan and India. It presents zoonotic transmission, made mainly through rodent reservoirs. *Leishmania tropica* is also an agent of CL however, it is fundamentally of urban areas and probably restricted to humans. It is present in Greece, Serbia, Romania, Turkey, Afghanistan, northern Africa, Middle East and some regions of western Asia. *Leishmania aethiopica* can be found in Ethiopia, Kenya and Uganda. It can cause diffuse CL and is maintained in rodents and cattle. *Leishmania infantum* and *L. donovani* are causative agents of VL. In the Mediterranean basin, northern Africa, central Asia and northwest China infections are caused by *L. infantum* and dogs represent their main reservoirs. *Leishmania donovani* is present in India, Pakistan, west of China, Sudan, Somalia, Ethiopia and Kenya. The *L. donovani* transmission is essentially anthroponotic, although rodents and cattle might also function as parasite reservoirs (Lainson and Shaw, 1987; Bryceson, 1996).

In the New World the only known agent for VL is *L. chagasi*. Different approaches show that *L. chagasi* cannot be clearly distinguished from *L. infantum* at the species level (Momen et al., 1987; Maurício et al., 1999), for this reason some authors divided the two parasites into two subspecies, *L. infantum chagasi* and *L. infantum infantum* (Lainson and Rangel, 2005). *Leishmania chagasi* is present in Central and South America, fundamentally in rural areas where dogs are the main reservoir. The New World CL can be originated by *L. mexicana*, *L. amazonensis*, *L. braziliensis*, among others (Grimaldi and

Tesh, 1993). *Leishmania mexicana* spreads from the south of USA to Colombia while *Leishmania amozonensis* is more restricted to South America. *Leishmania braziliensis* is also responsible for an important number of MCL cases in Central and South America. All three species have rodents as the most important reservoir. New World *Leishmania* spp. are essentially transmitted by *Lutzomyia* sandflies.

Table 1. Summary of the epidemiology of leishmaniasis

	Species	Disease type	Geographical distribution	Insect hosts	Mammalian hosts
Old World	*L. infantum*	VL	Mediterranean regions, northern Africa, central Asia and northwest China	*Phlebotomus* spp.	Dogs and humans
	L. donovani	VL	India, Pakistan, west of China, Sudan, Somalia, Ethiopia and Kenya		Humans, rodents and cattle
	L. major	CL	Northern Africa, Middle East, Arabian Peninsula, Kenya, Sudan and India		Rodents
	L. tropica	CL	Mediterranean regions, Afghanistan, northern Africa, Middle East, India and Pakistan		Humans
	L. aethiopica	CL	Ethiopia, Kenya and Uganda		Rodents
New World	*L. chagasi* *	VL	Central and South America	*Lutzomyia* spp.	Canids and humans
	L. mexicana	CL	Central and South America and south regions of USA		Rodents
	L. amazonensis	CL	South America		Rodents and marsupials
	L. braziliensis	MCL	Central and South America		Canids and horses

**L. chagasi* is synonymous to *L. infantum*. *L.*, *Leishmania*; VL, Visceral Leishmaniasis; CL, Cutaneous Leishmaniasis; MCL, Mucocutaneous leishmaniasis. Only the most common species are referred in the table. (Lainson and Shaw, 1987; Grimaldi and Tesh, 1993; Bryceson, 1996; Dedet and Pratlong, 2000)

2. *Leishmania* parasites

2.1. Taxonomy and evolution

Parasitic protozoa represent an important group of human and animal eukaryotic pathogens including diverse genera of microorganisms such as *Giardia*, *Trichomonas*, *Plasmodium*, *Trypanosoma* and *Leishmania*. The *Leishmania* and *Trypanosoma* genera belong to the same order (Kinetoplastida) and family (Trypanosomatidae), which are included in the Protozoa phylum (Sarcomastigophora subphylum), a large group of flagellated protozoans evolutionarily well separated from other eukaryotic groups. Within this phylum, the trypanosomatids are a late-emerging group of strictly parasitic uniflagellated protozoans. Trypanosomatid phylogenies based on small subunit ribosomal RNA present a division of the group into two different clades, one comprising the trypanosomes and another *Leishmania*, *Crithidia*, *Leptomonas*, *Herpetomonas*, *Phytomonas* and *Blastocrithidia*. Initial analysis estimated that trypanosomes and the *Leishmania*/*Crithidia* branch split approximately 100 million years (My) before present (Lake et al., 1988). However, more recent studies proposed an earliest division, around 300 to 600 My ago, suggesting that *Trypanosoma* and *Leishmania* genera might present different evolutionary histories, being more distantly related than it was originally considered (Overath et al., 2001; Stevens et al., 2001; Simpson et al., 2002).

The origin of the genus *Trypanosoma* has been for long a matter of debate. Some phylogenetic reconstructions based on ribosomal RNA sequences showed that *Trypanosoma brucei* and *Trypanosoma vivax* fell outside the groups of all other trypanosomes, supporting, therefore, a paraphyletic origin (Maslov et al., 1996; Hughes and Piontkivska, 2003). Other authors, using both ribosomal RNA sequences and glyceraldehyde phosphate dehydrogenase genes, concluded that the *Trypanosoma* genus is

monophyletic, even though Salivarian parasites (e.g. *T. brucei*, *T. vivax*, *T. congolense*) clearly branched off earlier, supporting a higher divergence of this group towards all other non-Salivarian trypanosomes (Haag et al., 1998; Stevens et al., 2001; Hamilton et al., 2004).

The *Leishmania* and *Crithidia* genera diverged after the *Trypanosoma* genus separation, probably relatively recently (Kerr, 2000). It is broadly well accepted that *Leishmania* species had a single evolutionary ancestral, i.e. they have monophyletic origin (Thomaz-Soccol et al., 1993; Stevens et al., 2001). Their ancestral geographical origin is however less consensual. In this respect, two hypothesis have been proposed to the origin of the genus, one defending New World or Neotropical origin (Noyes, 1998) and another Old World or Paleartic origin (Kerr, 2000). Consequently, speculations about the evolutionary relationship of the different *Leishmania* species will depend on the author's viewpoint. We can however analyze the proximity between species in terms of enzyme electrophoretic profiles, DNA restriction fragment patterns or DNA sequencing. For example, *L. infantum* and *L. chagasi* are currently considered to be synonymous, which indicates a very recent separation of these species (Maurício et al., 2000; Dantas-Torres, 2006). Based on the phylogeny of the genes encoding the glyceraldehyde phosphate dehydrogenase and the RNA polymerase II, the estimated divergence time of the visceralizing species *L. infantum* and *L. donovani* is approximately of 1 My (Lukes et al., 2007). In contrast, the *L. major* species probably diverged from the *L. donovani*/*L. infantum* complex approximately 20 My before present (Lukes et al., 2007). There is also a higher genetic distance between *L. major* and *L. braziliensis* than between *L. major* and *L. donovani*/*L. infantum*, probably reflecting a more ancient geographical separation (Momen and Cupolillo, 2000; Peacock et al., 2007).

In spite of the phenotypic differences between distinct *Leishmania* species, namely in what concerns disease symptoms and pathogenicity, chromosome synteny is maintained for more than 99% of the genes, amino acid identity is higher than 77% and very few genes are species-specific (analysis between *L. major*, *L. braziliensis* and *L. infantum* in Peacock et al., 2007). Interestingly, most of the genetic differences observed between *Leishmania* species are associated to pseudogene formation or gene loss rather than emergence of new genes. There are some examples for this in the literature, for instances, cysteine peptidase *PFP1*-like genes that are expressed in *L. major* but not in *L. mexicana*, *L. braziliensis* or *L. infantum* (Eschenlauer et al., 2006) and a putative phosphatidylcholine transfer protein SEC14 factor, encoded by a *L. infantum* gene, which is a pseudogene in *L. braziliensis* and is absent from the *L. major* genome (Peacock et al., 2007). In this respect, in Chapter 3 we discuss the emergence of *Leishmania*-specific tryparedoxin (*TXN*) genes and analyse the possibility of evolution of dysfunctional sequences in *L. infantum*. Besides some genetic variation, gene translation levels seem to fluctuate significantly within the *Leishmania* genus, which means that the specificity of each species might depend essentially on their protein expression patterns rather than on their genetic differences.

2.2. Biology

Kinetoplastids present typical eukaryote organelles, as for example, nucleus and nucleolus, endoplasmic reticulum, mitochondria and lysosomes. However, these organisms exhibit some characteristics that distinguish them from other eukaryotes, including specific metabolic pathways and organelles. One of their unique features is the presence of a mass of circular DNA molecules inside the single mitochondrion. This structure called the kinetoplast consists of two components, the maxicircles, which encode mitochondrial

genes, and the minicircles, present in higher number and apparently coding only for small guide RNAs implicated in RNA editing (Simpson et al., 2002). These protozoans present further biological differences towards other eukaryotes. Chromosome condensation is not detectable during cell cycle, most genes do not include introns or promoters and transcription occurs as large polycistronic units, which need trans-splicing and polyadenylation to generate individual messenger RNAs (reviewed in Stiles et al., 1999). Trypanosomatids, in particular, are characterized by having considerable genome plasticity that can be exemplified by their capacity to vary chromosome size and number or to amplify specific regions forming amplicons and minichoromosomes (Cruz et al., 1993; Britto et al., 1998; Genest et al., 2005).

Another important difference between kinetoplastids and other eukaryotes is the compartmentation of most of the glycolytic enzymes inside specific organelles named glycosomes. These spherical microbodies are rather numerous in the cell and are structurally similar to peroxisomes, an organelle that is absent in kinetoplastids. The confinement of the first part of the glycolytic pathway to a membrane-enclosed compartment is believed to be important to prevent an excessive ATP consumption, thus regulating glucose degradation in a distinctive manner (Bakker et al., 2000). Apart from glycolysis, other enzymatic activities are associated with glycosomes, namely pyrimidine biosynthesis, fatty acid oxidation and the purine salvage pathway (reviewed in Michels et al., 2000). Interestingly, some organelles that were thought to be specific to kinetoplastids were later described in other organims. An example of these are the acidocalcisomes, acidic organelles, which store cations and phosphates that recently were identified in prokaryotic cells (Seufferheld et al., 2003) and in human platelets (Ruiz et al., 2004). In kinetoplastids, these structures can be linked to calcium homeostasis, osmoregulation, cell signaling and pH control (Docampo et al., 2005).

The antioxidant defense system of trypanosomatids has also particularities that have been a matter of interest for parasitologists and other researchers. The most striking difference is the substitution of glutathione by trypanothione (a glutathione-spermidine conjugate) in vital redox pathways. One of these is hydroperoxide metabolism that in trypanosomatids is largely assured by trypanothione-dependent enzymes. Furthermore, trypanosomatids lack classic eukaryotic antioxidant enzymes, such as catalase or selenocysteine-containing glutathione peroxidases, and they also do not possess glutathione reductase and thioredoxin reductase (reviewed in Castro and Tomás, 2008; Krauth-Siegel and Comini, 2008). Since antioxidant defense is the core subject of this dissertation, it will be later detailed in this introduction chapter.

2.3. Life cycle

Trypanosomatids are digenic pathogens, which alternate between different hosts and differentiation stages to complete their life cycles. Depending on the geographical area, *Leishmania* spp. parasitize sandflies of the genus *Phlebotomus* or *Lutzomyia,* as extracellular flagellated promastigotes. The intracellular non-motile amastigotes develop inside phagocytic cells of vertebrate hosts, predominantly mammalians (rodents, marsupials, sloths, canids, cattle, horses, primates and humans).

The *Leishmania* life cycle is schematically represented in Figure 1. During the insect blood meal in an infected vertebrate host, the sandfly ingests parasitized macrophages and the amastigotes are released in the insect midgut, where they rapidly transform into promastigotes. Throughout a period of 6 to 10 days promastigotes undergo a series of replications and differentiations, which culminate in their migration to the foregut as infective metacyclic promastigotes. When the sandfly next feeds on a vertebrate host,

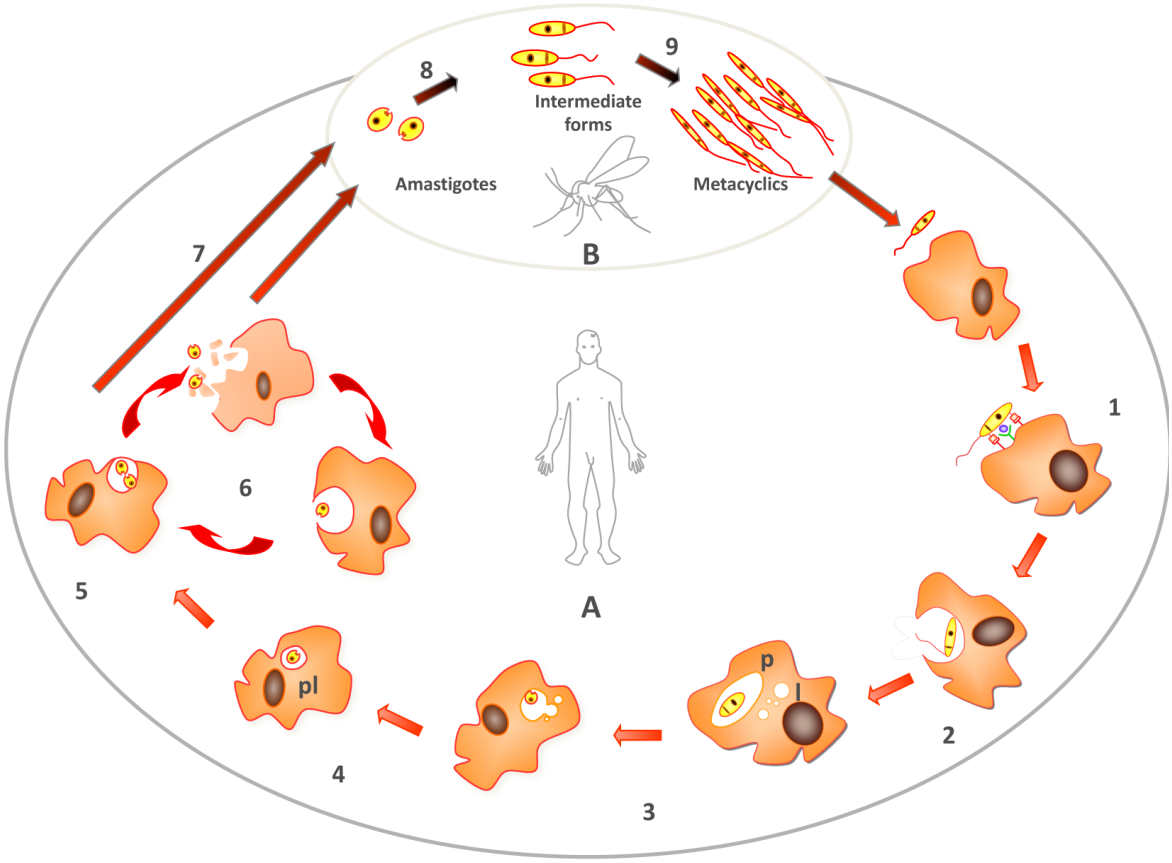


Fig. 1. The *Leishmania* life cycle. Once inside the vertebrate host (A) metacyclic promastigotes are internalized by phagocytes through receptor-mediated phagocytosis (1 and 2). The promastigote-containing phagosome (p) fuses with lysosomes (l) forming the phagolysosome (pl), where promastigotes differentiate into amastigotes (3 and 4), which then replicate (5), and invade new cells (6). During a blood meal, the insect host (B) ingests parasitized macrophages (7) and amastigotes are released into the insect midgut wherein they revert to the promastigote stage (8). Promastigotes go through a number of differentiation phases that culminate in the development of the infective metacyclic forms (9). Adapted from Tomás and Romao (2009).

metacyclic promastigotes are injected into the bite wound together with insect saliva (Sacks and Perkins, 1985; Alexander et al., 1999; Bates and Rogers, 2004). Once in the host skin, promastigotes are taken up by phagocytic cells namely, neutrophils, dendritic cells (DCs) and macrophages. The latter are considered to be *Leishmania* final host cells, since in an infected organism, macrophages are the most frequent parasite-harboring cells. After phagocytosis, promastigotes revert into amastigotes inside phagolysosomes, compartments that result from the fusion of phagosomes with lysosomes. It is within these acidic vacuoles, enriched with hydrolytic enzymes, that *Leishmania* amastigotes proliferate. Multiplying amastigotes spread to new cells through the burst of infected

macrophages or by exocytosis or, also, as a result of phagocytosis of infected cells by new macrophages. Finally, after a new sandfly bite, infected cells enter the insect host and the cycle is completed.

The promastigote-amastigote reversion involves a series of drastic modifications, including significant morphological and physiological changes. Among these is the loss of the flagellum, the acquisition of a different cellular shape, alteration of cell surface components and of protein expression patterns. This process is the starting point for the adaptation to the vertebrate host and can be, to a certain point, mimicked in the laboratory (Zilberstein and Shapira, 1994). Thus, it is possible to cultivate the parasite insect stage and revert it to axenic amastigotes and vice-versa.

2.4. Immune response

The outcome of *Leishmania* infections depends on parasite species or strain, host genetic background, host immune status, number of inoculated parasites and route of inoculation (Honoré et al., 1998; Rolão et al., 2004). Control of the infection relies on non-specific innate immune responses, initiated promptly after parasite invasion, and on adaptive immune responses by means of T-cell mediation (Laskay et al., 1995; Bogdan and Rollinghoff, 1998). Experimental infections using murine models have been extensively investigated, particularly CL caused by *L. major* in which the immune response is clearly bipolarized between expansion of Th1 or Th2 cell subsets. In this case, Type 1 response (Th1) is correlated with disease resistance and with production of interleukin 12 (IL-12) and interferon-γ (IFN-γ), whereas Type 2 response (Th2) is linked to a phenotype of susceptibility to infection and to IL-4, IL-10 and IL-13 secretion (Gumy et al., 2004 and Fig. 2). In recent years, visceralizing *Leishmania* species have been shown to induce

different immunological response patterns, presenting tissue-specific immunity and lacking Th1/Th2 dichotomy in experimental animal models (Wilson et al., 2005). Interestingly, and in contrast to *L. major* infection, susceptible mouse strains (e.g. C57BL/6, BALB/c) resolve *L. donovani* and *L. infantum* infections spontaneously over time. Moreover, *L. donovani* and *L. infantum* present tissue tropism, replicating and expanding differently through the infected organs (Wilson et al., 2005). These observations indicate that immune response to *Leishmania* parasites might vary depending on parasite location in the mammalian host (Engwerda and Kaye, 2000). Also, it is noteworthy that in the case of *L. infantum* the spleen seems to be a more susceptible organ and infected animals present a mixed Th1/Th2 immune response with simultaneous production of IFN-γ, IL-4 and IL-10 (Rolão et al., 2007). Other study revealed that higher parasite loads correlate with TGF-β production, low levels of Th2 cytokines and absence of Th1 response (Gomes-Pereira et al., 2004). Altogether, research on *L. infantum* murine infection suggests that differential production of Th1 and Th2 cytokines cannot fully explain the disease outcome. However, in view of the present knowledge, cure phenotypes of CL and VL are invariably linked to IFN-γ production, thus correlating Type 1 response to resistance to infection, while susceptibility does not always rely on Type 2 cytokines (Honoré et al., 1998; Wilson et al., 2005).

3. *Leishmania* survival strategies inside the macrophage

3.1. General strategies

Macrophages are central cells of the immune system, responsible for the detection and elimination of pathogens and also for the initiation of an immune response against invading microorganisms. Amongst the diverse macrophage microbicidal mechanisms four

different strategies may be taken as the most relevant for pathogen destruction: i) vacuole acidification, ii) phagosome enrichment in lysosomal enzymes, iii) restriction of essential nutrients/cations and iv) production of highly reactive oxidants (detailed in section 3.2. of this chapter). In terms of the development of an effective immune response, macrophages can present antigens of intracellular pathogens through the major histocompatibility complex (MHC) class II, leading to $CD4^+$ helper T cells proliferation and differentiation with consequent microbicidal activities. Or, alternatively, macrophages present *Leishmania* antigens through MHC class I molecules, resulting in the $CD8^+$ cytotoxic T cell activation. In addition, macrophages may secrete a number of pro- and anti-inflammatory cytokines, which, depending on the type and the target-cell will stimulate immune cell proliferation and differentiation, antibody secretion, chemotaxis and/or cell death. Many pathogens like *Leishmania*, *Coxiella*, *Toxoplasma*, *Mycobacterium*, *Salmonella* and *Legionella* have, nevertheless, developed sophisticated mechanisms to survive inside macrophage vacuoles (Amer and Swanson, 2002). However, it is noteworthy that only *Leishmania* spp. and the bacterium *Coxiella burnetti* can actually replicate inside mature phagolysosomes (Naderer and McConville, 2008).

In general, the infection outcome will be the result of a very dynamic pathogen-host interaction, where both have evolved survival and adaptation tools. In this matter, *Leishmania* strategies for survival within the macrophage can be regarded as an example for intracellular parasitism as detailed next. The cell surface components of *Leishmania* metacyclic promastigotes are crucial to secure their fate in the initial steps of the infection, particularly the glycosylphosphatidylinositol (GPI)-anchored lipophosphoglycan (LPG) and glycoprotein 63 (GP63). GP63 is a zinc metalloprotease, which is generally upregulated in stationary-phase promastigotes (Schneider et al., 1992; Streit et al., 1996). This glycoprotein has been implicated in parasite-macrophage binding in two ways. First,

since it contains the specific fibronectin-like motif Ser-Arg-Tyr-Asp, it allows the parasite to enter the phagocytes directly through their integrin receptor (Soteriadou et al., 1992). Second, GP63 might indirectly attach to macrophage complement receptors, in an enzymatic-depend manner. Thus, GP63 catalyzes the cleavage of host complement factor C3b into an inactive form, iC3b, which bridges with surface GP63 molecules, conferring parasite resistance to complement-mediated cell lysis and, at the same time, access to the phagocytic cell via complement receptor CR3 (Mosser and Edelson, 1985). Alternatively, the parasite might also enter phagocytes through the mannose-fucose receptor, found exclusively on macrophages (Wilson and Pearson, 1986). *Leishmania* invasion by receptor-mediated phagocytosis can hence be regarded as a passive process for the parasite. Still, it might involve several different receptor systems, which ultimately may favor parasite phagocytosis (Wilson and Pearson, 1986). Readily after parasite engulfment, the phagosome fuses with endosomes and lysosomes and as a result it becomes severely acidified (pH 5 to 6) and filled with hydrolases. *Leishmania donovani* promastigotes were shown to delay phagosomal maturation thus, avoiding the initial acidification and enzymatic digestion (Desjardins and Descoteaux, 1997). The reduction of phagosome fusion properties was suggested to be mediated by the multifunctional LPG molecule, in a process involving F-actin disassembly inhibition (Holm et al., 2001). *Leishmania* amastigotes, however, do not impair phagolysosomal biogenesis but can deal directly with vacuole acidification since they present plasma membrane $H^{(+)}$-ATPases that pump the hydrogen cations out of the parasite cytosol, keeping it neutral (Marchesini and Docampo, 2002). Parasite resistance to enzymatic digestion is in part due to the protease activity of GP63, which degrades lysosomal acid hydrolases (Chaudhuri et al., 1989). Other parasite-derived proteases, such as cysteine peptidases, can also be associated to *Leishmania* virulence (Mottram et al., 2004).

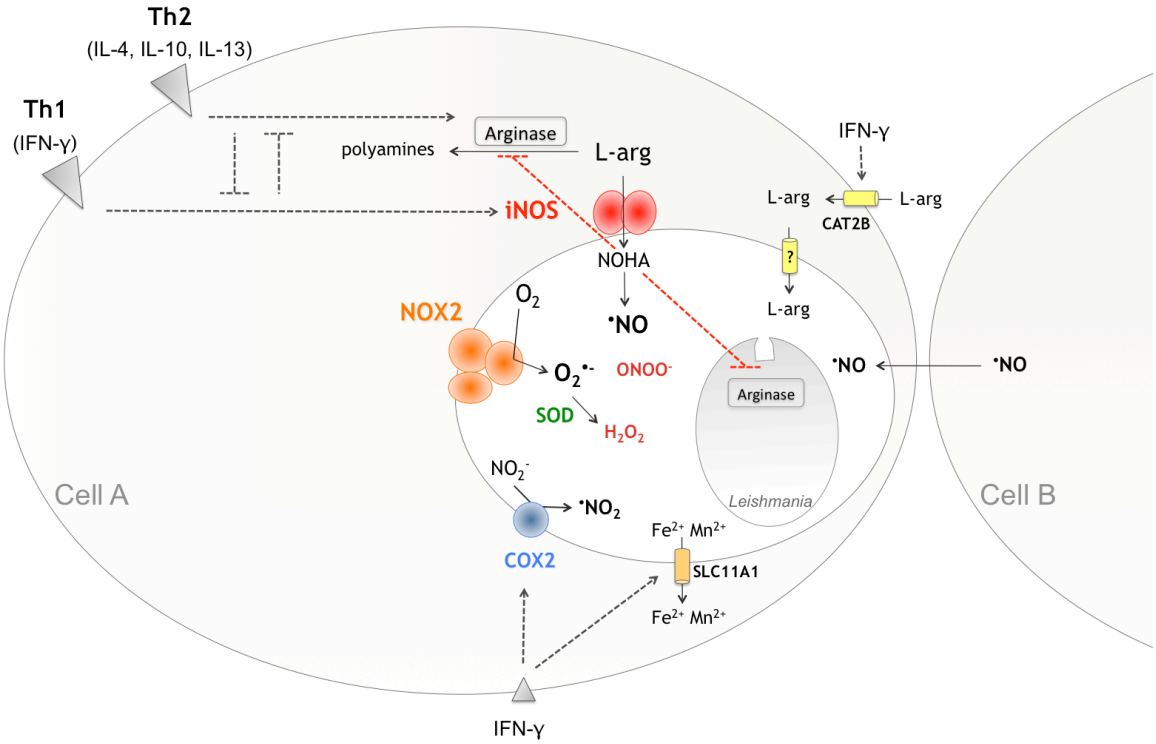


Fig. 2. Strategies to control *Leishmania* growth and survival inside murine macrophages. Upon parasite phagocytosis NADPH oxidase 2 (NOX2) is assembled on the phagosome membrane and consequently superoxide radical anion ($O_2^{\bullet-}$) is formed inside the parasite containing vacuole. Superoxide dismutase (SOD) catalyzes the conversion of $O_2^{\bullet-}$ into hydrogen peroxide (H_2O_2). Type 1 immune response (Th1) will activate inducible nitric oxide synthase (iNOS) through interferon-γ (IFN-γ) signaling pathways. iNOS catalyzes the two-step oxidation of L-arginine (L-arg), yielding first an intermediate compound, N^{ω}-OH-L-arginine (NOHA), which is further oxidized to citrulline and nitric oxide radical ($^{\bullet}NO$). Due to its physical-chemical properties $^{\bullet}NO$ can diffuse through cell membranes, reaching distant compartments or even neighbor cells. Intraphagosomal $O_2^{\bullet-}$ and $^{\bullet}NO$ can rapidly react to produce peroxynitrite anion ($ONOO^-$). An immunological response of Type 2 (Th2) is typically associated with IL-4, IL-10 and IL-13 production and activation of macrophage arginase. Arginase competes with iNOS for L-arg, catalyzing its conversion to L-ornithine and ultimately leading to polyamine generation. The $^{\bullet}NO$ intermediate NOHA was shown to block macrophage and parasite arginase activity, therefore impairing polyamine synthesis in both cells. Th1 cytokines, such as IFN-γ, might inhibit Th2 response. Conversely, Th2 IL-4 and IL-10 cytokines can hinder Th1 cells proliferation. IFN-γ stimulation has also been associated with i) production of nitrogen dioxide radical ($^{\bullet}NO_2$) via activation of cyclooxygenase 2 (COX2), ii) enhancement of L-arg uptake by up-regulating the expression of cationic amino acid transporter 2B (CAT2B) and iii) regulation of expression of the proton efflux pump SLC11A1, which seems to be crucial for iron homeostasis inside the phagolysosome. This illustration is a simplified schematic representation of different events probably occurring after parasite engulfment (Bogdan and Rollinghoff, 1998; Iniesta et al., 2001; Fritsche et al., 2007; Palazzolo-Ballance et al., 2007; Wanasen et al., 2007; Huynh and Andrews, 2008), it is not on scale and it does not include all possible macrophage responses. Gray triangles represent receptor-mediated processes.

Despite proliferating inside a vesicle, *Leishmania* amastigotes probably have facilitated access to most of the essential nutrients, since the phagolysosome lumen contains a variety of macromolecules, such as amino acids, polyamines, vitamins, fatty

acids, lipids, cations, heme and purines (Burchmore and Barrett, 2001). To scavange the nutrients from the medium of non-activated cells, *Leishmania* possess different transport systems, some of which are upregulated in the intracellular stage, thus facilitating the uptake (Naderer and McConville, 2008). However, the activation of macrophages by cytokines might modify the nutrient environment. For example, Th1 activation might lead to arginine (Chaturvedi et al., 2007) and tryptophan depletion (Murray et al., 1989) and/or to alterations in iron homeostasis (Fritsche et al., 2007) as illustrated in Fig. 2. Further research is required in this field to add information about nutrient supplies in cytokine-activated cells and clarify its relevance in intracellular parasite killing.

In addition to the mechanisms presented above, several studies demonstrate that *Leishmania* parasites promote inhibition of cytokine secretion, degradation of MHC class II molecules and subversion of host cell signaling pathways (reviewed in Gregory and Olivier, 2005). This active modulation of macrophage functions, might also contribute to the impairment of an adequate antiparasitic immune response.

3.2. Antioxidant defense

3.2.1. Generation of reactive oxygen and nitrogen species in macrophages

Macrophage production of highly reactive molecules is regarded as a major microbicidal mechanism. Superoxide ($O_2^{\bullet -}$) and nitric oxide ($^{\bullet}NO$) radicals are the macrophage front line effector molecules from which all other oxidants will derive (Fig. 2 and 3). The first evidence for generation of reactive oxygen species (ROS) came up with the discovery of the phagocyte respiratory burst (Iyer et al., 1961), which was later associated with the production of superoxide radical anion ($O_2^{\bullet -}$) by NADPH oxidases

(NOX) (Babior et al., 1973). Stuehr and Marletta (1985) reported one of the earliest observations of macrophage production of reactive nitrogen species (RNS), which was soon correlated with $^{\bullet}NO$ release (Stuehr et al., 1989) as a result of inducible nitric oxide synthase (iNOS) activity (Kwon et al., 1989; Gross et al., 1990). Hence, macrophages possess the required enzymatic machinery to induce oxidative and nitrosative damage to intracellular pathogens.

The NOX family of enzymes comprises different isoforms with conserved structure and function but distinct tissue distribution (recently reviewed by Bedard and Krause, 2007). NOX2, also known as gp91phox, is a complex of five protein subunits highly expressed in phagocytic cells. Its activation depends on chemotactic factors or phagocytic stimuli that lead to the assemblage of all the membrane ($p22^{phox}$ and $gp91^{phox}$) and cytosolic subunits ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) to form an active enzyme. The assembled NOX2 then promotes electron passage from NADPH to molecular oxygen (O_2) to form $O_2^{\bullet-}$. After phagocytosis, most of the NOX2 enzymes are present in the macrophage phagosome membrane, therefore generating $O_2^{\bullet-}$ towards the vacuole lumen. Consequently, formation of ROS will be specifically directed to phagocytized microorganisms and, because it is confined to a small compartment, it might potentiate oxidative damage.

Functional nitric oxide synthases consist of two NOS monomers and two calmodulin proteins, forming an homodimeric structure with tightly bound co-factors, namely tetrahydrobiopterin (BH_4), FAD, FMN and heme (reviewed in Alderton et al., 2001). Three distinct NOS isoforms have been identified: i) neuronal NOS (nNOS or NOS-1), found predominantly in neuronal tissue with constitutive expression; ii) inducible NOS (iNOS or NOS-2), present in a wide range of cells and tissues; iii) endothelial NOS (eNOS or NOS-3), constitutively expressed in vascular endothelial cells. Macrophages

express the inducible NOS isoform in the cell cytosol (Marletta, 1993) upon inflammatory stimulation (MacMicking et al., 1997). After activation, iNOS associates to the phagosome membrane (Vodovotz et al., 1995; Linares et al., 2001), wherein it catalyzes two-step oxidation of L-arginine to citrulline and $^{\bullet}NO$, with formation of N^{ω}-OH-L-arginine (NOHA) as an intermediate compound. Association of iNOS to the phagosome membrane will presumably favor diffusion of $^{\bullet}NO$ into the phagocytic compartment.

3.2.2. Characteristics of the different reactive oxygen and nitrogen species

An important number of molecules derived from $O_2^{\bullet-}$ and $^{\bullet}NO$ reactions are highly reactive compounds that can be classified according to their precursor into two different groups (Fig. 3). The ROS group includes $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), carbonate radical ($CO_3^{\bullet-}$) and hypochlorous acid (HOCl). Nitric oxide radical ($^{\bullet}NO$), peroxynitrite ($ONOO^-$) and nitrogen dioxide ($^{\bullet}NO_2$) belong to the RNS group. These distinct reactive species present different physical-chemical properties, which should be considered when assessing their oxidative power and relevance in biological systems.

3.2.2.1. Superoxide radical anion - $O_2^{\bullet-}$

Superoxide is a short-lived free radical with the dual capacity of oxidizing ($E^{0'}{}_{O2}{}^{\bullet-}{}_{/H2O2}$ = 0.94 V) or reducing ($E^{0'}{}_{O2/O2}{}^{\bullet-}$ = -0.33 V) different target molecules (Fee and Valentine, 1977). Being an anion molecule, $O_2^{\bullet-}$ can only move across membranes through anionic channels. However, in biological systems, $O_2^{\bullet-}$ diffusion is limited fundamentally by its rapid dismutation into H_2O_2 by superoxide dismutase (SOD) enzymes [Eq.1,

(Fielden et al., 1974)] or by its combination with •NO to form peroxynitrite [Eq.2, (Radi et al., 2001)]. As far as cellular oxidative damage is concerned, $O_2^{\bullet-}$ probably does not cause direct damage but is certainly crucial as a precursor of highly cytotoxic molecules (Fig. 3).

$$O_2^{\bullet-} + O_2^{\bullet-} \xrightarrow[SOD]{2H^+} H_2O_2 + O_2 \qquad k = 2 \times 10^9\ M^{-1}\ s^{-1} \qquad [1]$$

$$O_2^{\bullet-} + {}^{\bullet}NO \rightarrow ONOO^- \qquad k = 4.3 \text{ to } 19 \times 10^9\ M^{-1}\ s^{-1} \qquad [2]$$

3.2.2.2. Hydrogen peroxide - H_2O_2

Hydrogen peroxide is not a free radical, since it has no unpaired electrons, but it is one of the most important ROS. It is capable of crossing cell membranes and oxidize ($E^{0'}_{H2O2/{}^{\bullet}OH}$ = 0.38 V) important biotargets such as proteins, DNA and lipids (Hassan, 1997). Despite its cell damaging potential, H_2O_2 has also been implicated in redox signaling pathways that are essential to normal cell function (Rhee, 2006). Hydrogen peroxide is considered a very stable molecule, however, depending on the peroxidase-content of each cell type, H_2O_2 stability can be decreased as a result of its catalytic reduction to H_2O and O_2.

3.2.2.3. Hydroxyl radical - •OH

Hydroxyl radical presents the highest oxidative potential ($E^{0'}{}^{\bullet}_{OH/H2O}$ = 2.3 V) amongst ROS (Buettner, 1993). Due to its high reactivity, •OH has also a very short half-life and is impermeable to membranes. Thus, its damaging effects will only affect very nearby targets (Hassan, 1997). The generation of hydroxyl radical is achieved through the

Fenton's reaction [Eq.3] and involves reduced transition metals, such as Fe^{2+} or Cu^{+}, that are regenerated by the Haber-Weiss reaction [Eq.4].

$$H_2O_2 + Fe^{2+}/Cu^{+} \rightarrow {}^{\bullet}OH + OH^{-} + Fe^{3+}/Cu^{2+} \quad [3]$$

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \quad [4]$$

3.2.2.4. Nitric oxide radical - ${}^{\bullet}NO$

Nitric oxide is a free radical gas first described as a messenger molecule and later associated to cytotoxicity (Radi, 2004). It has an oxidant potential similar to H_2O_2 [$E^{0'}{}_{{}^{\bullet}NO/NO^{-}}$ = 0.39 V (Koppenol, 1998)], however, most of ${}^{\bullet}NO$-mediated toxicity is not due to ${}^{\bullet}NO$ direct effects but rather to its derivatives. Nitric oxide radical presents great tendency to interact with other free radicals, especially with $O_2^{\bullet-}$ yielding a powerful oxidant product, $ONOO^{-}$ [Eq.2 and Fig. 3]. In hydrophobic environments, such as cell membranes, ${}^{\bullet}NO$ might also react with O_2, producing important reactive intermediates such as ${}^{\bullet}NO_2$, and nitrite (NO_2^{-}) and nitrate (NO_3^{-}) as inert final products. Another worth mentioning property of ${}^{\bullet}NO$ is its capacity to freely diffuse in tissues, meaning that it can be produced in one cell and easily spread out to its vicinity (Quijano et al., 2005).

3.2.2.5. Nitrogen dioxide - ${}^{\bullet}NO_2$

Nitrogen dioxide is a relatively strong oxidant molecule [$E^{0'}{}_{{}^{\bullet}NO2/NO2^{-}}$= 0.99 V, (Stanbury, 1989)], which tends to accumulate in membranes and interact with a variety of free radicals and also with thyil radicals (Augusto et al., 2002; Ford et al., 2002). Since the

majority of cells possess a high intracellular thiol concentration, $^{\bullet}NO_2$ reactivity towards these molecules gains special relevance in biological systems. Nitrogen dioxide can be derived from $ONOO^-$, from oxidation of $^{\bullet}NO$ or from myeloperoxidase-mediated reaction of H_2O_2 with NO_2^- (Burner et al., 2000 and Fig. 3). Recently Palazzolo-Balance and co-workers (2007) suggestedd that $^{\bullet}NO_2$ could be generated via cyclooxygenase-mediated NO_2^- oxidation. Since the cyclooxygenase isoform COX2, is highly expressed in activated murine macrophages, these data propose a novel $^{\bullet}NO_2$ formation pathway, adding new players to the macrophage ROS/RNS generating arsenal (Fig. 2).

3.2.2.6. Peroxynitrite - $ONOO^-$

Peroxynitrite is a powerful oxidant [$E^{0'}_{(ONOOH,\ H^+/NO2^-,\ H2O)}$ = 1.4 V (Radi et al., 2000)] formed by the reaction between $O_2^{\bullet-}$ and $^{\bullet}NO$. Peroxynitrite anion can be protonated into peroxynitrous acid (ONOOH), a very unstable form that through homolytic cleavage decays to NO_3^- with formation of $^{\bullet}NO_2$ and $^{\bullet}OH$ (30% yields). Peroxynitrite is a reactive and short-lived molecule, which can passively diffuse through membranes in its protonated form (ONOOH) or be translocated via anionic channels ($ONOO^-$). It can directly interact with biologically relevant targets such as thiol compounds, carbon dioxide (CO_2) and metalloproteins. On the other hand, peroxynitrite oxidative damage might also be performed indirectly through the effects of its secondary derived radicals (Fig. 3). Peroxynitrite reacts with CO_2 as well, generating $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ (Radi et al., 2001). Given that, in biological systems CO_2 exists in relatively high concentrations (Alvarez, 2007), this interaction will probably represent one of the most important reactions taking place in vivo. Furthermore, $ONOO^-$ is known to promote tyrosine nitration, a process which involves inclusion of a nitro group in the tyrosine ring and that can alter protein function.

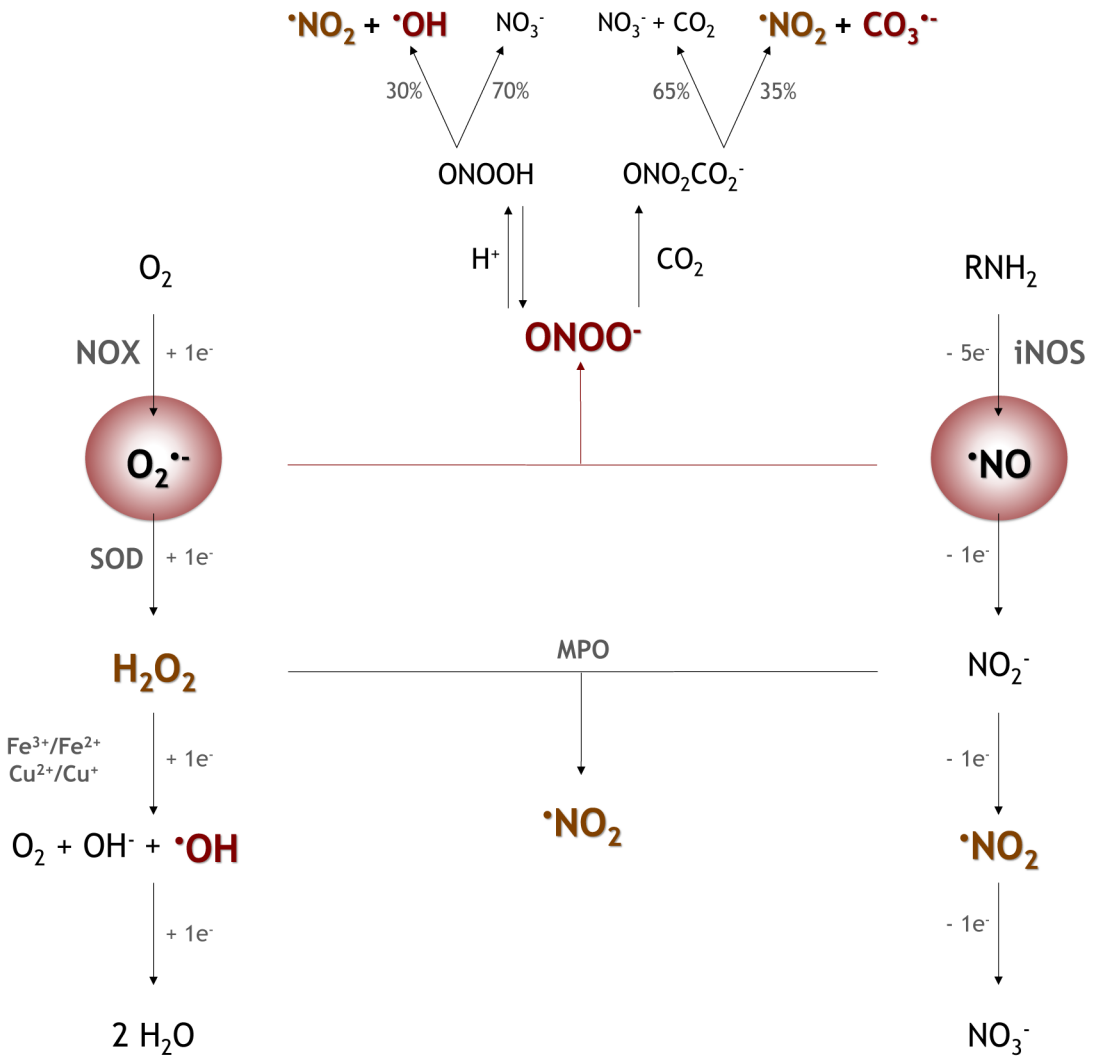


Fig. 3. Free radical reaction pathways in phagocytes. NADPH oxidase (NOX) catalyzes the monovalent reduction of molecular oxygen (O_2) to form superoxide radical anion ($O_2^{\bullet-}$). Superoxide dismutation into hydrogen peroxide (H_2O_2) might be held spontaneously (at low pH) or catalyzed by superoxide dismutase (SOD). Hydrogen peroxide can generate hydroxyl radical ($^{\bullet}OH$) via oxidation of a metal ion such as Fe^{2+} or Cu^{+} (Fenton's reaction). The regeneration of reduced metal ions can be achieved by $O_2^{\bullet-}$ reduction of Fe^{3+}/Cu^{2+} (Haber-Weiss reaction). Nitric oxide radical ($^{\bullet}NO$) is generated through the oxidation of the guanidine group of L-arginine (RNH_2), a process dependent on inducible nitric oxide synthase (iNOS) isoforms in macrophage cells. Superoxide and $^{\bullet}NO$ reaction yields peroxynitrite ($ONOO^-$). Peroxynitrite anion exists in protonation equilibrium with peroxynitrous acid (ONOOH) at a pH dependent ratio (at pH 7.4, 80% of peroxynitrite will be in the anionic form). Peroxynitrous acid may either form nitrogen dioxide radical ($^{\bullet}NO_2$) and $^{\bullet}OH$ in 30% yields, or form nitrate (NO_3^-). Alternatively, $ONOO^-$ might rapidly react with carbon dioxide (CO_2) forming a short-lived intermediate, nitroso-peroxocarboxylate ($ONO_2CO_2^-$), which is either converted in $^{\bullet}NO_2$ and carbonate radical ($CO_3^{\bullet-}$) or in NO_3^- and CO_2. Also, $^{\bullet}NO$ may be further oxidized to form $^{\bullet}NO_2$. Myeloperoxidase (MPO) mediates the reaction of H_2O_2 with NO_2^- to form $^{\bullet}NO_2$. Adapted from Nathan and Shiloh (2000).

This reaction is not directly conducted by $ONOO^-$ but depends on its derivatives, especially $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (Radi, 2004). The formation of $ONOO^-$ in biological systems has been demonstrated and correlated to a series of diseases (e.g., Haddad et al., 1994;

Kaur and Halliwell, 1994; Beal et al., 1997; Eliasson et al., 1999). Nevertheless, the relevance of this reactive species has raised some controversies, some of which, related to difficulties in detecting $ONOO^-$ in vivo (reviewed in Radi et al., 2001).

3.2.3. *Leishmania* antioxidant defense systems

Like any other cell, *Leishmania* parasites possess antioxidant protective systems to cope with pro-oxidants that result from normal cell metabolism. However, as a consequence of phagocyte parasitism, *Leishmania* can be exposed to unusually high concentrations of strong cytotoxic molecules. In fact, a single macrophage can produce 3 fmol/min of H_2O_2 (Tanaka et al., 1982) and present up to 30 pmol of H_2O_2 *per* infected cell (Melo et al., 2003). In the case of $ONOO^-$, it was estimated that approximately 0.002 to 0.003 fmol/min are released in the macrophage phagosome (Denicola et al., 1993; Alvarez et al., 2004). Therefore, to establish a persistent infection it is commonly inferred that *Leishmania* need antioxidant defense strategies to invade and persist inside the host cell. In this regard, antioxidant molecules located in the parasite cytosol are presumably the most well positioned to effectively eliminate oxidants from exogenous sources.

3.2.3.1. Non-enzymatic defense

A compound might exert antioxidant function by acting as a ROS/RNS scavenger, by modulating the production of reactive species or by enhancing antioxidant defenses in the cell (Halliwell, 2007). *Leishmania* surface LPG can act both as a direct oxidant scavenger (Chan et al., 1989) and as an inhibitor of NOX2 assemblage in the phagosome, thus preventing ROS formation (Lodge and Descoteaux, 2006). Other molecules, such as,

heat shock proteins, ascorbate or reduced pterins exhibit antioxidant function and have been suggested to act in trypanosomatid ROS defense (Miller et al., 2000; Wilkinson et al., 2005; Moreira et al., 2009). Resistance to oxidative stress is also associated to the presence of reduced intracellular thiols. Almost exclusive of trypanosomatids is a dithiol conjugate formed by two molecules of glutathione linked by a spermidine, named trypanothione [$T(SH)_2$]. The concentration of intracellular trypanothione has been shown to diverge between trypanosomatid species and parasite developmental stages (Ariyanayagam and Fairlamb, 2001). For example, in *L. major* and *L. donovani*, the $T(SH)_2$ content was reported to decrease considerably in intracellular amastigotes (Ariyanayagam and Fairlamb, 2001), while in *L. infantum*, it is suggested to be 3-fold more abundant in axenic amastigotes (M. Sousa-Silva and C. Cordeiro, unpublished results). Regardless of the differences among species, $T(SH)_2$ plays a crucial role in a variety of biological processes, including spontaneous scavenging of ROS and RNS (Ariyanayagam and Fairlamb, 2001; Thomson et al., 2003) and, more importantly, mediation of electron fueling to several antioxidant compounds and enzymes (see section 3.2.3.2.). Hence, it is not surprising that $T(SH)_2$ synthesis is essential for parasite survival (Comini et al., 2004; Ariyanayagam et al., 2005). Other low molecular mass thiols can be found in these parasites, namely glutathione (GSH), mono-glutathionylspermidine and ovothiol A (reviewed in Castro and Tomás, 2008; Krauth-Siegel and Comini, 2008). Glutathione and $T(SH)_2$ hold comparable reduction potentials, however, the reductant power of $T(SH)_2$ in the cell is kinetically favored, since i) due to the spermidine positively charged amino group, $T(SH)_2$ is more ionized at physiological pH and ii) being a dithiol it promotes generation of intramolecular disulfide bridges, in contrast with oxidation of two GSH molecules that will necessarily form intermolecular disulfides (Krauth-Siegel et al., 2007). In addition to $T(SH)_2$ and GHS, ovothiol A might also exert direct antioxidant effects in *Leishmania* spp.

(Ariyanayagam and Fairlamb, 2001; Steenkamp, 2002). Nonetheless, spontaneous reduction of ROS/RNS by reduced thiols is always less significant than the accelerated enzymatic antioxidant processes, which may question its physiological relevance inside the cell.

3.2.3.2. Enzymatic defense

As mentioned before, *Leishmania* lack catalase and typical glutathione-dependent peroxidases. However, they present other enzymatic antioxidant defenses, such as SOD and several types of peroxidases. SOD, which catalyzes the conversion of $O_2^{\bullet-}$ to H_2O_2 and O_2, is ubiquitous in every aerobic organism from bacteria to humans (Bowler et al., 1992). This metalloenzyme can be cofactored with copper and zinc, manganese, iron or nickel (reviewed in Fridovich, 1995). So far, only iron-containing SODs were isolated from *Leishmania* and other trypanosomatids, where they appear to predominantly localize in glycosomes and mitochondria (Le Trant et al., 1983; Temperton et al., 1996; Paramchuk et al., 1997; Kabiri and Steverding, 2001; Wilkinson et al., 2006). Peroxides resulting from SOD activity or from other sources also have to be readily eliminated by cells. In trypanosomatids, typical 2-cysteine peroxiredoxins (2-Cys PRXs) are most likely the key players in peroxide detoxification systems (reviewed in Castro and Tomás, 2008). They were found to be cytosolic and mitochondrial in *L. infantum* (Castro et al., 2002), *T. cruzi* (Wilkinson et al., 2000a) and *T. brucei* (Tetaud et al., 2001). In addition, another 2-Cys PRX coding sequence presenting a glycosome targeting motif exist in *Leishmania* genomes. Cytosolic 2-Cys PRXs are encoded by several nearly identical genes located in the same chromosomal locus, while mitochondrial 2-Cys PRXs are transcribed from single-copy genes. These enzymes present two separate redox active cysteines and are

obligate homodimers. Their catalytic cycle is initiated with peroxide oxidation of the peroxidatic cysteine that will in turn form an intermolecular disulfide bridge with the resolving cysteine residue from another subunit. This will finally be resolved by an oxidoreductase, thus completing the peroxidases catalytic cycle (Trujillo et al., 2007). Trypanosomatids 2-Cys PRXs can use as substrates H_2O_2 and, to a lesser extent, hydrophobic hydroperoxides, with catalytic efficiencies ranging from 3 x 10^4 to 1.7 x 10^7 M^{-1} s^{-1} (König and Fairlamb, 2007; Krauth-Siegel et al., 2007; Trujillo et al., 2007). Moreover, *T. brucei* and *T. cruzi* 2-Cys PRXs were also reported to exhibit $ONOO^-$ reductase activity, with estimated second order rate constants of 9 x 10^5 and 7.2 x 10^5 M^{-1} s^{-1}, respectively. Several studies estimated that trypanosomatids present relatively high concentrations of 2-Cys PRXs (Nogoceke et al., 1997; Atwood et al., 2005; Comini et al., 2007; König and Fairlamb, 2007) and others confirmed that the proteins were active within *Leishmania* and *T. cruzi* (Wilkinson et al., 2000a; Castro et al., 2002), which strengthens the relevance of these type of enzymes in the parasites' antioxidant machinery. More importantly, cytosolic 2-Cys PRX down-regulation in *T. brucei* drastically affected parasitic survival (Wilkinson et al., 2003) and in *L. amazonensis* significantly increased H_2O_2 susceptibility (Lin et al., 2005). Conversely, up-regulation of cytosolic 2-Cys PRX augmented *T. cruzi*, *L. donovani* and *L. chagasi* resistance to H_2O_2 produced by host macrophages (Barr and Gedamu, 2003; Iyer et al., 2008; Piñeyro et al., 2008) and, in the case of *T. cruzi*, also enhanced macrophage-derived $ONOO^-$ reduction (Piacenza et al., 2008).

Trypanosomatids present other types of peroxidases, namely glutathione peroxidases (GPX)-like proteins, which differ from typical GPX in several features, for example the active site selenocysteine is substituted for a cysteine residue. These non-selenium GPX-like enzymes (nsGPX) distribute through the cytosol and mitochondrion in

T. brucei (Hillebrand et al., 2003; Schlecker et al., 2005) and in *T. cruzi* are found in the cytosol, glycosomes and endoplasmic reticulum (Wilkinson et al., 2000b; Wilkinson et al., 2002a; Wilkinson et al., 2002b). *Leishmania* genome databases present open reading frames for nsGPX, differing in the N- and C-terminal sequences, which suggests different subcellular localizations also in these organisms. The rate constants determined for nsGPX hydroperoxide and lipid hydroperoxide reduction are around 10^5 M^{-1} s^{-1} and despite the fact that their designation suggests affinity to GSH, these enzymes are preferably reduced by specific parasite oxidoreductases (König and Fairlamb, 2007; Castro and Tomás, 2008). Curiously, although *L. major* nsGPX1 is only slightly less effective against H_2O_2 than 2-Cys PRXs, it was estimated to be 40-fold less abundant in the parasites (König and Fairlamb, 2007). This difference may clearly compromise the hypothesis of redundancy between these type of *Leishmania* peroxidases. However, in *T. brucei*, knockdown of the nsGPX enzymes compromises parasite survival (Wilkinson et al., 2003; Schlecker et al., 2005). Together these data support that 2-Cys PRXs and nsGPXs cannot functionally replace each other.

An ascorbate-dependent hemeperoxidase (APX) has been described in *L. major* (Adak and Datta, 2005) and *T. cruzi* (Wilkinson et al., 2002c), but is not encoded by *T. brucei*. The *L. major* enzyme shows mitochondrial localization, being mainly restricted to the intermembrane space and might have ascorbate and ferrocytochrome c as reducing agents (Dolai et al., 2007; Dolai et al., 2008; Yadav et al., 2008). Overexpression of *Lm*APX seems to confer increased resistance to exogenously added H_2O_2 (Dolai et al., 2008). In the case of *T. cruzi*, APX is detected in the endoplasmic reticulum also presenting H_2O_2 reducing capacity in vitro (3.5×10^6 M^{-1} s^{-1}) and in the parasite context (Wilkinson et al., 2002c).

Finally, in *Leishmania*, the eukaryotic elongation factor 1B (eEF1B) displays trypanothione-dependent peroxidase activity (Vickers et al., 2004). Since, *L. major* eEF1B localizes to the endoplasmic reticulum and reacts with hydrophobic hydroperoxides but not with H_2O_2, a putative function in resistance to lipid peroxidation has been suggested for this molecule (Vickers et al., 2004).

3.2.3.3. $T(SH)_2$-dependent electron fueling of *Leishmania* peroxidases

The trypanosomatids unique dithiol, $T(SH)_2$, was linked to H_2O_2 reduction in the late 80's (Penketh and Klein, 1986; Henderson et al., 1987) and soon after this it was broadly accepted as a key antioxidant molecule in *Leishmania*, *Crithidia* and *Trypanosoma* (Fairlamb and Cerami, 1992). The existence of a $T(SH)_2$-mediated enzymatic cascade for hydroperoxide detoxification was unraveled for the first time in 1997 (Nogoceke et al.) in the insect parasite *Crithidia fasciculata* and later extended to all medically relevant species of the Trypanosomatidae family (Levick et al., 1998; Lopez et al., 2000; Tetaud et al., 2001; Flohé et al., 2002; Castro et al., 2004). As schematically represented in Fig. 4, the system includes $T(SH)_2$, three redox enzymes [trypanothione reductase (TR), tryparedoxin (TXN) and 2-Cys PRX] and uses NADPH as the primary electron source. Trypanothione reductase is the NADPH-dependent flavoenzyme which is responsible for keeping the pool of reduced $T(SH)_2$ in the cell (Krauth-Siegel and Schoneck, 1995). A decrease in TR activity was shown to have no influence in *Leishmania* promastigote survival in culture, however, it severely reduced intracellular parasite growth in macrophages (Dumas et al., 1997; Tovar et al., 1998). As for TXN enzymes, these are also exclusive of the Kinetoplastida group and consist of a particular type of oxidoreductases, distantly related to thioredoxins. TXNs are characterized by a typical Trp-Cys-Pro-Pro-Cys-Arg active site

signature and by being specifically reduced by $T(SH)_2$. All trypanosomatids present at least two TXN genes, one of which encodes a cytosolic protein (Steinert et al., 1999; Tetaud et al., 2001; Castro et al., 2004). The existence of a mitochondrial TXN was, so far, only experimentally demonstrated in *L. infantum*, suggesting that in this species two $TR/T(SH)_2$/TXN systems operate in distinct subcellular compartments. Similarly to what happens with other eukaryotic multifunctional thioredoxins, TXNs might display additional cell functions apart from participating in the aforementioned trypanosomatid antioxidant defense system. Moreover, TXNs were suggested to serve as electron sources for the ribonucleotide reductase (Dormeyer et al., 2001) and the universal minicircle sequence binding protein (Onn et al., 2004), further implicating these enzymes in parasite replication processes. The essential role anticipated for parasite survival was proven true for the *T. brucei* cytosolic TXN (Comini et al., 2007).

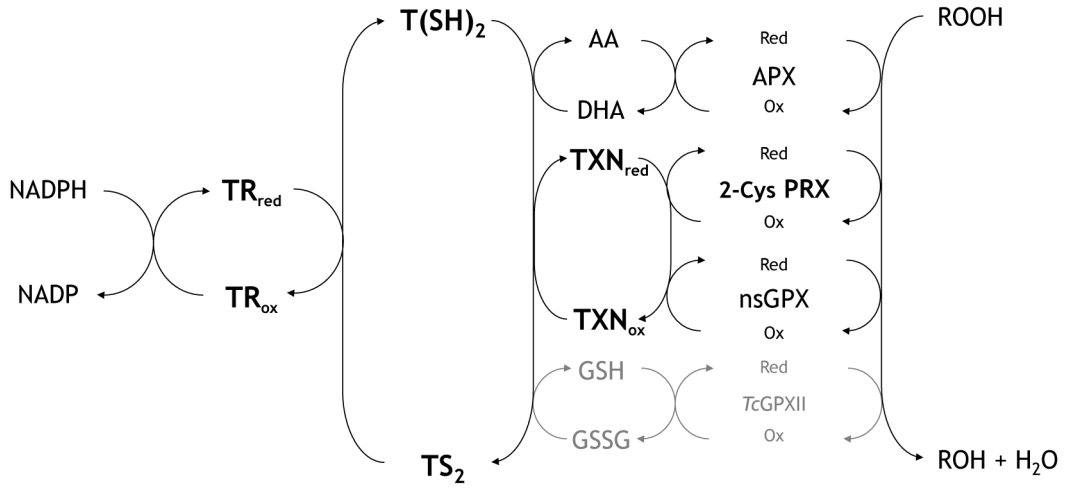


Fig. 4. Trypanothione-dependent pathways for hydroperoxide detoxification in trypanosomatids. Elimination of hydroperoxides in trypanosomatids can be catalyzed by three types of peroxidases, 2-cysteine peroxiredoxins (2-Cys PRX), non-selenium glutathione peroxidases (nsGPX) and ascorbate peroxidases (APX). Trypanothione disulfide (TS_2) is reduced to dihydrotrypanothione [$T(SH)_2$] by the NADPH-dependent flavoprotein, trypanothione reductase (TR). Dihydrotrypanothione transfers the reducing equivalents to tryparedoxin (TXN) that in turn reduces 2-Cys PRX and nsGPX. Alternatively, $T(SH)_2$ can reduce dehydroascorbate (DHA) to ascorbate (AA) and the latter drives the electron transfer to APX. Dihydrotrypanothione-mediated reduction of glutathione disulfide (GSSG) to glutathione (GSH) can also occur and further reduce nsGPXs. The subscripts red and ox refer to the dithiol and disulfide forms of the proteins, respectively. The pathways/enzymes that have not been characterized in *Leishmania* spp. are shown in gray. Adapted from Krauth-Siegel et al. (2007).

In the same manner as for 2-Cys PRXs, reduction of almost all reported nsGPX enzymes also depends on the TR/$T(SH)_2$/TXN system (Wilkinson et al., 2000b; Wilkinson et al., 2002a; Hillebrand et al., 2003; Schlecker et al., 2005 and Fig.4). In fact, only *T. cruzi* GPXII was reported to be TXN independent, reacting directly with GSH but with very low affinity (Wilkinson et al., 2002b). Still, the maintenance of reduced GSH pools in the parasite are also assured by the TR/$T(SH)_2$ redox couple (Moutiez et al., 1995). Moreover, as illustrated in Fig.4, even alternative antioxidant systems involving APXs might depend on TR/$T(SH)_2$. In this case, $T(SH)_2$ reduces dehydroascorbate to ascorbate, which in turn delivers the electrons to APX that finally reduces the hydroperoxides (Wilkinson et al., 2002c; Dolai et al., 2007).

In conclusion, maintenance of the *Leishmania* redox status largely depends on $T(SH)_2$, particularly on its involvement in multi-component enzymatic pathways. In fact, all the three types of peroxidases described in *Leishmania* parasites depend on the TR/$T(SH)_2$/TXN system (2-Cys PRX and nsGPX) or simply on the TR/$T(SH)_2$ redox couple (APX) to sustain their reduction throughout time.

4. Different hosts different strategies?

The vast majority of critical milestones in the field of infection biology and immunology were accomplished using experimental mouse models and *Leishmania* research is no exception to this. Nevertheless, care must be taken when extrapolating from infection mouse models to human, dog or even to close relative models such as the hamster. In fact, as mentioned before, apart from intrinsic factors of *Leishmania* species and strains, the infection course will also depend on host factors, namely at the immunological level. A pattern of resistance to *Leishmania* infection in murine and human

cells has been consistently associated with cellular stimulation with Th1 cytokine IFN-γ (Honoré et al., 1998; Wilson et al., 2005). Since, macrophage activation by IFN-γ induces direct microbicidal actions that can be associated to enhancement of NOX and induction of iNOS (Schroder et al., 2004), parasite killing might primarily result from macrophage-induced oxidative/nitrosative stress (Fig. 2). However, conflicting opinions remain with regard to the relative contribution of ROS/RNS in different mammalian organisms.

Murine and human phagocytic NOX2 enzyme were characterized and shown to share the same basic structural and functional features (Bedard and Krause, 2007). Indeed, upon *Leishmania* promastigote phagocytosis, both murine and human macrophages generate detectable amounts of $O_2^{\bullet-}$ (Gantt et al., 2001), suggesting that the oxidative burst process is identical in the two cell types. On the contrary, production of iNOS derived $^{\bullet}NO$ is well documented in murine cells but it is still controversial in human macrophages (Table 2 and 3). For example, two independent groups successfully induced $^{\bullet}NO$ in monocyte-derived human macrophages in the presence of *Leishmania* and different cytokines (Vouldoukis et al., 1995; Panaro et al., 2003), but using similar conditions Gantt et al (2001) failed to detect $^{\bullet}NO$ production in the same cell type. Inconsistent data in the literature might result from unawareness of the inductive signals for iNOS activation in human cells, lack of accurate $^{\bullet}NO$ probes with the possibility of in vivo application and/or low-output of $^{\bullet}NO$ formation in human macrophages. In fact, murine and human iNOS promoters contrast markedly, meaning that classical IFN-γ/LPS combination might not suffice to express the human enzyme (Taylor and Geller, 2000). On the other hand, although a number of reports demonstrate $^{\bullet}NO$ production by human phagocytes, their levels are generally much lower than the ones of rodent cells and often present high inter-individual variability (Albina, 1995). Regarding this issue, some authors speculate that human macrophages might have suffered evolutionary pressure to down-regulate iNOS

expression and with this avoid exposure to highly toxic levels of •NO (Albina, 1995; Taylor and Geller, 2000). Nitric oxide implication in human immune response is nonetheless strengthened by in vivo studies in which human tissue analyses reveal expression of functionally active iNOS in macrophage-enriched areas during inflammatory processes (Bachmaier et al., 1997; Facchetti et al., 1999; Fang and Vazquez-Torres, 2002). Based on the arguments above-mentioned it is possible to conclude that, although each organism may mount a specific immune response against *Leishmania* parasitosis, i) ROS are likely to be widespread microbicidal effector molecules and ii) even if implication of RNS is not always clear in vitro, control of natural human infections may also involve •NO-derived toxicity.

Table 2. Direct evidence for nitric oxide production by human monocytes/macrophages

Year	Cell type	Cell stimulus	Assay for NO detection	Reference
1991	PBMC (macrophages)	*M. avium* + 100 U TNF-α + GM-CSF	Griess assay (7 days p.i.)	(Denis)
1992	PBMC (monocytes)	*T. cruzi* + 100 U.ml^{-1} IFN-γ (+300 U.ml^{-1} TNF-α)	Griess assay (72 h after stimuli)	(Muñoz-Fernandez et al.)
1993	PBMC (macrophages)	No stimulus	Griess assay (after 7 days in culture)	(Martin and Edwards)
1993	Bone marrow monocytes	No stimulus	Griess assay (24 h in culture)	(Wickramasinghe and Hasan)
1994	PBMC (monocytes)	10 μg.ml^{-1} anti-CD69	Griess assay (4 to 48 h after stimulus)	(De Maria et al.)
1994	PBMC (macrophages)	HIV envelope glycoprotein (gp120)	Griess assay (72 h after stimulus); Spin trapping (DMPO)	(Pietraforte et al.)
1994	PBMC (macrophages)	10^6 *M. avium*	Griess assay (72 and 144 h p.i.)	(Dumarey et al.)
1995	PBMC (macrophages)	*L. major* + (IL-4 + anti-CD23 added 48 h p.i.)	iNOS activity; Griess assay (72 h p.i.)	(Vouldoukis et al.)
1995	Peritoneal macrophages	200 U.ml^{-1} IFN-γ + 1 μg.ml^{-1} LPS	iNOS activity; Griess assay (96h after stimuli)	(Weinberg et al.)
1996	PBMC from RA patients (macrophages)	500 U.ml^{-1} IFN-γ + 1 μg.ml^{-1} LPS	iNOS activity (5 days after stimuli); Griess assay (120 h after stimuli)	(St Clair et al.)
1996	PBMC (monocytes)	0.1 – 10 μM Morphine	NO-specific amperometric probe	(Magazine et al.)
1997	PBMC (monocytes)	100 μg.ml^{-1} MPL	Griess assay (18 h after stimulus)	(Saha et al.)
1998	PBMC (macrophages)	125 ng.ml^{-1} LPS	Griess assay (2 to 18 days after stimulus)	(Tunçtan et al.)
1998	PBMC (macrophages)	T. cruzi alone or + cysteine-cysteine chemokines	Griess assay (48 h after stimulus)	(Villalta et al.)
1999	PBMC (monocytes)	Lymphocytes + anti-CD3	Detection of protein nitration (anti-nitrotyrosine)	(Brito et al.)
2002	Alveolar macrophages (from patients with transplanted lungs)	25 μg.ml^{-1} SP-A or *Klebsiella*	Griess assay; DAN fluorescent probe	(Hickman-Davis et al.)
2003	PBMC (macrophages)	*L. infantum* alone or *L. infantum* + 10 U.ml^{-1} IFN-γ + 100 ng.ml^{-1} LPS	Griess assay (72 h after stimuli)	(Panaro et al.)

DAN, 2,3-diaminonaphthalene; GM-CSF, Granulocyte Macrophage Colony Stimulating Factor; IFN, Interferon; IL, Interleukin; iNOS, inducible Nitric Oxide Synthase *L. major*, *Leishmania major*; *L. infantum*, *Leishmania infantum*; LPS, Lipopolysaccharide; *M. avium*, *Mycobacterium avium*; MPL, Monophosphoryl Lipid A; PBMC, Peripheral Blood Mononuclear Cells; RA, Rheumatoid Arthritis; SP-A, Human Surface Protein-A; *T. cruzi*, *Trypanosoma cruzi*; TNF, Tumor Necrosis Factor.

Table 3. No evidence for nitric oxide production by human monocytes/macrophages

Year	Cell type	Cell stimulus	Assay for NO detection	Reference
1990	Alveolar and peritoneal macrophages	*C. neoformans* + 100 $U.ml^{-1}$ IFN-γ + 1 $\mu g.ml^{-1}$ LPS	iNOS activity; Griess assay	(Cameron et al.)
1991	PBMC (macrophages) and THP-1 cell line	IFN-γ	Griess assay	(Werner et al.)
1992	PBMC (macrophages)	IFN-γ	Griess assay	(Murray and Teitelbaum)
1992	PBMC (macrophages)	500 $U.ml^{-1}$ IFN-γ or 1 $\mu g.ml^{-1}$ LPS or IFN-γ + LPS or Zymosan or Zymosan + PMA or IFN-γ + PMA	Griess assay (24 h and 48 h after stimuli)	(Padgett and Pruett)
1993	PBMC (macrophages)	*M. avium* + TNF-α + GM-CSF + 100 $U.ml^{-1}$ IFN-γ	Griess assay (72 h p.i.)	(Bermudez)
1993	Peritoneal macrophages of renal patients	1.5 μM PMA or 5 $\mu g.ml^{-1}$ LPS or PMA+LPS	Griess assay (24 h after stimuli)	(Ben-Efraim et al.)
1993	PBMC (macrophage)	IFN-γ or IFN-γ + LPS or IFN-γ + LPS + BH_4 or GM-CSF or TNF-α or *L. monocytogenes*	iNOS activity; Griess assay	(Schneemann et al.)
1994	PBMC (monocytes)	10 $\mu g.ml^{-1}$ LPS + IFN-γ	Griess assay (4 to 48 h after stimulus)	(De Maria et al.)
1997	Alveolar macrophages	1-1000 $ng.ml^{-1}$ LPS + 1-100 $U.ml^{-1}$ IFN-γ	Griess assay	(Jesch et al.)
2001	Alveolar macrophages and PBMC (macrophages)	10 $\mu g.ml^{-1}$ LPS + 200 $U.ml^{-1}$ IFN-γ or 200 $U.ml^{-1}$ TNF-α or 200 $U.ml^{-1}$ GM-CSF or IL-1 or IL-4	Griess assay (24 h after stimuli)	(Muijsers et al.)
2001	PBMC (macrophages)	400 $U.ml^{-1}$ IFN-γ + 1 $ng.ml^{-1}$ LPS + 10 $\mu g.ml^{-1}$ IL-1 (+ *L. chagasi*)	Griess assay (72 h after stimuli)	(Gantt et al.)
2002	Alveolar macrophages	25 $\mu g.ml^{-1}$ SP-A or *Klebsiella*	Griess assay; DAN fluorescent probe	(Hickman-Davis et al.)
2007	PBMC (monocytes)	*P. falciparum* or 100 $U.ml^{-1}$ IFN-γ or 400 $U.ml^{-1}$ IFN-γ + 500 $U.ml^{-1}$ TNF-α + IL-1 + 20 $\mu g.ml^{-1}$ LPS	Griess assay (24 h after stimuli)	(Skorokhod et al.)
2008	PBMC (macrophages) and THP-1 cell line	50 $U.ml^{-1}$ IFN-γ (12 h before infection) + M. bovis or M. bovis BCG	Griess assay (3 to 72 h after stimuli)	(Jordão et al.)
2009	PBMC (macrophages)	5 $\mu g.ml^{-1}$ LPS + 500 $U.ml^{-1}$ IFN-γ (+ *L. infantum*)	Griess assay (24 to 72 h); DAF-2DA fluorescent probe	(This work, chapter 4)

BH_4, tetrahydrobiopterin; *C. neoformans*, *Cryptococcus neoformans*; DAN, 2,3-diaminonaphthalene; GM-CSF, Granulocyte Macrophage Colony Stimulating Factor; IFN, Interferon; iNOS, inducible Nitric Oxide Synthase; LPS, Lipopolysaccharide; *L. chagasi*, *Leishmania chagasi*; *L. infantum*, *Leishmania infantum*; *L. monocytogenes*, *Listeria monocytogenes*; *M. avium*, *Mycobacterium avium*; *M. bovis*, *Mycobacterium bovis*; *P. falciparum*, *Plasmodium falciparum*; PBMC, Peripheral Blood Mononuclear Cells; PMA, Phorbol Myristate Acetate; TNF, Tumor Necrosis Factor.

5. Aims of the project

This PhD dissertation deals with an antioxidant defense system of *Leishmania infantum*, the $T(SH)_2$/TXN/2-Cys PRX peroxide detoxification pathway. Our interest in this field of research is related with two main aspects. First, due to the uniqueness and importance for parasite metabolism of the trypanothione-dependent system for peroxide detoxification, it is worthy to explore the potential as drug targets of its components. Second, in the face of controversial reports concerning the significance of oxidative stress in *Leishmania* infection control, it is important to better understand the implication of antioxidant defense in host-parasite interaction. More specifically, our project is focused on the two final components of the peroxide removal pathway above-mentioned, the tryparedoxins (TXNs) and the 2-cysteine peroxiredoxins (2-Cys PRXs). We defined three main goals:

1) To genetically validate the *L. infantum* cytosolic TXN as a drug target by establishing its essentiality to parasite survival.

2) To analyze the uncharacterized *TXN*-like sequences of *L. infantum*, determining if they encode active TXN enzymes.

3) To investigate whether the *L. infantum* 2-Cys PRX-dependent antioxidant system is implicated in parasite survival in the host cell, by protecting the parasite from macrophage-derived ROS/RNS.

The results for each topic are organized in independent chapters. Thus, Chapter 2 addresses the essentiality of *Li*TXN1 in the insect and mammalian stages of the parasite. Further characterization of the enzyme is provided by assessment of *Li*TXN1 active secretion in infective parasite forms. Chapter 3 characterizes *Li*TXN3 and *Li*TXN5, including the analysis of the amino acid sequences, enzymatic activity and subcellular

localization. In addition, the phylogeny of the trypanosomatids *TXN* genes is also discussed in this chapter. Finally**,** Chapter 4 reports the phenotypic analysis of parasites overexpressing the cytosolic *Li*TXNPx2, including the evaluation of their infectivity levels in two different host cells.

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Chapter 2

The cytosolic tryparedoxin of *Leishmania infantum* is essential for parasite survival

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Abstract

Leishmania infantum cytosolic tryparedoxin (*Li*TXN1) can be regarded as a potential candidate for drug targeting. This redox active molecule, which belongs to the thioredoxin superfamily, is one constituent of the hydroperoxide elimination cascade in *L. infantum* and may also be involved in other cellular processes such as DNA synthesis or host-parasite interaction. In order to validate *Li*TXN1 as a drug target we have employed a gene replacement strategy. We observed that substitution of both chromosomal *LiTXN*1 alleles was only possible upon parasite complementation with an episomal copy of the gene. Furthermore, contrary to control parasites carrying the empty vector, both the insect and the mammalian stages of *L. infantum* retained the episomal copy of *LiTXN*1 in the absence of drug pressure. These results confirm the essentiality of *LiTXN*1 throughout the life cycle of the parasite, namely in the disease-causing amastigote stage. In addition, the data obtained showed that disruption of one allele of this gene leads only to a 25% reduction in the expression of *Li*TXN1. Even though this does not affect promastigote growth and susceptibility to hydrogen peroxide, ex vivo infection assays suggest that wild-type levels of *Li*TXN1 are required for optimal *L. infantum* virulence.

1. Introduction

Safeguarding the redox status in a cell largely depends on thiol-based mechanisms involving, among other molecules, thioredoxins. Members of the thioredoxin family are present in virtually all organisms (Eklund et al., 1991). These oxidoreductases can be found in different intracellular compartments and extracellularly, where they exhibit diverse functions, from DNA synthesis to immunomodulation (Arnér and Holmgren, 2000). In 1997 a thioredoxin-related protein was described in the protozoan trypanosomatid *Crithidia fasciculata* (Nogoceke et al., 1997), which presented a number

of differences from classical thioredoxins. This enzyme was designated tryparedoxin (TXN) because it was shown to be specifically reduced by trypanothione, a thiol found almost exclusively in trypanosomatids. TXNs are proteins characterized by a typical WCPPCR active site motif and known to be present in all trypanosomatids, including *Trypanosoma brucei* (Lüdemann et al., 1998), *Trypanosoma cruzi* (Lopez et al., 2000) and *Leishmania infantum* (Castro et al., 2004), the agents of sleeping sickness, Chagas' disease and leishmaniasis, respectively. Subsequent biochemical analysis of several TXNs showed that they can transfer reducing equivalents from trypanothione to different acceptor molecules. In this way, TXNs might be implicated in distinct cell functions, namely hydroperoxide and peroxynitrite elimination when the reduced proteins are peroxidases (Wilkinson et al., 2000; Trujillo et al., 2004), DNA synthesis if the acceptor is ribonucleotide reductase (Dormeyer et al., 2001) or kinetoplast replication when TXNs interact with the universal minicircle sequence binding protein (Onn et al., 2004). The postulated involvement of TXNs in different aspects of parasite development, together with these enzymes' specificity for trypanothione, suggest that their inhibition may be exploited for chemotherapy of trypanosomatidal diseases. For this, however, TXNs must be confirmed as essential to the various trypanosomatids. To date, this has only been shown in *T. brucei* using RNA interference (Wilkinson et al., 2003; Comini et al., 2007), a methodology that lacks the robustness of classical gene knockout.

The genome of *L. infantum*, the causative agent of visceral leishmaniasis in Mediterranean areas, presents several tryparedoxin-like sequences, two of which have been previously characterized, namely *Li*TXN2, a mitochondrial enzyme and *Li*TXN1, present in the cytosol (Castro et al., 2004). *Li*TXN1 is the only known molecular partner of the parasite cytosolic peroxidases and occurs both in the vector and in the mammalian stages of *L. infantum* being up-regulated in stationary-phase promastigotes and in

amastigotes (Castro et al., 2004). As previously suggested, this expression pattern may reflect *Li*TXN1 involvement in host-parasite interaction. As a consequence of the macrophage defense response to infection, parasites become exposed to hydroperoxides and to peroxynitrite (Gantt et al., 2001; Mukbel et al., 2007). Increasing the level of *Li*TXN1, and possibly of other components of the parasite hydroperoxide elimination apparatus in the cytosol, may thus facilitate infection. Alternatively, as observed for thioredoxins of other cells (Rubartelli et al., 1992; Billiet et al., 2005), *Li*TXN1 may be released by infective parasites and, directly or indirectly, modulate macrophage response to the parasite. In this report we addressed the essentiality of *Li*TXN1 in the insect and mammalian stages of *L. infantum*, our main objective being to genetically validate *Li*TXN1 as a drug target. In addition, we investigated whether infective parasites are capable of actively secreting this protein.

2. Materials and methods

2.1. Parasite culture

Leishmania infantum promastigotes (strain MHOM/MA/67/ITMAP-263) were cultured at 25ºC in RPMI 1640 medium with Glutamax, supplemented with 10% heat inactivated FBS (iFBS), 50 $U.ml^{-1}$ penicillin, 50 $\mu g.ml^{-1}$ streptomycin (complete RPMI medium) and 20 mM HEPES sodium salt pH 7.4. To obtain axenic amastigotes, 5 x 10^6 stationary phase promastigotes were resuspended in 5 ml of medium for axenic amastigotes (MAA) medium supplemented with 20% iFBS, 2 mM Glutamax, 11.5 μM hemin (MAA20 medium adapted from Sereno and Lemesre, 1997) and kept for 5 to 7 days at 37ºC, 5% CO_2. Axenically grown amastigotes were maintained in MAA20 medium at 37ºC, 5% CO_2.

2.2. Transfection constructs

The *LiTXN*1 single knockout construct was generated in pGL345 vector (Mottram et al., 1996a). PCR amplification of part of the 3' untranslated region (UTR) of *LiTXN*1 was performed using as template a cosmid clone containing the *LiTXN*1 locus (Castro et al., 2004), and primers 5'-CGC**GGATCC**GGCGACTGTGCGGGTGA-3' and 5'-GGA**AGATCT**<u>**GAGCTC**</u>GAAGCTGTGTCGGCGTGT-3', (restriction sites in bold; the second restriction site is underlined). The 932 bp PCR fragment was subcloned downstream to the hygromycin-resistance gene (HYG). A fragment of the 5'UTR of *LiTXN*1 was obtained from the same template with primers 5'-CCC**AAGCTT**GTTATCCTTGCCGTCCTCT-3' and 5'-CGG**ACTAGT**TGCTGTGCACGGGGAGG-3'. The resulting 1100 bp PCR product was subcloned upstream of the HYG gene. The construct to target the second *LiTXN*1 allele was obtained by replacing the HYG gene by the phleomycin-resistance gene (BLE), excised from pGL726 (Mottram et al., 1996a) by *Spe*I/*Bam*HI digestion. Previous to transfection both constructs were linearized with *Hind*III and *Sac*I restriction enzymes. The episomal version of *LiTXN*1, used for complementation studies, was generated in the pTEX-NEO vector (Kelly et al., 1992). The *LiTXN*1 open reading frame (ORF) was PCR-amplified from the cosmid clone referred to above with primers 5'-CGC**GGATCC**ATGTCCGGTGTCAGCAAG-3' and 5'-CACCG**CTCGAG**TTACTCGTCTCTCCACGG-3'.

2.3. Electroporation of L. infantum

Log-phase promastigotes were electroporated at 450 V and 350-400 μF with 1 to 5 μg of linearized or circular DNA (for gene replacements or episomes, respectively) as described elsewhere (Beverley and Clayton, 1993). Selective plates contained 15 $\mu g.ml^{-1}$

geneticin (G418), 15 μg.ml^{-1} hygromycin B or/and 17.5 μg.ml^{-1} phleomycin. Individual colonies were picked and transferred to 24-well plates containing 1 ml complete RPMI medium with selective drugs (50 μg.ml^{-1} G418, 15 μg.ml^{-1} hygromycin B, 17.5 μg.ml^{-1} phleomycin) and cultured for further analysis.

2.4. Genomic DNA analysis

Genomic DNA was isolated from *L. infantum* cells following the proteinase K protocol (Kelly, 1993). Southern blots were done according to standard protocols (Sambrook, 1989). Probe labeling, hybridizations and washings followed the Gene Images AlkPhos Direct Labeling protocol (GE Healthcare). Detection was carried out with the CDP-Star Detection reagent (GE Healthcare).

2.5. Western blotting

Leishmania infantum protein extracts were obtained as described elsewhere (Castro et al., 2004), resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk and probed against the adequate primary antibodies. Horseradish peroxidase (HRP)-labeled imunoglobulins were used as secondary antibodies and detection was achieved using ECL detection reagents (GE Healthcare).

2.6. Estimation of the concentration of LiTXN1 in L. infantum

Extracts of *L. infantum* promastigotes and amastigotes and increasing quantities of *Li*TXN1 recombinant protein (to be used as standards) were separated on a 12% SDS-polyacrilamide gel and electroblotted onto nitrocellulose membranes. The blot was then incubated with an anti-*Li*TXN1 antibody previously adsorbed with 20 μg of His-tagged *Li*TXN2 recombinant protein to prevent cross reactivity both with the His-tag epitope in

recombinant *Li*TXN1 and with endogenous *Li*TXN2 in the parasite protein extracts. The intensity of the bands was quantified using a GS-800 densitometer and the Quantity One software (BioRad). The cellular concentration of TXN1 in the different parasite extracts was estimated considering cell volumes of 75 femtoliters for promastigotes (Sousa Silva et al., 2005) and of 15 femtoliters for amastigotes (based on cell measurements).

2.7. Flow cytometry

Leishmania infantum promastigotes were fixed with 4% paraformaldehyde (w/v) in PBS and permeabilized with 0.1% Triton X-100 in PBS. Subsequently, cells were incubated with anti-*Li*TXN1 (previously adsorbed with recombinant *Li*TXN2, Castro et al., 2004) or anti-*Li*mTXNPx (Castro et al., 2002) primary antibodies, followed by FITC-conjugated secondary antibodies. Flow cytometry analysis was performed in a FACSCalibur (BD Biosciences) and at least 30.000 events were analyzed using the CellQuest software.

2.8. Hydrogen peroxide sensitivity assay

The growth inhibitory effect of H_2O_2 on wild-type and transformed parasites was analyzed as previously described (Castro et al., 2002). Briefly, exponentially grown cultures were seeded at 10^6 ml^{-1} in 2 ml complete RPMI medium in 24-well plates in the absence of selective drugs and incubated for 24 h at 25ºC. Different H_2O_2 concentrations were then added to each well. Four to 5 days later parasite growth was measured by absorbance reading at 600 nm.

2.9. Macrophage infections

Peritoneal macrophages were obtained from National Marine Research Institute (NMRI) mice (Charles River, France) by peritoneal lavage. Animals were housed under specific pathogen-free conditions at the Instituto de Biologia Molecular e Celular animal facility (Porto, Portugal). Euthanasia was performed in a 20% isofluorane atmosphere. Macrophages, recovered from the peritoneal cavity, were seeded at 4 x 10^5 cells per well in 24-well plates and allowed to adhere for at least 2 h. The culture medium consisted of DMEM Glutamax complemented with 20% iFBS, 50 $U.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin. Upon washing with pre-warmed Hanks Balanced Salt Solution (HBSS) , macrophages were incubated with stationary-phase promastigotes at a parasite:macrophage ratio of 10:1, for 3 h at 37ºC, 5% CO_2. Non-internalized parasites were removed by gentle washing with pre-warmed HBSS medium and the macrophages were further cultured for 24 h and 144 h. Cells were then fixed with methanol, stained with Diff Quick, and the number of infected cells and amastigotes per cell determined by optical microscopy. A minimum of 400 macrophages was counted for each experiment.

2.10. NMRI in vivo infections

NMRI mice were infected by i.p. injection with 10^8 stationary-phase promastigotes in PBS. At determined time points, animals were sacrificed, their spleens and livers homogenized in complete RPMI medium and differentiated promastigotes were cultured for further analysis. Animal experiments were approved by IBMC ethics committee and by Direcção Geral de Veterinária (DGV).

2.11. Metabolic labeling and immunoprecipitation

For metabolic labeling and subsequent immunoprecipitation, 1.2 x 10^8 parasites were washed three times in PBS and resuspended in methionine-free RPMI medium for 1 h at 25ºC. After starvation, cells were washed in methionine-free RPMI medium, labeled for 1 h at 25ºC with 125 μCi/ml [^{35}S]-methionine and cultured for 2 h, 4 h and 6 h in complete medium at 25ºC. Upon centrifugation, supernatants were collected (400 μl) and cell pellets lysed in an appropriate buffer containing protease inhibitors [50 mM Tris-HCl pH7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 2 mM Phenylmethylsulfonylfluoride (PMSF), 1 μM Pepstatin A]. Resulting cell lysates and cell supernatants were further processed to immunopurifiy *Li*TXN1. Briefly, following pre-clearing with a mixture of Protein A, Protein G and normal rat serum for 1 h at 4ºC, samples were incubated with 4 μl of anti-*Li*TXN1 polyclonal antibody in immunoprecipitation buffer (10 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, 0.5% NP-40, 0.2 mM PMSF) for 30 min at 4ºC. A mixture of Protein A and Protein G was added to the samples and these were further incubated for 4 h at 4ºC. Upon centrifugation, beads were washed three times in immunoprecipitation buffer. Finally, reducing sample buffer was added to the beads, these were boiled for 5 min and proteins separated on a 15% polyacrylamide SDS-PAGE gel. After fixation (40% methanol, 10% acetic acid) the radioactive signal was enhanced using Amplify solution (GE Healthcare). Labeled proteins were visualized on dried gels using a Phosphor Storage System (Typhoon). The relative amounts of *Li*TXN1 were determined by densitometry using the Quantity One software (BioRad). To calculate the number of parasites lysed during metabolic labeling, the activity of glucose-6-phosphate dehydrogenase (GPDH) detected in cell supernatants was compared with that of cell lysates prepared from known amounts of parasites. Briefly, GPDH activity was measured by monitoring NADPH formation at 340 nm in a reaction

mixture containing cell samples and 0.6 mM $NADP^+$, 10 mM D-glucose-6-phosphate in 25 mM KH_2PO_4/K_2HPO_4 pH 7.4 buffer.

2.12. Statistical analysis

Data sets were compared by independent-samples *t*-test using SPSS software 16.0.

3. Results

3.1. Estimation of the expression of LiTXN1 in L. infantum promastigotes and amastigotes

In a previous report we observed that the expression of *Li*TXN1 increases throughout the *L. infantum* life cycle (Castro et al., 2004). Here we used Western blot analysis to investigate the concentration of the protein in the different parasite phases. For this, the cellular protein extracts (Fig. 1A) were compared with increasing amounts of recombinant *Li*TXN1 protein (Fig. 1B). To prevent recognition of the His-tag in the recombinant protein by the anti-*Li*TXN1 serum, this was adsorbed with the recombinant His-tagged *Li*TXN2 protein (Fig. 1C) before use. This treatment also prevented recognition of *Li*TXN2 in the parasite extracts. As shown in Fig. 1, a protein content varying from 1 to 2.5 μM and of 40.7 μM was estimated for promastigotes and amastigotes, respectively. As expected, the concentration of *Li*TXN1 is lower in the insect stage than in the mammalian stage.

3.2. Production of LiTXN1 null mutants requires previous complementation with an extrachromosomal copy of the gene

To investigate the relevance of *Li*TXN1 in *L. infantum*, we attempted to generate homozygous deletion mutants. Considering that *LiTXN*1 is a single copy gene, two linear DNA fragments containing either the HYG or the BLE genes, flanked by part of the

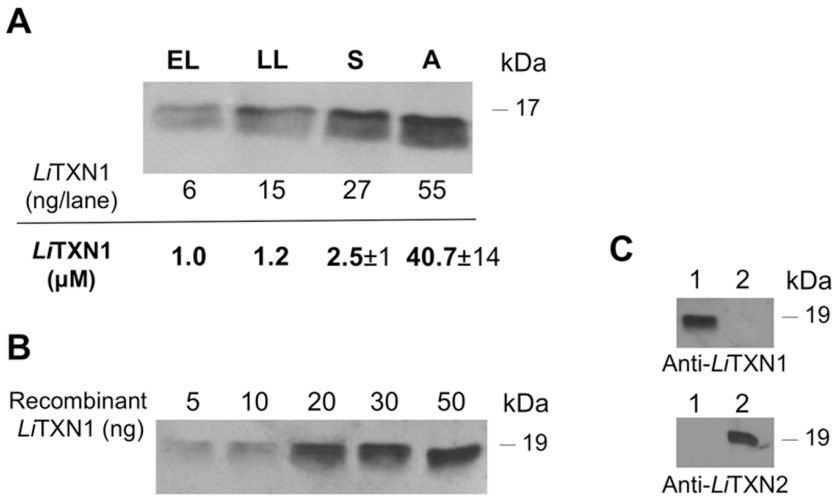


Fig. 1. Estimation of *Li*TXN1 protein concentration in *Leishmania infantum* parasites. (A) Western blot comparing 20 µg of protein extract from early log- (EL), late log- (LL) and stationary-phase (S) promastigotes and from axenic amastigotes (A). (B) Increasing quantities of recombinant *Li*TXN1 were used as calibration standards to estimate the concentration of protein in parasites. Protein bands were analyzed by densitometry and protein concentration was assessed based on parasite volumes of 75 and 15 femtoliters for promastigotes and amastigotes, respectively. (C) Adsorbed anti-*Li*TXN1 serum was tested for cross-reactivity by Western blotting performed on 50 ng of recombinant *Li*TXN1 (lane 1) and *Li*TXN2 (lane 2). Adsorption of the anti-*Li*TXN1 antibody was performed with 20 µg of recombinant *Li*TXN2. After stripping the blot was re-hybridized with anti-*Li*TXN2 antibody previously adsorbed with 20 µg of recombinant *Li*TXN1. Protein concentration in early- and log-phase promastigotes was obtained with one experiment, while values for late promastigotes and amastigotes refer to the mean ± S.D. of three different assays.

upstream and downstream regions of *LiTXN*1, were assembled and introduced successively into promastigotes by electroporation (Fig. 2A). After the first transfection round with the HYG construct several hygromycin-resistant clones were selected. Southern blot analysis of genomic DNA from these clones confirmed the correct integration of the marker gene into the chromosome and effective substitution of one *LiTXN*1 allele (data not shown). The morphology of these single knockout mutant cells (Δ*txn*1*::HYG*/*TXN*1, hereafter referred to as SK, for single knockout) and their growth rate presented no apparent differences towards wild-type WT parasites, indicating that one copy of the *LiTXN*1 gene is sufficient to ensure promastigote growth in culture. Next, the remaining allele was replaced by the BLE targeting fragment. Southern blotting of genomic DNA from six clones resistant to hygromycin and phleomycin revealed that both selection markers were integrated in the

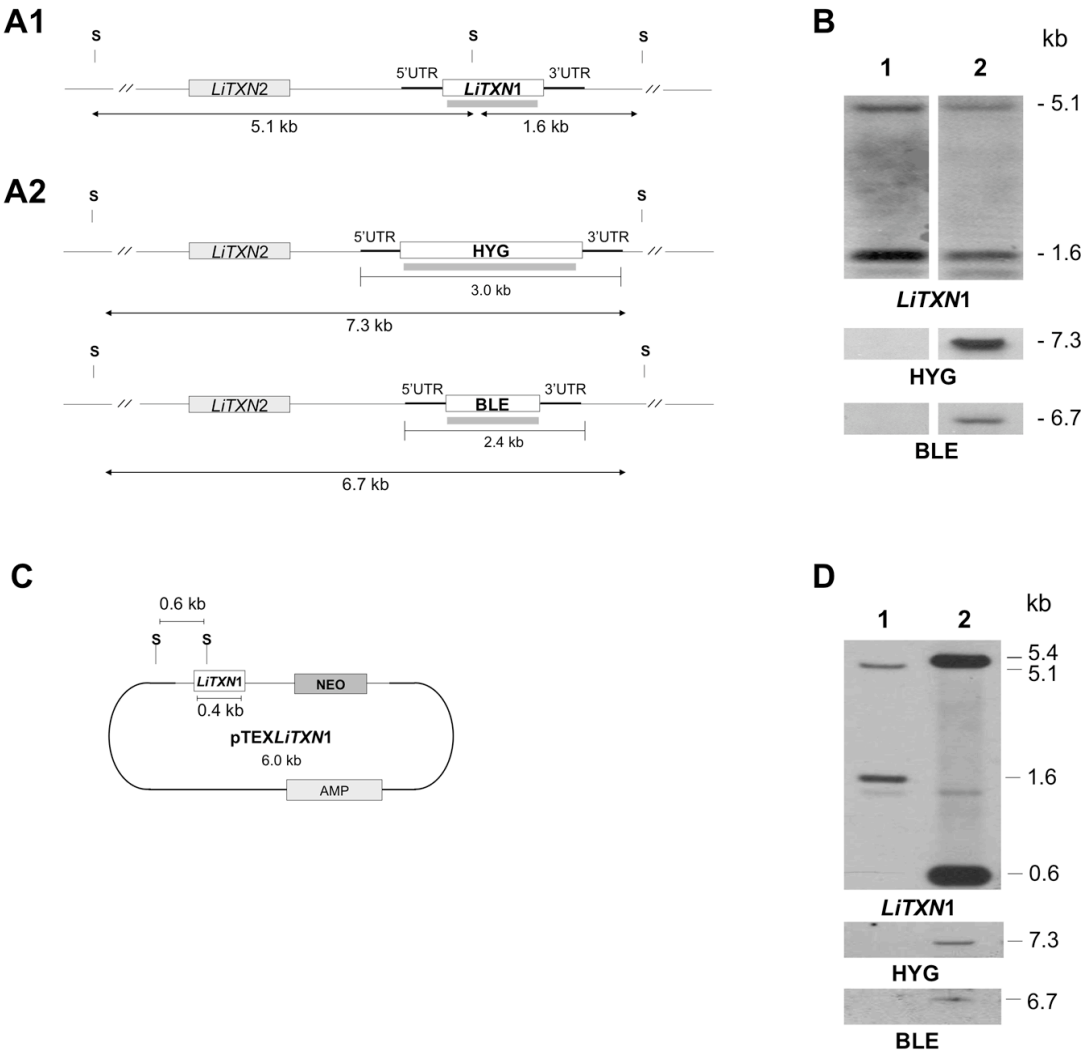


Fig. 2. Deletion of the second *LiTXN*1 allele from the *Leishmania infantum* genome requires previous complementation with an extrachromosomal copy of *LiTXN*1. (A) Map of the *LiTXN*1 locus before (A1) and after (A2) gene replacement. Part of the 5´- untranslated region (UTR) and 3´-UTR sequences of the *LiTXN*1 gene were used to flank the hygromicin (HYG) and the phleomycin (BLE) selection markers. Sizes of the transfection fragments are shown below each resistance cassette. The HYG construct was used to generate single knockout mutants for *LiTXN*1. The BLE construct was introduced into the single knockout cell line to target the second allele. Predicted restriction fragments sizes for diagnosis are indicated. Grey boxes below *LiTXN*1, HYG and BLE represent the gene-specific probes used in Southern blot analysis. (B) Southern blot of *Sac*I digested genomic DNA of wild-type (WT) *L. infantum* promastigotes (lane 1) and a representative mutant clone obtained after transfection with both drug resistance constructs (lane 2). The blot was successively hybridized with the *LiTXN*1, HYG and BLE probes as indicated. When hybridized with the *LiTXN*1 probe, both WT and mutant parasites presented two bands of ~5.1 kb and ~1.6 kb, respectively, indicating preservation of the *LiTXN*1 gene in the mutant clone. Integration of the selection markers into the *LiTXN*1 locus of the mutant cell line was confirmed by the appearance of a ~7.3 kb and a ~6.7 kb band upon hybridization with HYG and BLE probes, respectively. (C) Map of the pTEX*LiTXN*1 episome. (D) Southern blot analysis of *Sac*I digested genomic DNA of WT (lane 1) and complemented double knockout (CDK) promastigotes (lane 2) (one representative clone out of six is shown). The blot was successively hybridized with the *LiTXN*1, HYG and BLE probes as indicated. WT parasites present two specific bands of ~5.1 kb and ~1.6 kb, when hybridized with the *LiTXN*1 probe, which correspond to the genomic copy of the gene. The CDK clone presents two bands of ~5.4 kb and ~0.6 kb that correspond to the pTEX*LiTXN*1 copy. Integration of both selection markers into the *LiTXN*1 locus of the mutant clone was confirmed by the appearance of the ~7.3 kb and ~6.7 kb bands resulting from hybridization with HYG and BLE probes, respectively. *Sac*I (S) restriction sites are indicated on both maps.

*LiTXN*1 locus, as expected (Fig. 2A and B). Despite this, one copy of the gene persisted in these parasites (Fig. 2B). This genotype provided the first indication that *LiTXN*1 is essential to *L. infantum* promastigotes.

To discard the possibility that technical difficulties were preventing the generation of *LiTXN*1 null mutants, SK promastigotes were transfected with the trypanosomatid expression vector pTEX carrying a copy of the *LiTXN*1 gene, prior to targeting the second allele (Fig. 2C). In this case, triple drug-resistant clones were readily obtained. These mutants (Δ*txn*1*::HYG*/Δ*txn*1*::BLE* [pTEX*LiTXN*1], hereafter referred to as CDK, for complemented double knockout) had successfully substituted the second *LiTXN*1 allele by the phleomycin-resistance marker (Fig. 2D). From this set of experiments we concluded that the presence of the episomic *LiTXN*1 gene is necessary to eliminate both endogenous alleles.

3.3. Maintenance of the pTEXLiTXN1 vector in the absence of drug pressure confirms gene essentiality in promastigotes

As described for other *Leishmania* episomes (Boucher et al., 2004), pTEX is an unstable vector which is lost over time when parasites are cultured in the absence of selective drug (Kelly et al., 1992). Here, we made use of this feature of pTEX to assess the essentiality of *LiTXN*1 to *L. infantum* promastigotes. To that end, CDK clones were grown without G418 for several months and were then evaluated for the presence of pTEX*LiTXN*1 by Southern blotting and by G418-resistance assays. As expected, after 2 months in the absence of drug, control parasites (carrying the empty vector) had lost a significant part of their pTEX copies (Fig. 3A and B). On the contrary, CDK clones kept the vector even after 10 months of culture without G418 (fig. 3A). During this period CDK promastigotes were continuously kept in log-phase (by 3 weekly passages), a stage in

which *Li*TXN1 expression is downregulated (Fig. 1 and Castro et al., 2004). Expression of *Li*TXN1 in CDK clones was further confirmed by Western blot analysis (Fig. 3C). Altogether, these results suggest that *Li*TXN1 expression provides the pressure required for CDK cells to maintain the pTEX*LiTXN1* plasmid in the absence of G418 and, thus, confirm previous suggestions that *Li*TXN1 is essential for *L. infantum* promastigotes. From these experiments we can further conclude that even in a life stage in which *Li*TXN1 is poorly expressed, the protein is crucial for survival.

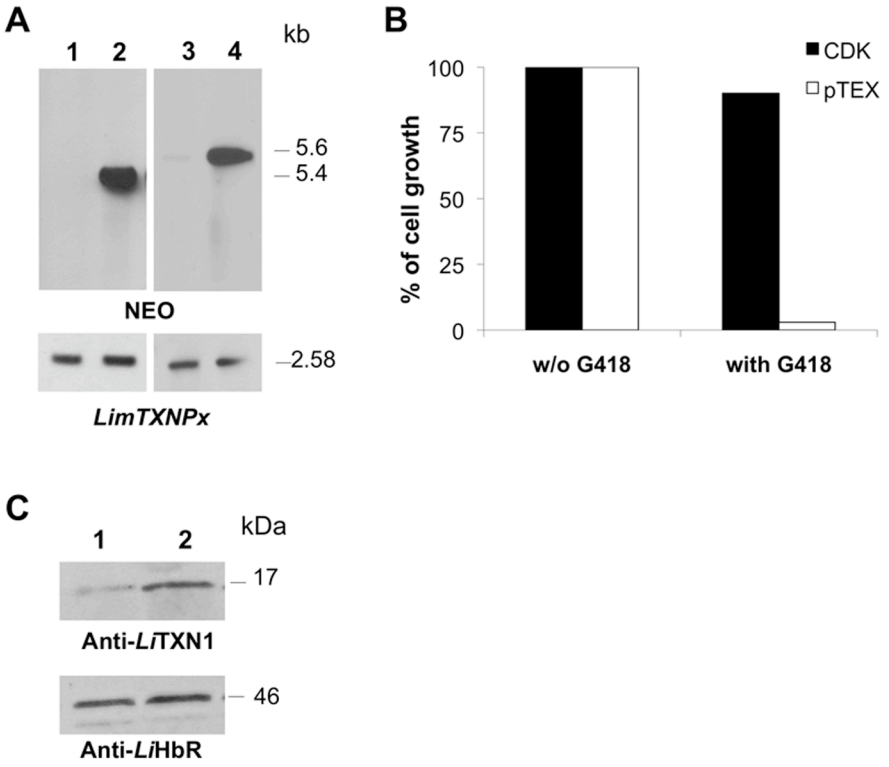


Fig. 3. Complemented double knockout (CDK) *Leishmania infantum* promastigotes maintain the episomic copy of *LiTXN*1 gene in the absence of drug pressure. (A) Southern blot analysis of *Sac*I digested genomic DNA from wild-type (WT) promastigotes (lane 1), CDK cultured without G418 drug for 6 months (lane 2) and pTEX control promastigotes carrying the empty vector, maintained for 2 months without (lane 3) and with G418 (lane 4), using the neomycin resistance gene (NEO) and the *LimTXNPx* gene (loading control) as probes. (B) Effect of G418 (50 μg.ml^{-1}) on the replication of CDK (black bars) and pTEX control promastigotes (white bars) that had been kept in the absence of drug pressure for 10 and 5 months, respectively. Four to 6 days after the addition of G418, the number of promastigotes was counted and the data expressed as a percentage of parasite replication in control cultures without drug addition. One representative experiment out of two is shown. (C) Western blot analysis of *Li*TXN1 expression on WT (lane 1) and CDK cells cultured without G418 for 6 months (lane 2). The blot was successively hybridized with antibodies anti-*Li*TXN1 and anti-*Li*HbR as loading control (Carvalho et al., 2009).

3.4. LiTXN1 is essential to the amastigote stage

For any anti-*Leishmania* drug to be effective, it must target the amastigote stage. Therefore, *LiTXN*1 essentiality was also assessed in the parasite mammalian stage. In a first set of experiments NMRI mice were infected with two CDK mutant clones (previously maintained without G418 for 4 months). At 3 months p.i. parasites were recovered from spleens and livers, differentiated to promastigotes, and tested for the presence of the pTEX*LiTXN*1 vector. As shown by Southern blot analysis, the episome is conserved in the CDK mutant clones recovered from both organs (Fig. 4), suggesting that *Li*TXN1 is also essential to amastigotes. Corroborating these results is the observation that CDK axenically grown amastigotes maintained the plasmid when cultured for over 7 months in the absence of selective drug (data not shown).

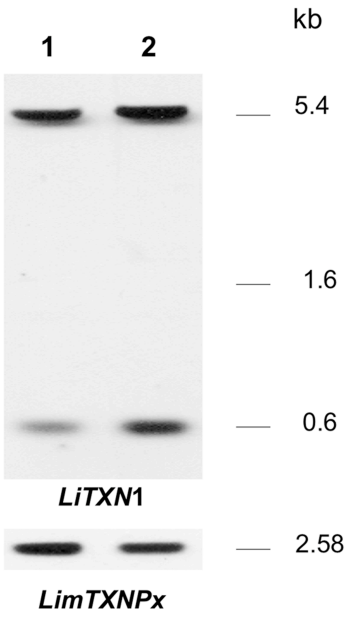


Fig. 4. Complemented double knockout (CDK) *Leishmania infantum* amastigotes retain the *LiTXN*1 gene as an episomal copy throughout a murine infection. Southern blot analysis of *Sac*I digested genomic DNA of a representative CDK clone recovered from the spleen (lane 1) and the liver (lane 2) of mice, three months p.i., using the *LiTXN*1 and the *LimTXNPx* (loading control) genes as probes.

3.5. Phenotypic analysis of LiTXN1 single knockout parasites

To gain insight into the function of *Li*TXN1, we carried out a phenotypical analysis of knockout mutants. Given the impossibility to produce double knockouts, this analysis was performed with heterozygous parasites. For other single copy genes of trypanosomatids, abrogation of one allele often leads to a 50% decrease in protein expression, which can translate into a particular phenotype (Dumas et al., 1997; Allaoui et al., 1999). Before proceeding with the phenotypical characterization of *LiTXN*1 SK mutants, we quantified the relative amount of *Li*TXN1 present in these parasites by flow cytometry. The results, shown in Fig. 5, revealed that these mutants express only 25% less *Li*TXN1 than WT parasites. An antibody directed to *Li*mTXNPx (Castro et al., 2002) was used as a control and no differences in the protein level were observed between both parasite lines (Fig. 5, inset). Similar results were apparent from Western blot analysis (data not shown), further confirming that SK mutants have diminished *Li*TXN1 expression, albeit not to half of WT levels.

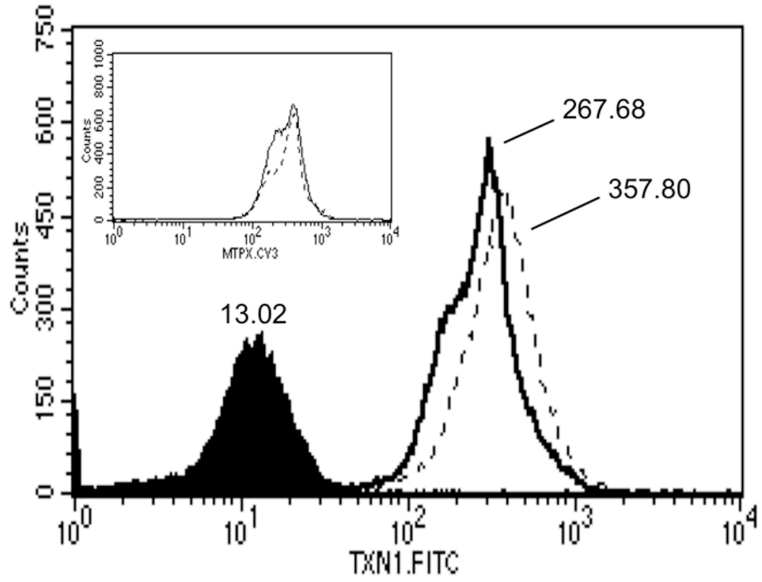


Fig. 5. Comparison of *Li*TXN1 protein expression between single knockout (SK) and wild-type (WT) *Leishmania infantum* parasites. Flow cytometry showing the levels of *Li*TXN1 in stationary-phase WT (dashed line) and SK (filled line) promastigotes. Parasites were fixed, permeabilized and incubated with anti-*Li*TXN1 antibody followed by FITC-conjugated goat anti-rat. Mean values for fluorescence are shown next to each curve. Normal rat serum (NRS) was used as negative control (filled curve) and anti-*Li*mTXNPx as internal control (inset).

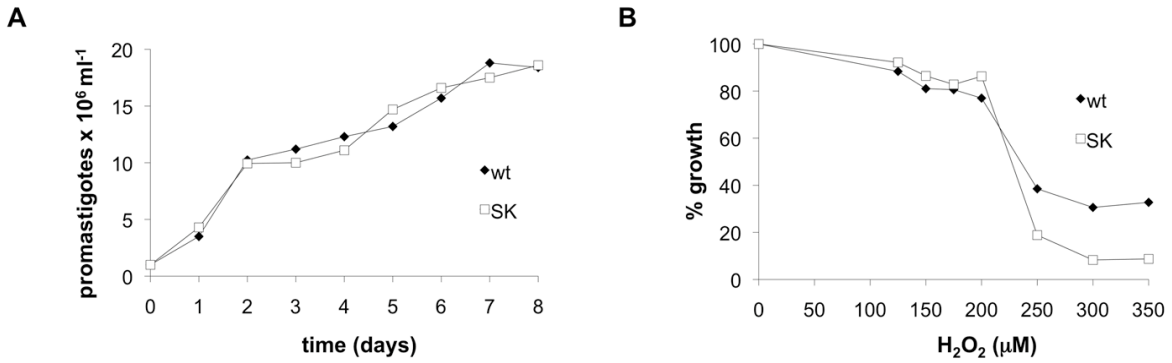


Fig. 6. Single knockout (SK) and wild-type (WT) promastigotes of *Leishmania infantum* are similar in terms of growth rate and resistance to hydrogen peroxide. (A) Cell growth was registered daily for 8 days by counting promastigotes in culture. (B) Parasites were exposed to a bolus of H_2O_2 and 5 days later cell culture density was assessed spectrophotometrically at 600 nm. Data are expressed as a percentage of parasite growth in cultures with no H_2O_2. Graphs in (A) and (B) refer to one representative experiment out of two.

For the phenotypical analysis of SK mutants, these were compared with WT parasites in terms of growth rate, sensitivity to H_2O_2 and virulence in an ex vivo infection assay. As shown in Fig. 6A, the growth curve of SK promastigotes, recorded throughout 8 days, was the same as that of WT parasites. Likewise, SK and WT promastigotes behaved similarly upon exposure to a bolus of H_2O_2 (Fig. 6B). A distinct phenotype for SK mutants only became apparent when parasites were analyzed for their ability to invade and persist in NMRI peritoneal macrophages. In this case, SK mutants exhibited decreased infectivity compared with WT cells, which was evident both at the level of the percentage of infected macrophages and at the number of amastigotes per macrophage (Fig. 7A and B). From this group of experiments we can conclude that decreasing *Li*TXN1 expression levels to 75% of that of WT cells has no impact on promastigote growth rate and susceptibility to H_2O_2. However, ex vivo infection assays suggest that WT levels of *Li*TXN1 are required for optimal *L. infantum* virulence.

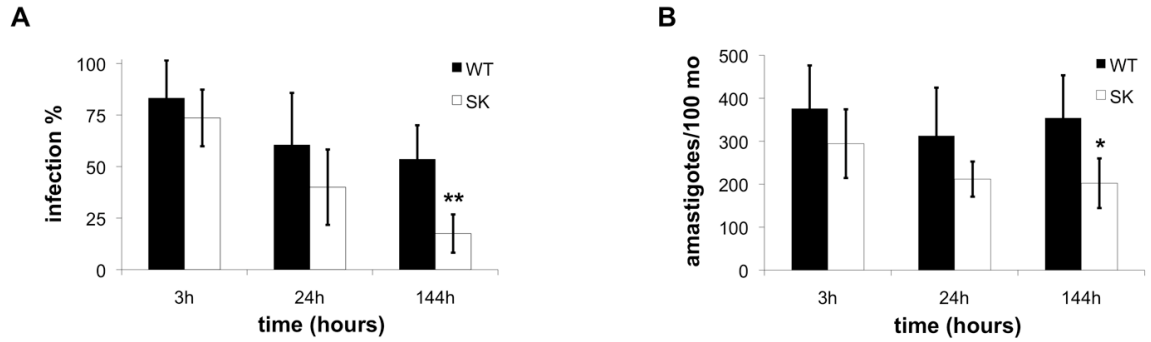


Fig. 7. Single knockout (SK) *Leishmania infantum* parasites are less infective than wild-type (WT) cells in an ex vivo infection model. National Marine Research institute (NMRI) mice macrophages were incubated with stationary-phase WT (black bars) or SK promastigotes (white bars) at a ratio of 10 parasites per macrophage. At 3, 24 and 144 h after infection cells were stained with Diff Quick and the percentage of infected macrophages (A) and the total number of amastigotes within 100 infected macrophages (B) was assessed microscopically. Results are the mean of the values obtained in three independent experiments ± S.D. Statistical significance $^{**}P < 0.01$ and $^{*}P < 0.05$.

3.6. Infective promastigotes do not secrete LiTXN1

Following the evidence that thioredoxins can be secreted by a variety of cells (Rubartelli et al., 1992), we analyzed the possibility of *Li*TXN1 active secretion by *L. infantum*. To this end, parasites were labeled with [^{35}S]-methionine and, at different time points, cell supernatants were tested for the presence of the protein. Using this approach, we could not find evidence for secretion of *Li*TXN1. As depicted in Fig. 8, only very low amounts of *Li*TXN1 could be detected in cell supernatants which likely result from cell lysis. In fact, in these experiments, at least 1.5 to 3% of cells lysed and released their cytosolic content into the medium. This percentage was calculated based on the activity of GPDH, a strictly cytosolic and glycosomal enzyme, detected in cell supernatants. Another important observation is that the levels of *Li*TXN1 present in the medium did not increase over time, as expected for a protein that is being actively secreted (Fig. 8A). Moreover, control experiments in which the supernatants of 0 h post-labeling were analyzed already showed the presence of *Li*TXN1 (data not shown). Together these observations argue against *Li*TXN1 secretion by *L. infantum* promastigotes.

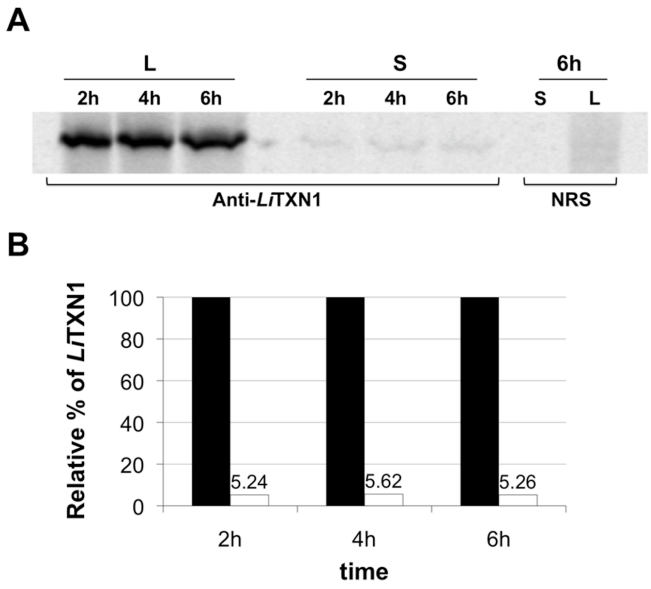


Fig. 8. Stationary-phase *Leishmania infantum* promastigotes do not secrete *Li*TXN1 protein. (A) Promastigotes were labeled with [^{35}S]-methionine and *Li*TXN1 immunoprecipitated with a specific anti-*Li*TXN1 polyclonal antibody. The specificity of the antibody was controlled with normal rat serum (NRS). Cell supernatants (S) contain proteins released to the medium at different time points (2 h, 4 h and 6 h). Cell lysates (L) at the same time points were used as controls. Each lane corresponds to 4 x 10^7 parasites. (B) Levels of *Li*TXN1 detected in cell supernatants (white bars), obtained from scanning densitometry and expressed as a percentage of the protein present in cell lysates (black bars). Data labels are shown for cell supernatants.

4. Discussion

The currently available chemotherapy for leishmaniasis and other trypanosomatidal diseases presents a number of limitations including lack of efficiency, toxicity and development of resistance. As a consequence, new drugs need to be developed (Chappuis et al., 2007). To prevent unwanted cross-reactivity with host proteins, these should preferentially exploit differences between the biochemistry of trypanosomatids and their hosts. One peculiarity of these parasites is the use of trypanothione (Fairlamb et al., 1985; Fairlamb and Cerami, 1992). This dithiol replaces many of the functions of glutathione and is fundamental for *T. brucei* survival (Comini et al., 2004; Ariyanayagam et al., 2005). Therefore, enzymes interacting with trypanothione have long been suggested to provide adequate targets for drug design against the three medically relevant trypanosomatids. An

example is trypanothione reductase, the flavoprotein that maintains trypanothione in its reduced state, which is undergoing extensive study with a view to develop adequate inhibitors (e.g. Martyn et al., 2007). Tryparedoxin is an enzyme that in trypanosomatids also reacts with trypanothione, working as a redox shuttle between the dithiol and enzymes that display distinct functions, namely TXN-dependent peroxidases, ribonucleotide reductase and universal minicircle sequence binding protein. This work is focused on the cytosolic tryparedoxin of *L. infantum*, *Li*TXN1.

For *Li*TXN1 to enter the pipeline of inhibitor production it must to be amenable to specific inactivation. Previous studies have addressed this issue, using either *Li*TXN1 or homologous enzymes of other trypanosomatids. One feature that distinguishes TXNs from host cell thioredoxins is their dependence on trypanothione as a source of reducing equivalents. Other reductants present in the host, such as glutathione, glutathione reductase, thioredoxin and thioredoxin reductase react poorly or not at all with TXNs (Gommel et al., 1997; Nogoceke et al., 1997; Steinert et al., 2000; Castro et al., 2004). The molecular basis of TXNs specificity towards trypanothione has been dissected for the TXN enzymes of *C. fasciculata* (Alphey et al., 1999; Hofmann et al., 2001) and of *T. brucei* (Alphey et al., 2003), either by three-dimensional analysis or by site-directed mutagenesis (Steinert et al., 2000). These studies pinpointed differences between TXNs and thioredoxins and highlighted the key residues involved in the reactivity of TXNs with trypanothione. Despite TXNs' unique features, their inactivation by specific drugs may be challenging. Indeed, as argued by Comini and coworkers (2007), the active site of TXNs located on the surface of the protein may prevent inhibitors from binding tightly to the enzyme. Nevertheless, pharmacological inhibition of thioredoxin, whose overall structure and mechanism of action are similar to those of TXNs, has been reported and is considered one valid goal in cancer research (Pallis et al., 2003).

Apart from presenting unique traits, an enzyme can only be accepted as a drug target if the parasite is unable to induce compensatory strategies to overcome its absence. Here we unequivocally demonstrate that this is the case for the cytosolic tryparedoxin of *L. infantum* in both life cycle stages. Firstly, all attempts to delete the second *LiTXN*1 allele were unsuccessful despite the fact that the targeting construct correctly integrated into the genome. It is well-known that *Leishmania* can avoid full deletion of essential genes by undergoing genetic rearrangements such as chromosome duplication (Cruz et al., 1993; Mottram et al., 1996b; Dumas et al., 1997) or amplification of the targeted locus (Grondin et al., 1998; Genest et al., 2005). Although we did not dissect the mechanisms responsible for persistence of *LiTXN*1 in these parasites, it is likely that one of those has occurred in this case as a means to counteract the detrimental consequences of the loss of the enzyme. Corroborating this hypothesis, the second allele of the gene could easily be deleted upon complementation with an extrachromosomal copy of *LiTXN*1. Definitive evidence for the essential role of this protein in the parasite mammalian stage of *L. infantum* came from the observation that the plasmid bearing the gene is maintained in the absence of drug pressure in both axenic amastigotes and amastigotes recovered from mice 3 months after infection. In conclusion, our data show that no alternative pathways exist in *L. infantum* to compensate the loss of *LiTXN*1 and thus genetically validate the protein as a drug target.

When considering the likelihood of a protein as a drug target it is relevant to know the level to which it is present and how much it must be inhibited in order to eliminate the parasite. In *L. infantum* the concentration of *Li*TXN1 was observed to be lower than the ones referred to for other Kinetoplastida (Nogoceke et al., 1997; Wilkinson et al., 2002; Comini et al., 2007; König and Fairlamb, 2007). Still, in amastigotes in particular, *Li*TXN1 is expressed at an elevated level. The non-existence of RNA interference (RNAi) systems in *Leishmania* precluded us from investigating the minimum levels of protein compatible

with parasite survival. We observed, however, that deletion of one *Li*TXN1 allele was accompanied by a reduction of only 25% in the level of protein and not the usual 50% (Dumas et al., 1997; Allaoui et al., 1999). This finding indicates that *L. infantum* parasites can, to a certain extent, modulate the level of this enzyme and may introduce a challenge to the successful therapeutic inhibition of the enzyme. At present we cannot attribute the increased *Li*TXN1 expression in SK cells to specific regulatory mechanisms as we have not investigated its molecular basis. However it is of interest that a previous study suggested the occurrence of coordinated regulation between several components of the hydroperoxide detoxification system (Hsu et al., 2008).

In addition to validating an enzyme as a drug target, gene abrogation studies can help establish the function(s) of the protein in parasite development. In biochemical assays, the cytosolic TXN of *T. brucei* functions as a reductant of the enzyme ribonucleotide reductase (Dormeyer et al., 2001). Experimental evidence proving this in *Leishmania* spp. is lacking, however the similarity between both organisms suggests that the same occurs with *Li*TXN1. In an attempt to directly test whether *Li*TXN1 is also implicated in the synthesis of DNA precursors and hence in *L. infantum* replication, SK mutants with diminished protein expression were compared with WT parasites in terms of growth rate. The results, however, showed no differences between both parasite strains probably because it was impossible to downregulate *Li*TXN1 expression below 75% and, therefore, to reach the protein threshold under which cell division becomes impaired. It is noteworthy that as log-phase dividing cells, CDK promastigotes and amastigotes did not lose the *LiTXN*1-carrying episome even when maintained for long periods in the absence of selective drug. This could be indicative that this enzyme is involved in cell division.

Cytosolic tryparedoxins have been shown to function as key players in the reduction of different peroxidases. Contrary to what happens with cell division,

involvement of *Li*TXN1 in hydroperoxides elimination is well documented (Castro et al., 2004). Based on the pattern of expression and on the localization of this enzyme, we have previously proposed that the major function of *Li*TXN1 would be to shield parasites against host-derived hydroperoxides, such as H_2O_2, produced as a consequence of parasite phagocytosis by macrophages (reviewed in Lambeth et al., 2007). Under such conditions, there will be an increased demand for the activity of the parasite cytosolic peroxidases and the level of *Li*TXN1 present in log-phase promastigotes may not be sufficient to reduce those. Upregulation of *Li*TXN1 could thus constitute a developmental adaptation associated to the transition from infective promastigote to intracellular amastigote. Single knockout and WT stationary-phase promastigotes were therefore tested for their capacity to infect and survive in macrophages. However, although we did observe a trend towards a decreased infectivity of mutant parasites, these cells were not more susceptible towards H_2O_2 added exogenously at concentrations similar to those the parasites encounter in the macrophage (Melo et al., 2003). In fact, at 75% of its normal level, *Li*TXN1 does not limit the capacity of stationary-phase promastigotes to deal with an increase in hydroperoxides. Therefore, the data obtained do not permit attribution of decreased infectivity of SK promastigotes to a lower resistance to intramacrophagic H_2O_2. The reason why WT levels of *Li*TXN1 seem to be required for optimal parasite infectivity is also unlikely to be due to a decreased capacity of amastigotes to divide as, at 24 h p.i., parasites have not started replicating. Additional studies are thus necessary to understand the basis for this phenotype. At this time we cannot discard the possibility that *Li*TXN1 participates in cell processes other than ribonucleotide reductase and hydroperoxide reduction.

Thioredoxin is well known to be released by some cells (fibroblasts, epithelial cells, B and T cells) through a non-classical pathway (Rubartelli et al., 1992). Secreted thioredoxin can act as a chemoattractant to cells of the immune system such as

macrophages, neutrophils and T cells, therefore functioning in a cytokine/chemokine analogous way. In the case of *Li*TXN1, we wondered whether upregulation in infective stage parasites could also be related to secretion of the protein. When investigating this premise, no evidence for *Li*TXN1 active secretion could be found. Firstly, a small percentage of *Li*TXN1 was detected in culture supernatants, but this was just above that of the control GPDH enzyme, a cytosolic protein with minor glycosomal location (Heise and Opperdoes, 1999). Second, the level of *Li*TXN1 detected extracellularly did not increase over time, further suggesting that it was due to cell lysis. The possibility that this molecule is released during phagocytosis and in this way modulates parasite outcome thus seems unlikely.

In conclusion, by demonstrating that *Li*TXN1 is essential to the mammalian stage of *L. infantum*, we genetically validated this oxidoreductase as a drug target. Although some features of this enzyme may pose a challenge to its inhibition, it would still be interesting to evaluate existing anti-cancer thioredoxin inhibitors for their capacity to suppress *Leishmania* infection.

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Chapter 3

Leishmania infantum encodes two membrane-bound tryparedoxins without typical enzymatic activity

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Unpublished results.

Abstract

Tryparedoxins (TXNs) are thioredoxin-related oxidoreductases found exclusively in organisms of the Order Kinetoplastida. One of the best characterized TXN-dependent pathways is the one culminating in hydroperoxide reduction. In this pathway, TXN is reduced by the parasites' unique dithiol, trypanothione, and then transfers its reducing equivalents to tryparedoxin-dependent peroxidases (TXNPx). The genome of *Leishmania infantum*, the causative agent of visceral leishmaniasis in the Mediterranean region, contains six tryparedoxin-like sequences. Two of them, *Li*TXN1 and *Li*TXN2, have been previously reported to encode redox-active enzymes with cytosolic and mitochondrial compartmentalization, respectively. Regarding the other *L. infantum* TXN-like sequences, only *Li*TXN3 and *Li*TXN5 have the potential to encode functional proteins, albeit with substantial differences from typical TXNs described so far. Both present an hydrophobic tail at their C-terminus and some amino acid modifications at sites which are critical for TXN interaction with their substrates (trypanothione and TXNPx). Here we report that these proteins have no reductase activity towards several *L. infantum* TXNPxs. Nonetheless, *Li*TXN3, but not *Li*TXN5, preserves oxidoreductase activity, being reduced by trypanothione, although at a slow rate. Immunofluorescence assays and membrane protein fractionations suggest that both *Li*TXN3 and *Li*TXN5 are membrane-anchored proteins, *Li*TXN3 is associated to the mitochondrial membrane and *Li*TXN5 to the endoplasmic reticulum. In spite of being a mitochondrial protein, *Li*TXN3 is unlikely to interact with inner mitochondrial enzymes. The exact physiological role of these atypical TXNs in *L. infantum* remains to be elucidated.

1. Introduction

Tryparedoxins (TXNs) are a particular type of oxidoreductases that, up to now, have only been found in organisms of the Order Kinetoplastida. They are distant homologues of thioredoxins, with low overall sequence similarities (identity scores around 13%), higher molecular weight and a distinct active site signature that reads WCPPCR instead of WCGPCK. TXNs are believed to be key elements of the antioxidant machinery of trypanosomatids, a group of Kinetoplastida comprising medically relevant parasites such as *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* spp. This assumption is based on the fact that these organisms lack conventional eukaryotic antioxidant enzymes like catalase and selenium-containing glutathione peroxidases. In addition, contrary to other eukaryotic cells, these parasites do not use glutathione as their main intracellular reducing thiol, but instead a bis-glutathionyl-spermidine conjugate, known as trypanothione [$T(SH)_2$] (Fairlamb and Cerami, 1992). The dithiol directly reduces TXN and this transfers the reducing equivalents either to 2-cysteine peroxiredoxins (2-Cys PRX) or non-selenium glutathione peroxidase-like enzymes (nsGPX), which will ultimately reduce hydroperoxides (reviewed in Castro and Tomás, 2008). Tryparedoxins might as well display additional cell functions, which in other organisms are fulfilled by thioredoxins. For example, TXNs were suggested to be implicated in parasite division through their interaction with ribonucleotide reductase (Dormeyer et al., 2001) and with universal minicircle sequence binding protein (Onn et al., 2004). Therefore, the potential multifunctional nature of TXNs added to their distinctive feature of interaction with $T(SH)_2$, turns these type of enzymes into potential targets for the development of new antiparasitic drugs.

Trypanosomatids present several *TXN*-like sequences in their genomes. An overview of these genes reveals that the predicted proteins they encode can be separated

into two main groups, according to the absence/presence of an hydrophobic stretch at the C-terminal region. The first type of TXN (type I) has been characterized in several species as being a redox active cytosolic protein (generally designated as TXN1). Since true orthologues for *TXN*1 gene exist in all known trypanosomatid genomes, it is assumed that the corresponding protein displays the same enzymatic activity and localization in all organisms. In fact, in *Crithidia fasciculata*, *T. brucei* and *L. infantum*, TXN1 was similarly reported to participate in hydroperoxide elimination pathways (Steinert et al., 1999; Tetaud et al., 2001; Castro et al., 2004). Furthermore, for both *T. brucei* and *L. infantum*, TXN1 was demonstrated to be essential to parasite survival (Comini et al., 2007; Romao et al., 2009), suggesting that the presence of an active TXN in the parasite cytosol is vital to the normal cell metabolism of these organisms. Interestingly, *Leishmania*, but not *Trypanosoma* species, present another type I TXN, named TXN2. *Leishmania infantum* TXN2 was shown to be a functionally active TXN enzyme, in all equivalent to *Li*TXN1 except for its mitochondrial localization (Castro et al., 2004). In addition, contrary to the cytosolic *Li*TXN1, *Li*TXN2 is a redundant enzyme in *L. infantum* (H. Castro and A.M. Tomás, manuscript in preparation), suggesting that *Li*TXN2 function can be compensated by an alternative molecule or that the existence of a TXN in the mitochondrion is dispensable for parasite survival and infectivity.

The second TXN type (type II) is much less studied. *Trypanosoma* species encode one gene (usually denominated as *TXN*2), whereas *Leishmania* species present three to four type II *TXN* genes. There are indirect evidences suggesting that in *T. brucei* TXN2 is present in the parasites' mitochondrion (Tetaud et al., 2001; Motyka et al., 2006), but since no biochemical characterization has thus far been reported, it remains to be proven whether *Tb*TXN2 is indeed an active oxidoreductase. In *L. infantum*, sequence analysis revealed

that only two out of the four uncharacterized type II *TXN* genes annotated on the genome have the potential to encode full-length proteins, *Li*TXN3 and *Li*TXN5.

The purpose of the present study is thus to biochemically characterize *L. infantum* type II TXNs, the ultimate objective being to know if any of these molecules can replace *Li*TXN2 in supplying reducing equivalents to mitochondrial peroxidases. To this end, we aimed at analyzing the enzymatic activity of both *Li*TXN3 and *Li*TXN5 and to investigate their subcellular localizations. In addition, in an attempt to understand the diversity of these proteins in *Leishmania* and remaining trypanosomatids, we performed an evolutionary analysis of all known Kinetoplastida TXN sequences. Our results suggest that none of *L. infantum* type II TXNs is likely to function in mitochondrial defense to hydroperoxide toxicity.

2. Materials and methods

2.1. Parasite culture

Leishmania infantum promastigotes (strain MHOM/MA/67/ITMAP-263) were cultured in RPMI 1640 medium with Glutamax supplemented with 10% heat inactivated fetal bovine serum (iFBS), 50 $U.ml^{-1}$ penicillin, 50 $\mu g.ml^{-1}$ streptomycin and 20 mM Hepes sodium salt pH 7.4 (complete RPMI medium) at 25ºC.

2.2. Amplification of LiTXN sequences

Amplification of the *TXN*4 sequence of *L. infantum* was achieved by reverse transcription-PCR and rapid amplification of cDNA 3'-ends (3'-RACE). One microgram of promastigote total RNA was reverse transcribed with superscript II using the adapter primer 5'-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3'. The 5' side of the *TXN* sequences was PCR amplified from 1 µl of cDNA employing oligonucleotides corresponding to the spliced leader sequence of *L. donovani* 5'-ggg**ggatcc**TCAGTTTCTG-TACTTTATTG-3' (restriction site in bold) and complementary to the active site of known tryparedoxins 5'-gggaattccC(G/T)(A/G)CAIGGIGG(A/G)CACCA-3' (I refers to inosine). The resulting products were cloned and sequenced. 3'RACE allowed to isolate the remaining *LiTXN*4

ORF. For this, the primer 5'-ccgcgca**catATG**TCGCATCTGTTCGAGAA-3' was used in conjunction with the abridged universal amplification primer 5'-GGCCACGCGTCGACTAGTAC-3', similar to the adapter primer but devoided of the poly-T tail. The *LiTXN*3 gene was PCR amplified (5'-cgcgca**catATG**GAGCCCAACTTCTTCAAC-3' and 5'-caccg**ctcgag**CAAGCTGCGGCTGATCAGT-3' oligonucleotides based on the homologous sequence of *L. major*) from a cosmid clone previously isolated from a library (Castro et al., 2002) using *LiTXN*4 as a probe. The *TXN*5 sequence from *L. infantum* was PCR amplified (5'-ccgcgca**catATG**CCCTTGCGTCACAA-3' and 5'-caccg**ctcgag**CTAATAATACCAAAAGTAG-3') from a previously isolated cosmid clone which also contains the *LiTXN1* and *LiTXN*2 genes (Castro et al., 2004).

2.3. Construction of vectors for parasite transfection

To overexpress *Li*TXN3, *L. infantum* promastigotes were transformed with pTEX6HTXN3 vector. Briefly, for pTEX6HTXN3 construction, the PCR amplified *TXN*3 ORF (5'-cgcgca**catATG**GAGCCCAACTTCTTCAAC-3' and 5'-caccg**ctcgag**TCACAAGCTGCGGCTGAT-3') was cloned into the *Nde*I/*Xho*I restriction sites of pET28a (Novagen). The 6His-TXN3 ORF (presenting the His tag at the TXN3 amino-terminal) was excised with *Bgl*II/*Xho*I and cloned into the corresponding sites of pTEX (Kelly et al., 1992). To overexpress *Li*TXN5, the pTEX6HTXN5 vector was constructed following a similar strategy with primers 5'-ccgcgca**catATG**TCGGGTTTGACGAAG-3' and 5'-caccg**ctcgag**TTACTTCTCCGACACGTTG-3'.

2.4. Parasite transfection

Promastigotes in logatithmic growth phase were electroporated at 450 V and 350-400 μF with 5 μg of plasmid DNA as described elsewhere (Beverley and Clayton, 1993). Isolated clones were picked from agar selective plates containing 15 $\mu g.ml^{-1}$ geneticin (G418) and further cultured in liquid medium with 50 to 200 $\mu g.ml^{-1}$ G418.

2.5. Expression and purification of complete and truncated versions of LiTXN3 and LiTXN5 in E. coli

To express both *Li*TXN3 and *Li*TXN5 recombinant proteins, the complete coding sequences were cloned into the pET28a expression vector (see section 2.3) and these used

to transform *E. coli* Tuner (Novagen). Protein expression was induced with 0.1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 3 h at 37ºC in the case of *Li*TXN3 and overnight at 28ºC for *Li*TXN5. The majority of the proteins was produced in the insoluble fractions of bacteria as inclusion bodies. To separate these from *E. coli* insoluble contents, pelleted bacteria were resuspended in 500 mM NaCl, 20 mM Tris-HCL pH 7.6, disrupted by sonication and centrifuged at 10.000 g for 30 min at 4ºC. The resulting pellet was then subjected to a series of consecutive sonications and centrifugations in 50 mM Tris pH 8.0, 1 mM EDTA, 100 mM NaCl and a cocktail of protease inhibitors, in 100 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM DTT, 1 M Ureia, 2% Triton X-100, in EDTA, Tris-HCl pH 8.0 and finally ressuspended in 0.27 M NaCl, 3.6 mM KCl, 4.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4 pH 7.2 (PBS). The small amount of soluble *Li*TXN3 and *Li*TXN5 that could be recovered from *E.coli* when this was induced with 0.01 mM IPTG for 1 h 30 min at 28ºC was purified and immediately used in the classical TXN activity assay. In order to obtain soluble *Li*TXN3 and *Li*TXN5 in high amounts, a truncated version of these proteins, without the hydrophobic C-terminus, was prepared. For this, the first 501 nucleotides of *Li*TXN3 and the first 768 nucleotides of *Li*TXN5 were amplified with PWO polymerase using the same sense primers as above (see section 2.3) and as antisense primers 5'-caccg**ctcgag**TCAACGGGTGTAGTCCAAA-3' for *Li*ΔTXN3, and 5'-caccg**ctcgag**ctaTTCTCGCCAGCCCTCGGT-3' for *Li*ΔTXN5. The PCR products were cloned into the *Nde*I/*Xho*I restriction sites of pET28a vector and the truncated proteins expressed in BL21 *E.coli* following IPTG induction (0.1 mM) for 3 h at 37ºC. After induction the bacteria were pelleted, ressuspended in 500 mM NaCl, 20 mM Tris-HCl pH 7.6, disrupted by sonication and centrifuged at 10.000 g for 30 min at 4ºC. The supernatants were applied to a His Bind resin (Novagen) column. The proteins were eluted with an imidazole gradient (5 to 1000 mM) at a flow rate of 2.5 $ml.min^{-1}$. Fractions confirmed to contain the protein by SDS-PAGE were pooled, applied to PD-10 columns (Amersham) and eluted with PBS pH 7.4.

2.6. Antibody production

Anti-*Li*TXN3 and anti-*Li*TXN5 polyclonal antibodies were raised in Wistar rats (Charles River, France) housed under specific pathogen-free conditions at the animal facility of the Instituto de Biologia Molecular e Celular (Porto, Portugal). Briefly, animals were immunized subcutaneously with 100 μg of each protein expressed as inclusion

bodies, previously sonicated in Freund's complete adjuvant. Four weeks later, 50 μg of the same proteins in Freund's incomplete adjuvant were again administrated by the same inoculation route. The boosts were repeated every 2 weeks for 3 months. Animal experiments were approved by the IBMC ethics committee.

2.7. Western blotting

Leishmania infantum protein extracts were obtained as described elsewhere (Castro et al., 2004), resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk and probed against the adequate primary antibodies. Secondary antibodies labeled with horseradish peroxidase (HRP) were used and signal detection was achieved using ECL detection reagents (GE Healthcare).

2.8. Indirect immunofluorescence assays (IFATs)

Immunofluorescence assays were performed as described elsewhere (Castro et al., 2002). Briefly, promastigotes were fixed with 4% paraformaldehyde (w/v) in PBS, permeabilized with 0.1% (v/v) TritonX-100 and spotted onto polylysine-coated microscope slides. Samples were blocked with 1% BSA and incubated first with primary antibody for 1 h at room temperature and then with adequate secondary antibodies (Alexa Fluor 488 or 568). Amastigotes inside peritoneal macrophages of BALB/c mice (Charles River, France) were stained in the same way. For macrophage infection, cells were recovered from BALB/c mice, seeded at 4 x 10^5 cells per well in 24-well plates and allowed to adhere for 3 h before infection. The culture medium consisted on DMEM Glutamax complemented with 20% iFBS, 50 $U.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin. Upon washing with prewarmed Hank's balanced salt solution (HBSS), macrophages were incubated with stationary-phase promastigotes at a parasite:macrophage ratio of 10:1, for 3 h at 37ºC, 5% CO_2. Non-internalized parasites were removed by gentle washing with prewarmed HBSS and the macrophages were further cultured for 24 h. All samples were mounted in VectaShield and analyzed with an Axioskop Zeiss microscope.

2.9. Immuno-electron microscopy

Promastigotes fixed in 2% (w/v) paraformaldehyde phosphate buffer pH 7.4 and 0.1% gluteraldehyde in RPMI medium were washed with RPMI medium and resuspended in 50mM NH_4Cl with 1% BSA. Parasites were transferred to capillary glass tubes and embedded in fixing solution. Upon dehydration in ascending ethanol solutions (70%, 90%

and 100%) the specimens were embedded in LR white resin overnight at 4ºC. Frozen sections (5 μm thick) were cut into 90 to 110 nm ultrathin sections, collected onto copper grids and blocked with 1% BSA, 1% Tween-20 and 1% FBS in PBS for 1 h. For immunolocalization, sections were incubated with anti-*Li*TXN5 primary antibody and anti-rat secondary antibody complexed with 10 nm gold particles. Contrast was obtained with uranyl acetate and lead citrate and samples analyzed with a Zeiss EM 10C transmission electron microscope.

2.10. Extraction of membrane proteins

Soluble and membrane proteins were differentially separated using two different procedures. In one of these (Bordier, 1981), *L. infantum* promastigotes were incubated with 1% TritonX-114 in 10 mM Tris-HCl pH 7.4, 150 mM NaCl and a cocktail of protease inhibitors for 10 min at 4ºC. Aqueous and detergent enriched phases were then separated at 37ºC for 10 min. In a second protocol, aliquots containing equal amounts of *L. infantum* promastigotes (5 x 10^8 per tube ≈ 1 mg of protein) were pelleted and resuspended in 2 ml of 10 mM Tris-HCl pH 7.4 buffer, 2 ml of 10 mM Tris-HCl, 0.5 M NaCl pH 7.4 buffer and 2 ml of 0.1 M Na_2CO_3 pH 11.5 buffer, respectively (a cocktail of protease inhibitors was added to all samples). Parasite suspensions were sonicated, incubated for 30 min at 4°C and sedimented by ultracentrifugation for 60 min at 100.000 g. Soluble and insoluble fractions obtained in both procedures were analyzed by Western blotting.

2.11. Enzymatic assays

Several enzymatic assays were carried out in this study. Classical TXN activity was measured at 25ºC according to Flohé et al. (2002) in 500 μl of total reaction mixture, containing 300 μM NADPH, 1 $U.ml^{-1}$ *T. cruzi* trypanothione reductase (TR), 50 μM trypanothione [$T(SH)_2$], 0.5-1 μM tryparedoxin (TXN), 10 μM peroxiredoxin (PRX) and 70 μM hydrogen peroxide (H_2O_2). After a 15 min pre-incubation period, the reaction was started by addition of H_2O_2 and followed at 340 nm by measuring NADPH consumption. The insulin reduction assay was performed at 25ºC according to Holmgren (1979) using 1 $mg.ml^{-1}$ insulin solution freshly prepared in 100 mM potassium phosphate pH 7.0, 2 mM EDTA (potassium phosphate buffer). The assay reaction mixture contained 125 μM insulin, 350 μM dithiothreitol (DTT) and 5 μM of TXN in potassium phosphate buffer. The increase in turbidity, due to formation of the insoluble insulin B chain, was followed at

650 nm. In another set of assays, insulin was also used as final electron acceptor but DTT was substituted by distinct TXN reductants. First, 300 μM NADPH, 2 $U.ml^{-1}$ TR, 100 μM $T(SH)_2$, 5 μM TXN and 125 μM insulin in potassium phosphate buffer were incubated at 25ºC and NADPH oxidation followed at 340 nm. Second, in a similar reaction mixture, the redox couple TR/T(SH)2 was replaced by 1.5 $U.ml^{-1}$ *E. coli* thioredoxin reductase, 1 $U.ml^{-1}$ yeast glutathione reductase/500 μM glutathione or 0.5 $U.ml^{-1}$ porcine lipoamide dehydrogenase/100 μM lipoamide and insulin reduction followed at 650 nm at 25ºC. The data was analyzed using the UVProbe software (Shimadzu Corporation).

2.12. Bioinformatics Analysis

TXN sequences were searched at the Sanger Institute GeneDB webpage (www.genedb.org), except for the *C. fasciculata* sequences, that were recovered from published work (Hofmann et al., 2001). TXNs designation follows the suggested genetic nomenclature for *Trypanosoma* and *Leishmania* (Clayton et al., 1998), mentioning the species abbreviation and the sequence number. Systematic names attributed by GeneDB to each gene are listed next (April 2009). *Li*TXN1: LinJ29.1400, *Li*TXN2: LinJ29.1390, *Li*TXN3: LinJ31.2320, *Li*TXN4: LinJ31.2330, *Li*TXN5: LinJ29.1380, *Li*TXN6: LinJ29.1370, *Lm*TXN1: LmjF29.1160, *Lm*TXN2: LmjF29.1150, *Lm*TXN3: LmjF31.1960, *Lm*TXN4: LmjF31.1970, *Lm*TXN5: LmjF29.1140, *Lm*TXN6: LmjF29.1130, *Lb*TXN1a: LbrM29_V2.1240, *Lb*TXN1b: LbrM29_V2.1230, *Lb*TXN2: LbrM29_V2.1220, *Lb*TXN3: LbrM31_V2.2190, *Lb*TXN5: LbrM29_V2.1210, *Tc*TXN1: Tc00.1047053509997.30, *Tc*TXN2: Tc00.1047053509997.20, *Tco*TXN1: congo1407h03.q1k_0, *Tco*TXN2: congo1407h03.q1k_2, *Tv*TXN1: tviv682b01.p1k_6, *Tv*TXN2: tviv682b01.p1k_1, *Tb*TXN1a: Tb927.3.3780, *Tb*TXN1b: Tb927.3.3760, *Tb*TXN2: Tb927.3.5090 (note that some are temporary names). Needle Emboss align software (www.ebi.ac.uk) was used to calculate pairwise identity and similarity scores. Protein sequence alignment was performed with the Ebi server ClustalW. ProSeq software (www.biology.ed.ac.uk/research/institutes/evolution) was employed for preparation and evolutionary analysis of DNA sequences. Phylogenetic trees were constructed by the MEGA4 program (Kumar et al., 2004).

3. Results

3.1. Isolation of LiTXN3, LiTXN4 and LiTXN5

To examine the *TXN*-like sequences of *L. infantum* each gene was isolated from the parasite's genome. *LiTXN*4 was isolated with a RT-PCR based strategy. Using a primer complementary to the spliced leader sequence of *L. donovani* and another to the TXN active site signature, we obtained several *TXN*-like sequences, one of which corresponded to the 5' region of *LiTXN*4. This allowed the amplification of its 3' region by 3'RACE. Analysis of the *L. major* genome revealed the presence of another *TXN* sequence upstream the *TXN*4 gene. Using *LiTXN*4 gene as probe, the *LiTXN*3 ORF was isolated through the screening of a *L. infantum* cosmid library (Castro et al., 2002). Sequencing of both genes revealed that *LiTXN*3 has the potential to encode a functional protein, but that *LiTXN*4 is a pseudogene, due to insertion of a nucleotide and deletion of another. Isolation of *LiTXN*5 also made use of information released by the *L. major* genome consortium, that showed a *TXN*-like sequence in the same locus as the *LiTXN*1 and *LiTXN*2 genes. Accordingly, the *LiTXN*5 gene was amplified from a cosmid clone previously obtained, which contained *LiTXN*1 and *LiTXN*2 (Castro et al., 2004). Recent progress in *L. infantum* genome sequencing further revealed the presence of a sixth *TXN*-like sequence. However, probably as the result of extensive nucleotide changes, the predicted gene product does not preserve the typical active site signature, thus it cannot be considered a true TXN-like molecule (Fig. 1). In short, the *L. infantum* genome harbors information to encode four distinct TXN proteins, two of which, *Li*TXN1 and *Li*TXN2, are already characterized and known to be redox active enzymes (Castro et al., 2004) and two other, *Li*TXN3 and *Li*TXN5, thus far awaiting biochemical characterization.

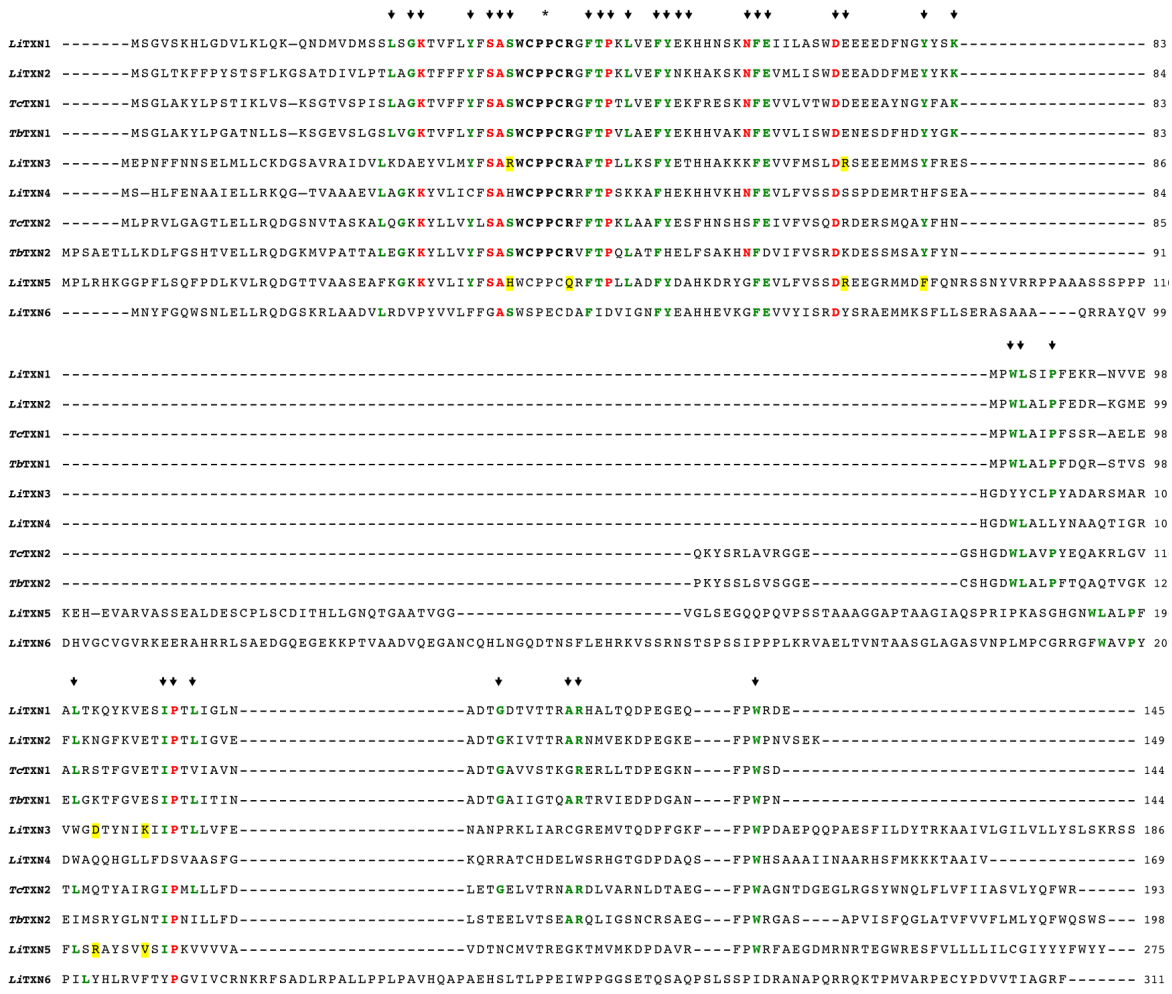


Fig. 1. Alignment of TXN-like sequences of different trypanosomatids. The active site signature is presented in black bold letters and an asterisk. Residues conserved in all TXNs are depicted in green and those also conserved in thioredoxins are shown in red (all indicated with arrows). Amino acid substitutions that might influence classical TXN activity of *Li*TXN3 and *Li*TXN5 are highlighted in yellow. The amino acid sequence of *Li*TXN4 corresponds to the one that would be predicted if no alterations occurred. *Li*: *L. infantum*, *Tb*: *T. brucei* and *Tc*: *T. cruzi*. To see the complete alignment of the 28 trypanosomatid TXN-like sequences please refer to Appendix 3.

3.2. Analysis of the predicted amino acid sequences of LiTXN3 and LiTXN5 and comparison to other TXN-like molecules

*LiTXN*3 and *LiTXN*5 are predicted to encode proteins with theoretical molecular weights of 21.6 and 30.4 kDa and pI's of 6.82 and 8.48, respectively. Alignment of *Li*TXN3 and *Li*TXN5 with TXN-like sequences of other trypanosomatids revealed that these two molecules carry amino acid substitutions that might affect their enzymatic activity (Fig. 1). These alterations can either be found i) at the active site motif, ii) at the

level of interaction with $T(SH)_2$ and/or iii) at the region of contact with PRXs. In fact, the tryparedoxin WCPPCR active site motif is conserved in *Li*TXN3 but not in *Li*TXN5, that presents a critical Arg to Gln exchange. As previously shown by Hofmann and colleagues (2001), this amino acid substitution is critical for $T(SH)_2$ binding to TXN (and hence for TXN activity) since interaction between both molecules requires the presence of a basic residue after the second redox active Cys. Moreover, Glu72 (negatively charged), also known to be an important residue for $T(SH)_2$/TXN interaction, is replaced by an Arg (positively charged) in both *Li*TXN3 and *Li*TXN5 (amino acid positions refer to *Li*TXN1). A similar charge reversion in *Cf*TXN2 and *Tb*TXN1 muteins reduced TXN activity to 17% and 50% of the wild-type enzyme levels, respectively (Hofmann et al., 2001; Budde et al., 2003). Ser38 is also substituted by an Arg in *Li*TXN3 and by an His in *Li*TXN5. This particular site is in the vicinity of an acidic area that was proposed to be involved in TXN/PRX interaction (Budde et al., 2003). Accordingly, simultaneous substitution of Ser38 and Glu72 might impair TXN contact with the trypanosomatids most abundant peroxidases, i.e. typical 2-cysteine peroxiredoxins (2-Cys PRX). Another class of peroxidases present in trypanosomatids are non-selenium glutathione peroxidases-like enzymes (nsGPX). Recently, Melchers and colleagues (2008) identified, by structural analysis, the residues that are likely to be involved in the interaction of TXN with nsGPX. The majority of these residues are conserved in *Li*TXN3 and *Li*TXN5 with the exception of Lys102 and Glu107. Furthermore, in *Li*TXN5 Tyr80 is replaced by a Phe. Since together Ser38 and Tyr80 contribute to the maintenance of the hydrogen bond system (Hofmann et al., 2001), non-synonymous substitutions of these residues are likely to affect the architecture of the *Li*TXN5 active site, further weakening its oxidoreductase activity. Another atypical feature of *Li*TXN3 and *Li*TXN5, also shared by *Trypanosoma* TXN2 sequences, is the presence of a C-terminal extension enriched in hydrophobic amino acids

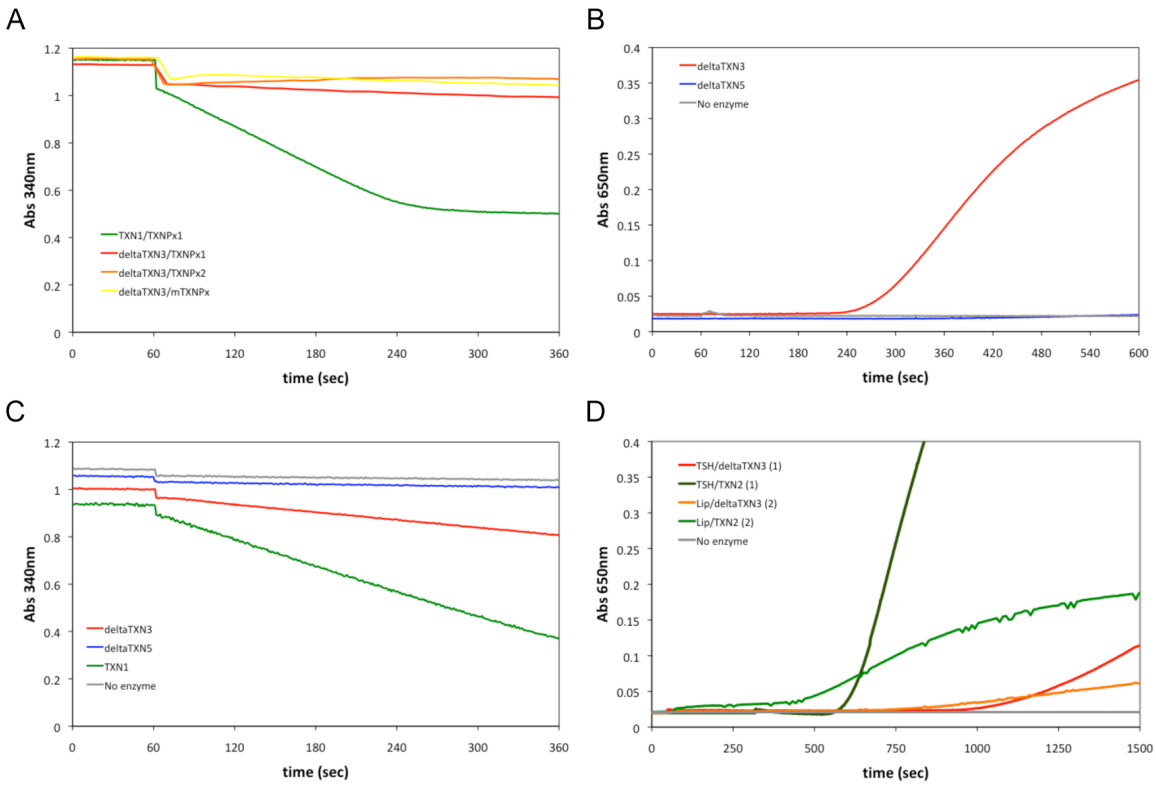


Fig. 2. Enzymatic assays to analyze *Li*TXN3 and *Li*TXN5 activity. (A) Reconstitution of the TR/T(SH)$_2$/TXN/PRX cascade. Positive control was performed with *Li*TXN1/*Lic*TXNPx1. *Li*ΔTXN3 was tested with *Leishmania infantum* 2-Cys PRXs (TXNPx1, TXNPx2 and mTXNPx). The reaction was initiated at the 60 seconds time point by adding H_2O_2 and NADPH consumption was followed at 340 nm at 25ºC. *Li*ΔTXN5, as well as full-length versions of the proteins were tested in the same conditions as *Li*ΔTXN3. Since similar data were obtained with the truncated and complete forms of both proteins, for simplicity only the reaction with *Li*ΔTXN3 is shown on the graph. (B) Insulin reduction assay. The proteins, *Li*ΔTXN3 and *Li*ΔTXN5, were reduced by DTT and the subsequent reduction of insulin was measured at 650 nm at 25ºC. Negative control was performed in the same reaction mixture without addition of TXN. (C) Reduction of *Li*ΔTXN3 and *Li*ΔTXN5 by T(SH)$_2$ using insulin as final electron acceptor. TXN reduction was measured in a mixture containing the reducing system NADPH, TR and T(SH)$_2$. NADPH consumption was registered at 340 nm at 25ºC. *Li*TXN1 was used as positive control. Negative control was done without adding TXN to the reaction mixture. The reaction was initiated at the 60 sec time point when TXN was added. (D) Reduction of *Li*ΔTXN3 by alternative molecules using insulin as the final electron acceptor. TXN activity was measured following insulin reduction at 650 nm at 25ºC. The reaction mixture consisted on (1) NADPH/TR/T(SH)$_2$ or, alternatively, on (2) NADPH/lipoamide dehydrogenase/lipoamide, (3) NADPH/glutathione reductase/glutathione and (4) NADPH/thioredoxin reductase. For each different reaction *Li*TXN2 was used as positive control. Negative controls, performed in the absence of TXN, overlap with the grey line depicted on the graph. The same happens with the reaction of *Li*ΔTXN3 with glutathione reductase/glutathione or with thioredoxin reductase that also overlaps with the negative control curve.

(Fig. 1). This suggests that these proteins might be membrane-anchored, an hypothesis supported by different software programs (e.g., TopPred, Das and TMpred). In addition, *Li*TXN5 and its orthologues in other *Leishmania* species show an insertion of approximately 90 amino acids in the middle of the sequence, which comprises a second putative transmembrane domain. *Trypanosoma* TXN2 members also present a mid-

insertion in their sequence, although much shorter than in *Leishmania* TXN5 molecules (approx. 15 amino acids). In conclusion, protein sequence alignments reveal that the likelihood of *Li*TXN5 and, to a lesser extent, *Li*TXN3, to operate as typical TXNs is reduced, yet experimental confirmation is required to draw a definitive conclusion about their enzymatic activity.

3.3. Analysis of LiTXN3 and LiTXN5 enzymatic activity

To investigate if *Li*TXN3 and *Li*TXN5 retained the enzymatic activity distinctive of TXNs, recombinant proteins were purified from bacteria by chelate chromatography as N-terminal 6-His fusion proteins, with and without the C-terminal hydrophobic sequences. TXN activity was tested by the classical assay first described by Nogoceke and coworkers (1997), whereby reduction of hydroperoxides is determined by NADPH consumption through an enzymatic cascade comprising trypanothione reductase (TR), $T(SH)_2$, TXN and 2-Cys PRX. As depicted on Fig. 2A, *Li*TXN3 and *Li*TXN5 were shown inactive in this assay, irrespectively of the 2-Cys PRX used (*Li*TXNPx1, *Li*TXNPx2 or *Li*mTXNPx, Castro et al., 2002) and of the TXN form assayed (either full-length or truncated). In addition, when the truncated version of *Li*TXN3 was tested for its ability to reduce *L. infantum* nsGPXs, it displayed no activity (in contrast to the positive controls *Li*TXN1 and *Li*TXN2, data not shown). All together, these findings show that *Li*TXN3 and *Li*TXN5 do not function as typical TXNs and are in line with what was predicted from the protein primary structures. Still, we should not exclude the possibility that the proteins maintain oxidoreductase capacity using substrates other than peroxidases.

To test the ability of *Li*TXN3 and *Li*TXN5 to function as oxidoreductases we used *Li*ΔTXN3 and *Li*ΔTXN5 in the spectrophotometric assay of Holmgren (1979), which measures aggregation of the A and B insulin chains upon reduction of intramolecular

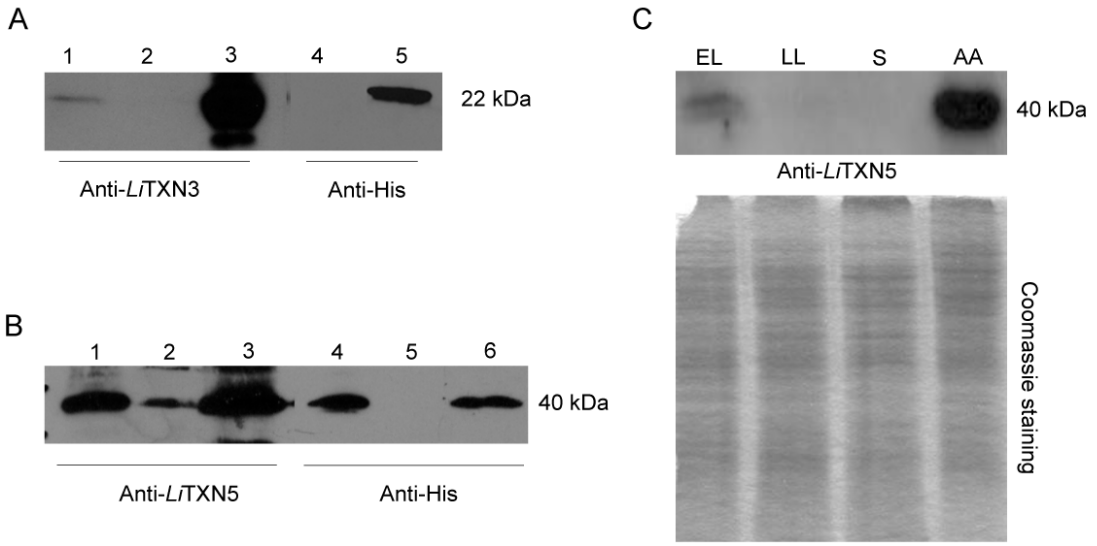


Fig. 3. Western blot detection of *Li*TXN3 and *Li*TXN5 with different antibodies. (A) Western blot analysis of 50 μg of total protein extract from pTEX6HTXN3 (lane 1 and 5) and wild-type (lane 2 and 4) promastigotes and of 2 μg of *Li*TXN3 recombinant protein (lane 3). The blot was hybridized with anti-*Li*TXN3 and anti-His antibodies as indicated. (B) Western blot analysis of 25 μg total protein extract from pTEX6HTXN5 (lane 1 and 6) and wild-type (lane 2 and 5) promastigotes and of 2 μg of *Li*TXN5 recombinant protein (lane 3 and 4). The blot was hybridized with anti-*Li*TXN5 and anti-His antibodies as indicated. (C) *Li*TXN5 is differentially expressed along the parasite life cycle. Each lane is loaded with 25 μg of total protein extract from early log-phase (EL), late log-phase (LL), stationary-phase promastigotes (S) and axenic amastigotes (AA). The blot was hybridized with anti-*Li*TXN5 antibody. Coomassie blue staining of an identical gel run in parallel was used as loading control.

disulfide bonds. As shown in Fig. 2B, *Li*ΔTXN3, but not *Li*ΔTXN5, is capable of catalyzing insulin reduction. Reaction with *Li*ΔTXN5 was similar to the negative control, performed in the absence of a control TXN. These results suggest that *Li*TXN3, although unlikely to react as a classical TXN, retains oxidoreductase activity and that, on the contrary, *Li*TXN5 is probably devoided of redox activity.

Since $T(SH)_2$ is the main trypanosomatid thiol, we verified whether *Li*ΔTXN3 could be reduced by this molecule. To this end, NADPH, TR, $T(SH)_2$ and insulin were incubated with *Li*ΔTXN3 and NADPH consumption was determined spetrophotometrically. Figure 2C shows that *Li*ΔTXN3 can be reduced by $T(SH)_2$ and subsequently reduce insulin. This, however, proceeds at a much lower efficiency in

comparison to the positive control enzyme (*Li*TXN1). As expected for *Li*ΔTXN5, it presents no reactivity in this assay.

Alternative reductants for *Li*ΔTXN3 were analyzed in order to test if the enzyme could catalyze insulin reduction more efficiently when using a different reducing system. As depicted in Fig. 2D, *Li*ΔTXN3 can also be reduced by the lipoamide dehydrogenase/lipoamide system, albeit at a rather slow rate. Thioredoxin reductase and the glutathione reductase/glutathione couple were also tested in this assay, but none was able to transfer reducing equivalents to *Li*ΔTXN3.

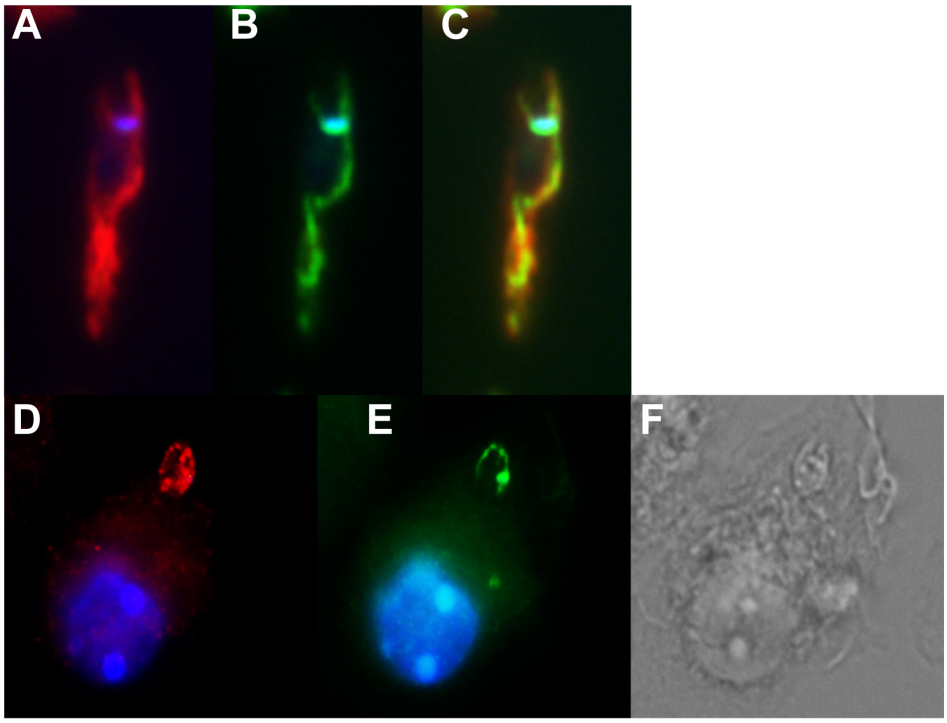


Fig. 4. Immunofluorescence assay showing mitochondrial localization of *Li*TXN3 in *L. infantum* promastigotes. pTEX6HTXN3 promastigotes (A to C) and amastigotes (D to F). *Li*TXN3 was detected with anti-His primary antibody (shown in red). *Li*mTXNPx (Castro et al., 2002) was used as mitochondrial protein control (shown in green) and DNA marked with DAPI (shown in blue). A merged image is shown in C. Parasites were photographed at 1000x magnification.

3.4. Expression of LiTXN3 and LiTXN5 and subcellular localization in L. infantum

To gain insight into the role that *Li*TXN3 and *Li*TXN5 display within the parasite, the expression and subcellular localization of these molecules was investigated. For this, anti-sera against *Li*TXN3 and *Li*TXN5 were produced and confirmed to recognize the corresponding recombinant proteins by Western blot analysis. However, despite exhaustive attempts, the anti-*Li*TXN3 antibody did not detect any band in protein extracts of promastigotes (at different growth stages) and of axenically grown amastigotes (data not shown). One possible reason for this negative result could be that within *L. infantum* parasites *Li*TXN3 is subjected to substantial post-translational modifications. This is, however, unlikely to be the case, since the tagged version of the protein (6HTXN3) cloned into the pTEX vector (Kelly et al., 1992) showed similar molecular weight as the recombinant protein expressed in *E. coli* (see Materials and methods and Fig. 3A). One alternative explanation could be that the anti-*Li*TXN3 antibody is weak. Supporting this premise is the observation that the 6HTXN3 chimera expressed by *L. infantum* transfectants is strongly recognized by the anti-His antibody but only very weakly by the anti-*Li*TXN3 serum. In the case of the antibody anti-*Li*TXN5, it detected a protein band in wild-type (WT) parasites (Fig. 3B), although with unexpected size. Instead of the predicted 30.4 kDa, *Li*TXN5 is recognized as a 40 kDa peptide on an SDS-PAGE gel. This aberrant mobility in gel might be explained by the high pI of *Li*TXN5 (8.48), as was observed for other proteins (Iakoucheva et al., 2001). Protein expression was also analyzed along the parasite life cycle and showed that *Li*TXN5 is differently expressed throughout *L. infantum* development. As depicted in Fig. 3C, the protein is predominantly expressed in axenic amastigotes, while in promastigotes it is only detected in log-phase cells. One of the characteristics that is shared between log-phase promastigotes and amastigotes is their enrichment in dividing cells. Accordingly, the *Li*TXN5 expression pattern might suggest

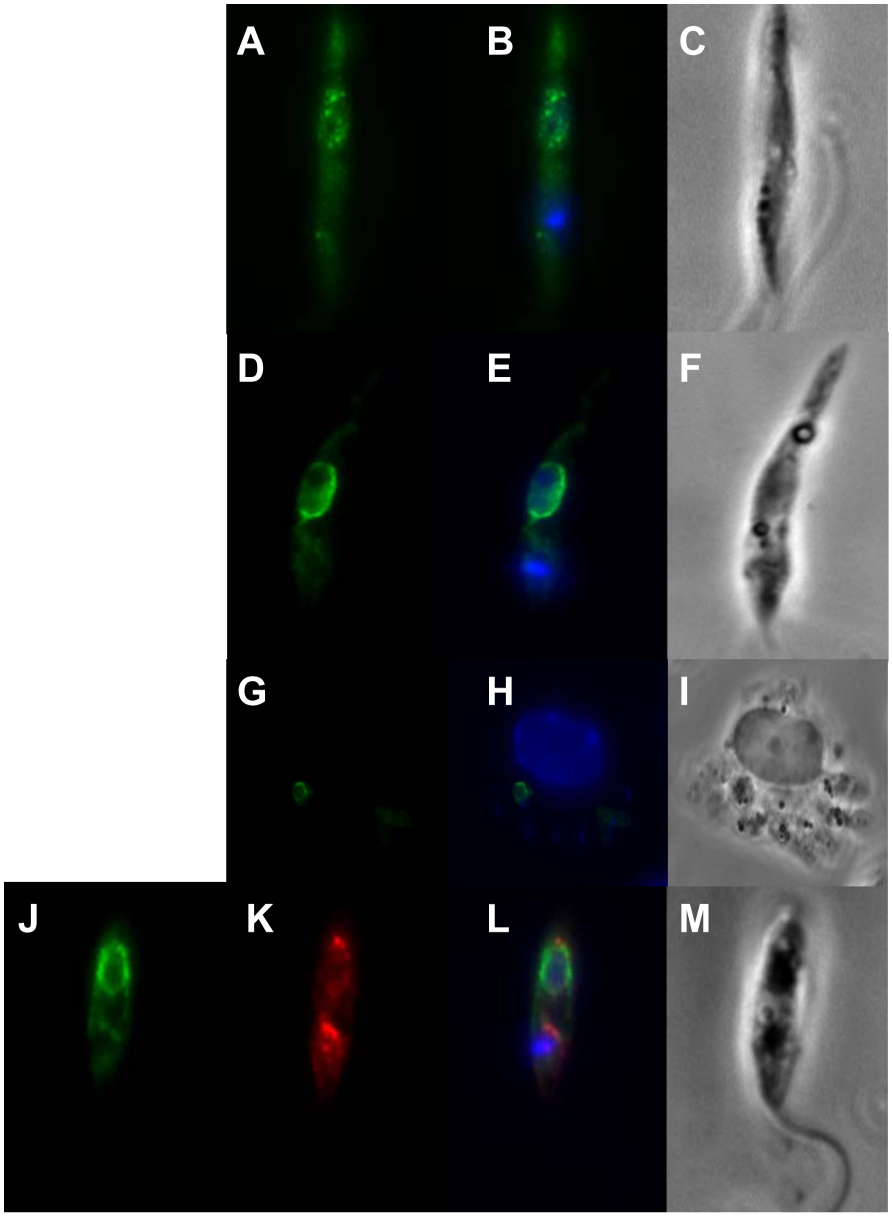


Fig. 5. Immunofluorescence assay showing ER localization of *Li*TXN5. Wild-type promastigotes (A to C), pTEX6HTXN5 promastigotes (D to F and J to M) and amastigotes (G to I). *Li*TXN5 detected with anti-*Li*TXN5 primary antibody is shown in green, DNA is shown in blue (DAPI) and ER control protein BiP, detected with anti-*Tb*BiP primary antibody, is shown in red. Merged images in B, E, H and L. Parasites were photographed at 1000x magnification.

protein involvement in cell replication processes. However, based on the enzymatic assays previously presented, it is important to mention that the *Li*TXN5 potential functions will probably not depend on its oxidoreductase activity.

To localize *Li*TXN3 and *Li*TXN5 in the parasites, immunofluorescence assays (IFATs) were conducted. In the same way as in Western blots, the anti-*Li*TXN3 antibody

failed to detect the protein in WT promastigotes and in intracellular amastigotes. For this reason, anti-His antibody was used to localize the protein in overexpressing parasites transformed with a 6His N-terminal tagged *Li*TXN3 (pTEX6HTXN3). Figure 4 shows that *Li*TXN3 is present in the mitochondrion of both promastigotes and intracellular amastigotes. The transformed parasites were co-stained with an antibody against a mitochondrial enzyme (*Li*mTXNPx, Castro et al., 2002), evidencing co-localization between the two proteins, clearer in the case of promastigotes than in amastigotes. The subcellular localization of *Li*TXN5 was performed in WT and pTEX6HTXN5 transformed parasites. Immunofluorescence assays show the protein mainly in a perinuclear region and as a tubular-like extension throughout the parasite body (Fig. 5), suggesting endoplasmic reticulum (ER) localization (Mullin et al., 2001; Descoteaux et al., 2002; Wilkinson et al., 2002). This pattern was particularly evident in pTEX6HTXN5 promastigotes (Fig. 5D to F), but the same perinuclear distribution is maintained in intracellular amastigotes (Fig. 5G to I). To confirm *Li*TXN5 ER localization, we used an antibody against a classical ER protein marker, BiP, and co-stained the parasites with anti-*Li*TXN5. The resulting images show that *Li*TXN5 co-localizes with BiP, although the two proteins present different signal intensities throughout the ER structure (Fig. 5J to M). To corroborate these immunofluorescence results, immunoelectron microscopy was performed with pTEX6HTXN5 promastigotes. As depicted in Fig. 6, the dots corresponding to gold-labeled *Li*TXN5 were observed mainly over the ER structure. Taken together, the localization assays indicate that the *L. infantum* TXNs under investigation present different subcellular distribution, *Li*TXN3 is mitochondrial, while *Li*TXN5 is associated to the ER.

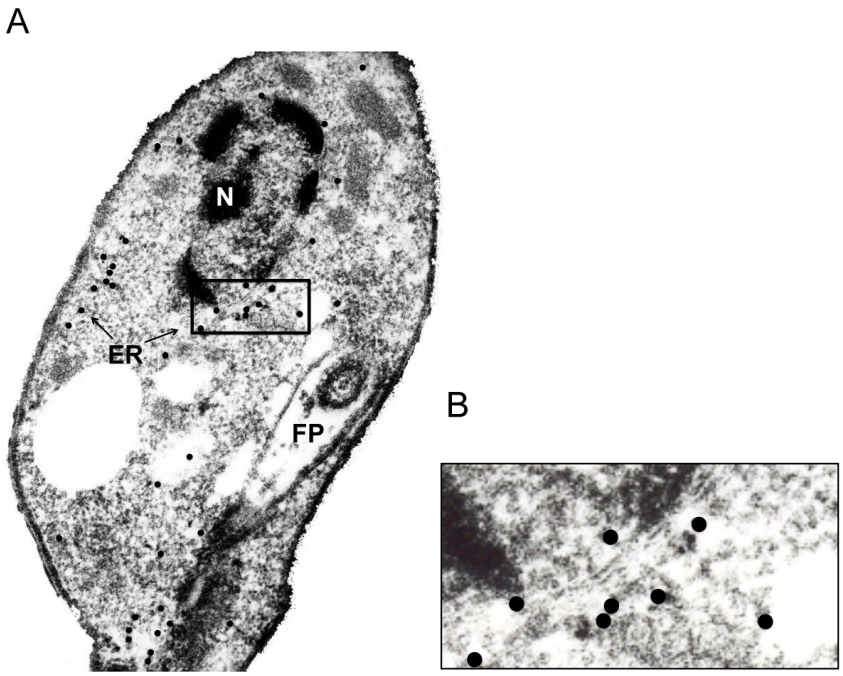


Fig. 6. Immunoelectron microscopy showing ER localization of *Li*TXN5. pTEX6HTXN5 promastigotes were used for the immunolocalization of the protein with anti-*Li*TXN5 primary antibody and gold-labeled anti-rat secondary antibody (black dots). The area limited by a black square in A was digitally magnified in B to better visualize the ER membranes. The nucleus (N), flagelar pocket (FP) and endoplasmic reticulum (ER) are marked in the photograph. The picture was taken at 20.000x magnification.

3.5. Membrane association of LiTXN3 and LiTXN5

As referred to above, the presence of hydrophobic extensions at the C-terminus of *Li*TXN3 and *Li*TXN5, suggests that these proteins may be membrane associated. To confirm this, two different approaches were employed. One was the Bordier method (1981) that uses TritonX-114 to separate hydrophilic proteins from integral membrane proteins at 37ºC. As shown in Fig. 7A, *Li*TXN5 appears only in the detergent phase, suggesting that it is indeed membrane associated. The major surface protease GP63 was used as a membrane protein marker, being predominantly present in the detergent phase, while the cytosolic *Li*TXN1, used as a control for hydrophilic proteins, appeared exclusively in the aqueous phase. The other method to assess protein membrane association consisted first in lysing pTEX6HTXN3 and pTEX6HTXN5 transformed parasites by sonication in low salt, high salt or alkaline sodium carbonate solutions, and then in separating soluble and insoluble proteins by centrifugation. The presence of

*Li*TXN3 and *Li*TXN5 in the resulting supernatants and pellets was subsequently analyzed by Western blot using the anti-His antibody (Fig. 7B). Again *Li*TXN5 was found to be an integral membrane protein, being recovered in insoluble pellets and only partially released from membranes with sodium carbonate solution. *Li*TXN3 showed a similar pattern to *Li*TXN5, which supports membrane-association for this protein too. These results were confirmed by the correct extraction of the marker proteins, GP63 (membrane protein) and *Li*TXNPx2 (soluble protein). Since *Li*TXN3 presents a single transmembrane hydrophobic stretch at the C-terminal region, it belongs to the group of tail-anchored membrane proteins, which generally present the hydrophilic N-terminal portion facing the cytosol (Wattenberg and Lithgow, 2001). *Li*TXN5 might however present a different membrane topology, since apart from the C-terminal transmembrane sequence it has a second putative transmembrane domain at the middle of the sequence (predicted by TopPred and Das software).

3.6. Phylogeny of the TXN genes in trypanosomatids

Studying evolutionary relations between the TXN members of the Trypanosomatidae group might allow us to understand the origin of these proteins and to have a global view about the role they play in these organisms. To compare the several trypanosomatid TXN molecules a phylogenetic tree was built using 26 *TXN*-like sequences from 8 different trypanosomatid species. Several different methods were followed to construct the phylogenetic trees using the Mega4 software and, although small differences could be observed in the resulting trees, the main features were conserved. As an example Fig. 8 shows the topology of the Neighbor-joining tree revealing that TXN sequences form two separate clades with highly significant support. The first clade (Clade I) groups a branch containing all TXNs without an hydrophobic tail (*Trypanosoma* TXN1 and

Leishmania/Crithidia TXN1 and TXN2). In contrast, Clade II contains *Li*TXN3 and *Li*TXN5, as well as all other trypanosomal TXN sequences which present a C-terminal hydrophobic extension (*Trypanosoma* TXN2 and *Leishmania* TXN3, TXN4 and TXN5). The TXN distribution into two different groups suggests that the trypanosomatid ancestor already contained the two types of TXN sequences in its genome. *Trypanosoma cruzi*, *T. congolense* and *T. vivax* maintained one representative of each TXN type, while *T. brucei* have recently duplicated one of its genes and *Leishmania* presently contain a total of six *TXN*-like sequences.

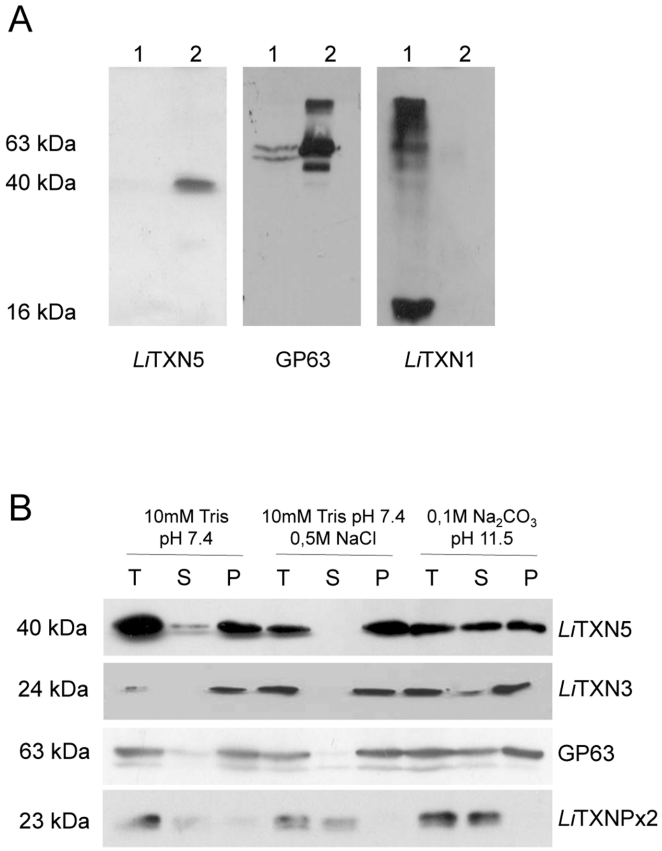


Fig. 7. Western blot analysis of *Li*TXN3 and *Li*TXN5 membrane association. (A) For detergent extraction of membrane proteins, $1x10^9$ pTEX6HTXN5 promastigotes were treated with TritonX-114 and the resulting aqueous (lane 1) and detergent (lane 2) phases were loaded on a 12% acrylamide gel. *Li*TXN5 was detected with the anti-*Li*TXN5 antibody as indicated. Anti-GP63 and anti-*Li*TXN1 were used as membrane and hydrophilic protein controls, respectively. (B) Alkaline carbonate extraction of membrane proteins. $5x10^8$ pTEX6HTXN3 or pTEX6HTXN5 promastigotes were treated with 10 mM Tris-HCl pH 7.4 or 10 mM Tris-HCl, 0.5 M NaCl pH 7.4 and 0.1 M Na_2CO_3 pH 11.5. Total extracts (T), supernatants (S) and pellets (P) were simultaneously run in polyacrilamide gels. Anti-His antibody detected episomal TXN3 and TXN5. Anti-GP63 and anti-*Li*TXNPx2 were used as membrane and hydrophilic protein controls, respectively (only the controls for the pTEX6HTXN5 extracts are shown, but similar results were obtained with pTEX6HTXN3 extracts).

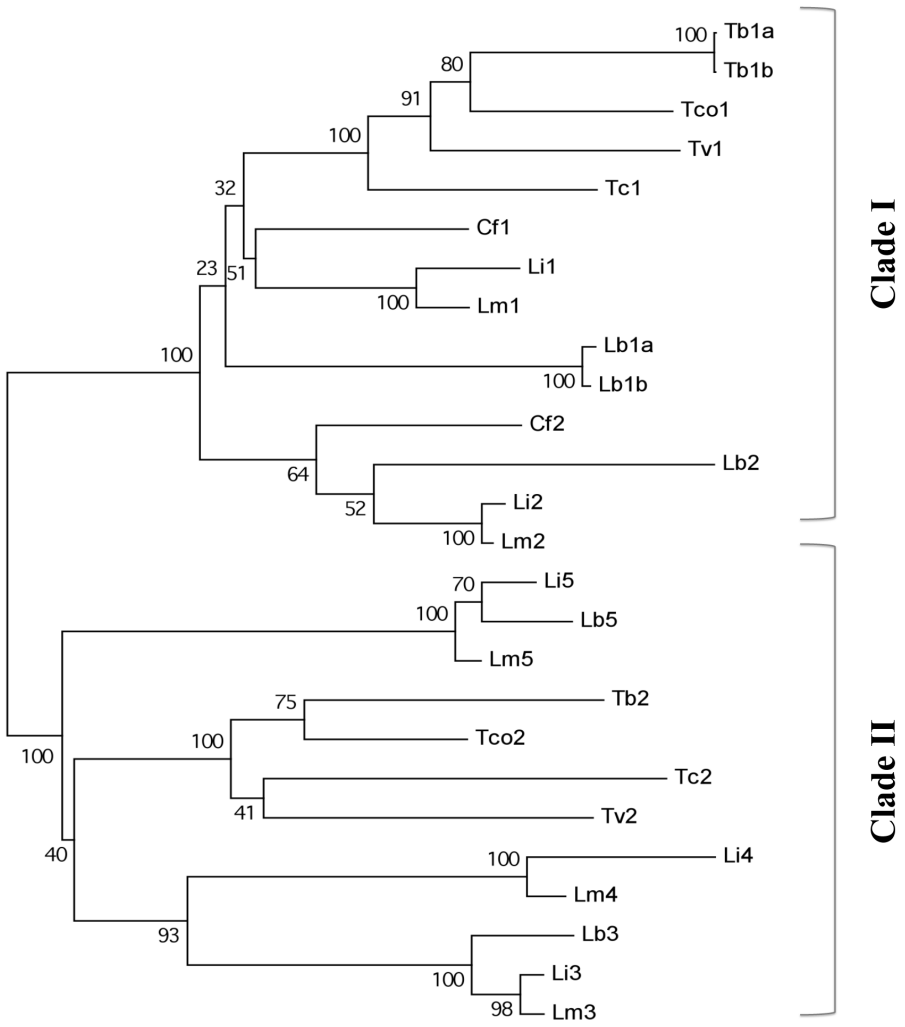


Fig. 8. Phylogenetic tree of the TXN genes in trypanosomatids. The tree was constructed by the bootstrapped (500 replicates) unrooted Neighbor-joining method using the MEGA4 program. Other algorithms were tested and generated similar tree topologies. Bootstrap values are shown next to the respective branch. Horizontal distances represent relative genetic distance. Li: *L. infantum*, Lm: *L. major*, Lb: *L. brazilienses*, Tb: *T. brucei*, Tc: *T. cruzi*, Tco: *T. congolense*, Tv: *T. vivax* and Cf: *C. fasciculata*.

In Clade I, it is also possible to observe the existence of two major subgroups separating all TXN1 sequences from TXN2 molecules of *Crithidia* and *Leishmania*. However, since the branches present poor bootstrap support (< 65%) the real significance of this distinct distribution remains unclear. When analyzing Clade II, it is noteworthy that all *Trypanosoma* TXN2 members group on a single branch, with 100% significance, which strongly suggests that these sequences share a common ancestor. Contrary to this, *Leishmania* TXN3 and TXN5, although in the same clade, group in different branches. On

one side, *Li*TXN5, *Lm*TXN5 and *Lb*TXN5 (100% bootstrap support) and on the other, *Li*TXN3, *Lm*TXN3, *Lb*TXN3, *Li*TXN4 and *Lm*TXN4 (93% bootstrap support). Also in this group, *L. infantum* and *L. major* TXN4 are shown to be closely related however, *Li*TXN4 presents a higher branch length than *Lm*TXN4, which is indicative of a sequence more prone to mutations. This might be explained by the fact that TXN4 is a pseudogene in *L. infantum* but not in *L. major* and, since dispensable proteins are believed to evolve more rapidly (Harrison and Gerstein, 2002), *Li*TXN4 is accumulating more mutations than *Lm*TXN4. In fact, the genomic region surrounding the *TXN*4 gene might be a particularly recombinogenic one. Interestingly, all *Leishmania* spp. keep the *TXN*3 gene without major recombinations. Contrary to *Li*TXN4, both *Li*TXN3 and *Li*TXN5 present identical relative genetic distance in comparison to their *L. major* orthologues.

3.7. Analysis of the TXN-containing loci in trypanosomatids

Syntenic map analysis allows a different view on TXNs evolution. For that reason, a schematic representation of the *TXN*-containing loci was drawn, based on GeneDB available information. The maps depicted on Fig. 9 support the phylogenetic conclusions presented above. Indeed, analysis of the trypanosomatid *TXN* loci suggests that the *TXN* disposition found in *T. cruzi* reflects the primitive situation, i.e., the presence of the two types of *TXN* sequences (coding for proteins with and without hydrophobic C-terminal extension) in a single locus. This is corroborated by the observation that the position of the genes flanking *TcTXN*1 and *TcTXN*2 (genes A and B) is retained in the *TXN* locus in chromosome 29 of *L. infantum*, *L. major* and *L. braziliensis*. Along with *Trypanosoma* speciation additional modifications occurred in these organism's *TXN* loci. Among these is the insertion of a DNA fragment containing genes C and D between gene B and the *TXN*2 coding sequence, as still perceived in *T. congolense*. Further rearrangements in *T. vivax*

and *T. brucei* led to a more complex pattern, as illustrated by the scheme in Fig. 9. Interestingly, in *L. infantum*, *L. major* and *L. braziliensis* the tryparedoxin sequences are disposed in two different genetic loci localized on chromosomes 29 and 31. One locus is composed by *TXN*1, *TXN*2, *TXN*5 and *TXN*6 and another by *TXN*3 and *TXN*4 (except in *L. braziliensis* that seems to have lost the *TXN*4 sequence). This arrangement is likely to have arisen from extensive genetic rearrangements within the chromosome 29 *TXN* locus, which originally should have contained only the two different *TXN* genes, and between this locus and an homologous sequence in chromosome 31. In conclusion, duplication and extensive rearrangement of TXN sequences probably took place in a primitive trypanosomatid. However, the majority of these rearrangements probably occurred after *Leishmania*/*Trypanosoma* separation. For this reason, only *Leishmania* spp. present several additional TXN sequences.

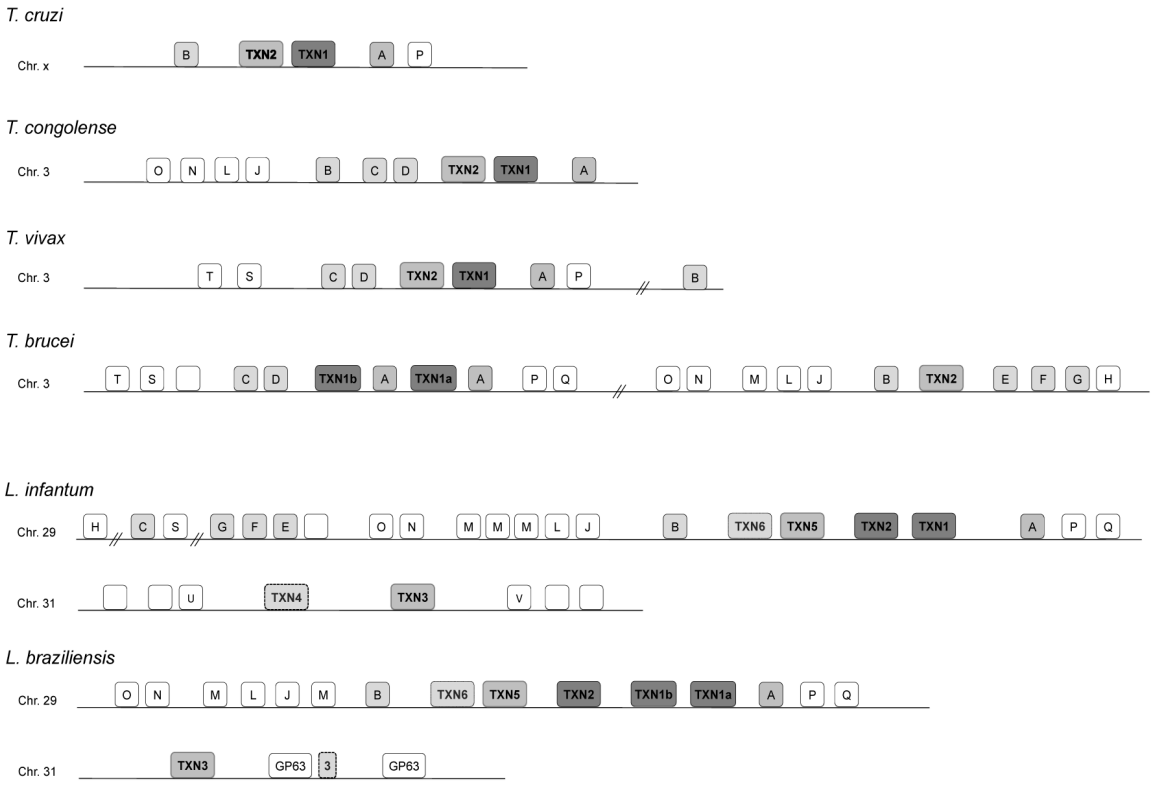


Fig. 9. Comparison of the TXN *loci* in *Leishmania* and *Trypanosoma* spp. Diagram design was based on genome database available for the represented trypanosomatids and schematically represents chromosomal organization. Genes delimited by a dashed line do not encode true TXNs. Distances between genes and gene lengths are not on scale. Since *L. infantum* and *L. major* have equivalent synteny maps, only one is represented. Chr.: chromosome.

4. Discussion

Tryparedoxins are proteins with a catalytically active disulfide group that belong to the thioredoxin superfamily. They were first described in *C. fasciculata*, an insect parasite commonly used as a model for pathogenic trypanosomatids (Nogoceke et al., 1997). In the years that followed this discovery, members of the TXN family were also characterized in *T. brucei* (Lüdemann et al., 1998), *T. cruzi* (Lopez et al., 2000) and *L. infantum* (Castro et al., 2004). Analyzing each known trypanosomatid genome we can observe that *TXN* sequences are divided into two groups, according to the absence or presence of a C-terminal hydrophobic extension. *Trypanosoma* spp. have one *TXN*-like gene in each group, while *Leishmania* spp. present two type I and three to four type II *TXN* genes. In this work we characterized *L. infantum* type II TXN-like proteins. In order to have an insight into their physiological function and in particular to determine whether they are part of the *L. infantum* mitochondrial antioxidant machinery, we investigated their enzymatic activity, as well as their subcellular compartmentalization. Moreover, phylogenetic analysis allowed us to position these proteins in relation to their homologues in other trypanosomatids.

We focused on the *LiTXN*3 and *LiTXN*5 genes since in *L. infantum* these were the only type II *TXN* sequences with the potential to encode functional proteins. Nevertheless, when compared to type I redox active *Li*TXN1 and *Li*TXN2, these proteins present a number of sequence alterations, including non-synonymous substitutions of residues implicated in classical TXN enzymatic activity. This indicated that *Li*TXN3 and *Li*TXN5 are unlikely to participate in typical TXN redox pathways. Among these, involvement in hydroperoxide elimination cascades is probably the most important TXN function (reviewed in Castro and Tomás, 2008). *Leishmania infantum* presents at least two peroxidases in its mitochondrion, which are TXN-dependent, a 2-Cys PRX and a nsGPX.

The former enzyme (*Li*mTXNPx) is essential for amastigote survival but *Li*TXN2, its presumable physiological reductant, is not (H. Castro and A.M. Tomás, manuscript in preparation). We thus tested whether *Li*TXN3 and/or *Li*TXN5 could substitute *Li*TXN2 in provision of reducing equivalents to the mitochondrial peroxidases. To this end, we first assayed the capacity of the recombinant proteins to reduce peroxidases in vitro. Production of full-length *Li*TXN3 and *Li*TXN5 in *E. coli*, for use in enzymatic assays, was nevertheless limited due to the high hydrophobicity of both proteins, which lead to their aggregation in inclusion bodies. To circumvent this, we produced truncated versions, without the hydrophobic C-terminus. Both full length and truncated forms of *Li*TXN3 and *Li*TXN5 were unable to display classical TXN activity, i.e. they were not capable of transferring reducing equivalents to 2-Cys PRX and nsGPX. These observations are in agreement with the analysis of their primary amino acid sequence that, as a result of amino acidic alterations, suggests a weaker association of *Li*TXN3 and *Li*TXN5 to the reaction substrates, $T(SH)_2$ and 2-Cys PRX or nsGPX. In the case of *Li*TXN3, this is at least partially due to the inability of the protein to react with the peroxidases, as this molecule was shown to retain oxidoreductase activity, being able to react with $T(SH)_2$ and then reduce insulin. Contrary to *Li*TXN3, *Li*TXN5 can neither react with *L. infantum* 2-Cys PRXs nor can it be reduced by $T(SH)_2$. These enzymatic data indicate that both *Li*TXN3 and *Li*TXN5 are unlikely to function as typical TXN enzymes in *L. infantum* and, thus, as part of the mitochondrial apparatus for hydroperoxide elimination. Although unlikely we cannot, however, discard the possibility that the forms of *Li*TXN3 and *Li*TXN5 used in the analysis (either full length or truncated) presented erroneous conformation and this affected the proteins enzymatic behavior. Nevertheless, for two molecules to interact they must share the same cell compartment, thus, if either *Li*TXN3 or *Li*TXN5 was to replace the redundant *Li*TXN2, it should be present within mitochondria. Our findings indicate

that whereas *Li*TXN5 is found in the ER, *Li*TXN3 is indeed a mitochondrial protein. More specifically, both of these proteins are integral membrane proteins of those organelles, as suggested by the presence of a C-terminal hydrophobic stretch and confirmed by protein extraction experiments in sodium carbonate buffer. Due to the characteristics of its anchoring region, *Li*TXN3 can be considered a C-tail membrane-anchored protein (Wattenberg and Lithgow, 2001; Borgese et al., 2003). These proteins use the C-tail region both as a targeting sequence and as a membrane anchor to their soluble domain, being always exposed to the cytosol. Thus, the redox active domain of *Li*TXN3 is unlikely to interact with mitochondrial peroxidases. Regarding *Li*TXN5, it is not possible to anticipate its topology, since it presents a second putative transmembrane domain in the middle of the sequence, but its localization in the ER membrane makes impossible its interaction with mitochondrial molecules. All together, these results demonstrate that *L. infantum* type II TXN proteins are not capable of functionally replacing the mitochondrial *Li*TXN2 in reduction of *Li*mTXNPx and remaining peroxidases. We might, therefore, conclude that the existence of a TXN molecule in the mitochondrion is dispensable for normal cell metabolism of *L. infantum* parasites.

In spite of the lack of direct experimental data, it is frequently assumed that type II TXN proteins of *Trypanosoma* spp. are localized to the mitochondrial compartment, where they interact with 2-Cys PRXs (Wilkinson et al., 2000; Motyka et al., 2006; Comini et al., 2007; Irigoín et al., 2008). A meticulous analysis of all known TXN-like sequences reveals that *Trypanosoma* TXN2 proteins share sequence similarities with *Li*TXN3. Indeed, TXN2 of *Trypanosoma* organisms also appear to be C-tail membrane-anchored proteins and, therefore, they are likely to present the redox active site facing the cytosol. In addition, *Trypanosoma* TXN2 sequences present alterations in some of the residues considered to be required for TXN activity. Thus, Glu72 is substituted by positively

charged amino acids in *Tb*TXN2, *Tc*TXN2 and *Tv*TXN2 and Lys102 and Glu107 are replaced by different-type amino acids in most of *Trypanosoma* TXN2 homologues. Except for Ser38, which is kept in *Trypanosoma* TXN2 sequences, all other alterations are identical to the ones observed in *Li*TXN3. Other amino acid changes shared by *Leishmania* type II TXNs and *Trypanosoma* TXN2, but for which no specific function is attributed are Leu26, Gly28, Tyr54, Asn62, Lys83, Leu87 and Gly120. Thus, it is likely that as *L. infantum* TXN3, *Trypanosoma* TXN2 proteins might also not act as typical mitochondrial TXN enzymes.

Given that *Li*TXN3 and *Li*TXN5 are not classical TXNs in *L. infantum*, we wondered what other functions might these proteins perform in the cell. Contrary to *Li*TXN5, *Li*TXN3 might display redox dependent function(s). In fact, this protein presents oxidoreductase activity and is capable of being reduced by $T(SH)_2$, the most abundant cell thiol. Of the other reducing systems tested only the lipoamide dehydrogenase/lipoamide redox couple was capable of reducing the enzyme, although less efficiently than $T(SH)_2$. However, in the parasite context, *Li*TXN3 reduction by the lipoamide system is improbable, since in eukaryotes, this system can only be physiologically relevant within mitochondria and, as mentioned before, although *Li*TXN3 is associated to the mitochondrial membrane, it is most likely interacting with cytosolic molecules. Independently of the reducing system used, *Li*TXN3 activity is considerably inferior to other TXNs which were used as control proteins (*Li*TXN1 and *Li*TXN2). It is possible that the substitution of some *Li*TXN3 amino acid residues implicated in $T(SH)_2$/TXN interaction, might not completely impair TXN reduction by the thiol, but render their association weaker. Eukaryotic thioredoxins were proved to be implicated in protein folding assistance, protease activation, intercellular and intracellular signaling, growth factor activity and transcription factor control, among other redox-dependent biological

processes (reviewed in Arnér and Holmgren, 2000; Vlamis-Gardikas, 2008). It might be that *Li*TXN3 also participates in one or more of these functions. Alternatively, *Li*TXN3 might be an inactive TXN in *L. infantum*, as indeed seems to be the case of *Li*TXN5, and thus be implicated in redox-independent functions. As an example, an enzymatic inactive phospholipid hydroperoxide glutathione peroxidase (PHGPx) was shown to be a major structural element of the mitochondrial sheath in sperm cells (Ursini et al., 1999). A redox-inactive thioredoxin was described in other eukaryotic cells and suggested to be important in the early secretory pathway apparently as an escort protein (Mkrtchian and Sandalova, 2006). In addition, a number of proteins with CXXC motifs were found within the ER, thus far without functional characterization (Matsuo et al., 2004). Finally, we have also considered the possibility that *Li*TXN3 and *Li*TXN5 are presently non-functional proteins. It is possible that the sequences observed in *L. infantum* might have been functional TXNs in a primitive ancestral that are now progressively losing function. In fact, other examples of maintenance of non-functional proteins can be found in trypanosomatid proteomes, namely the preservation of catalytically inactive protein kinases, which may represent around 8% of the total of phosphotransferases in the parasites (Parsons et al., 2005). Ghedin and colleagues (2004) proposed that the loss of dispensable sequences in trypanosomatids might be hindered by their mechanism of cotranscription of several genes in the same mRNA. In this way, the selective pressure will not be applied to a specific gene but rather to a transcriptional unit. Supporting this, trypanosomatids present unusual high levels of chromosome synteny in comparison to other eukaryotes with the same age of divergence (Ghedin et al., 2004). When we analyzed the genome organization of the *TXN* loci of different *Leishmania* species, we have also found a well-preserved distribution of the *TXN* genes. This could explain why *L. infantum* maintained inactive TXN copies, like TXN4 and TXN6, and also why TXN3 and

TXN5 are still being kept, if these too are proved to be non-functional proteins in these parasites. In conclusion, the data obtained herein, regarding *Li*TXN3 and *Li*TXN5, is still insufficient to propose what should be the most likely role for these proteins in *L. infantum*.

Along with the biochemical characterization of *Li*TXN3 and *Li*TXN5, we attempted to provide an explanation for the appearance of multiple *TXN*-like sequences in the *Leishmania* genomes. Gene duplication is an important mechanism for genome expansion (Betrán and Long, 2002) and in trypanosomatids it is a frequent process, which results in tandem arrangement of several copies of the same or similar genes (Thomashow et al., 1983; Clayton and Hotz, 1996; Levick et al., 1998). Since gene expression in these organisms relies on transcription of large polycistronic units, the tandem organization has the advantage of providing higher protein expression levels. Gene duplication is also a source of new proteins as the extra gene copies may suffer slight modifications that lead to a different localization, activity or specificity (Harrison and Gerstein, 2002). In addition, the occurrence of multiple copies of a specific gene might represent an advantage in evolutionary terms, as they guarantee higher genetic diversity, hence, improved adaptability and survival of a species throughout time. Phylogenetic analysis divides the trypanosomatid TXN-like sequences into two very well defined branches. This evolutionary topology reveals that TXNs are likely to have evolved from two separate primitive sequences. One of these probably resembled the currently existing *Trypanosoma* TXN1 and *Leishmania* TXN1 and TXN2 molecules (type I TXNs), while the other primitive TXN was likely to possess an hydrophobic tail similar to what presently happens with *Trypanosoma* TXN2 and *Leishmania* TXN3, TXN4 and TXN5 (type II TXNs). In terms of genome organization, the organism that best preserves the original gene arrangement is likely to be *T. cruzi*, with the two *TXN* type sequences positioned in the

same chromosome locus. The phylogenetic analysis also permits to conclude that both original *TXN* genes were duplicated in *Leishmania* parasites and that this probably occurred after their divergent evolution from the *Trypanosoma* genus. In addition, it is reasonable to speculate that the original TXN sequence, from which all type I TXNs derived, was likely to be a cytosolic protein. Nevertheless, the *L. infantum* mitochondrial TXN2 (Castro et al., 2004), as well as its homologues in other *Leishmania* species and in *C. fasciculata*, branche in the same group as cytosolic TXN1 members, which suggests that these proteins evolved from the same TXN ancestral. For *Li*TXN2 to gain a mitochondrial targeting sequence, the original *TXN* gene had, however, not only to duplicate but also to undergo a number of alterations, which eventually signaled this protein to parasite mitochondria. In the case of type II TXNs, their duplication in *Leishmania* genomes still remains to be elucidated.

In summary, maintenance of *TXN*-like sequences in *Leishmania* may imply the evolution of new advantageous protein functions. The *Li*TXN3 protein is present in the parasite mitochondrial membrane and seems to maintain minor oxidoreductase activity. However, it does not interact with classical 2-Cys PRX nor with nsGPX. *Li*TXN5 is a membrane-associated protein of the ER and seems to be redox-inactive. Either one of these proteins might exert different specific functions in the parasites yet unidentified. Alternatively, *Li*TXN3 and *Li*TXN5 may have lost their function and be in process of disappearance from the *L. infantum* genome.

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Chapter 4

Overexpression of a *Leishmania infantum* cytosolic 2-cysteine peroxiredoxin increases parasite resistance to oxidant challenge elicited by murine and human macrophages

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Abstract

Leishmania infantum can parasitize macrophages of different mammalian species causing visceral leishmaniasis in the Mediterranean region. Although disease progression has been associated to the capacity of the parasites to cope with macrophage-derived reactive oxygen and nitrogen species, the relevance of these oxidants to control *Leishmania* infections has been questioned. In *L. infantum*, 2-cysteine peroxiredoxins (2-Cys PRXs) are key elements of an enzymatic cascade for elimination of hydroperoxides, such as, hydrogen peroxide. In this study we show that the two cytosolic 2-Cys PRXs (*Li*TXNPx1 and *Li*TXNPx2) are also capable of rapidly reducing peroxynitrite ($k = 1 \times 10^6$ M^{-1} s^{-1} and $8.5 \pm 0.5 \times 10^5$ M^{-1} s^{-1}, respectively). Using a parasite line overexpressing a cytosolic 2-Cys PRX, we attempted to determine if hydrogen peroxide and/or peroxynitrite could exert leishmanicidal activity when produced by murine and human macrophages. At the same time, we aimed at evaluating if these parasites could serve as a suitable model to define the relevance of host oxidative challenge in the establishment of *L. infantum* infections in vivo. Our results show that upregulation of *Li*TXNPx2 enhances parasite infectivity in both host cells tested. In the case of murine macrophages, the phenotype observed can be associated to an increased capacity of *Li*TXNPx2 overexpressers to eliminate hydrogen peroxide and peroxynitrite. As for human macrophage infection, the increased infectivity can only be attributed to hydrogen peroxide toxicity, since these cells were unable to induce nitric oxide production. Our findings also support the use of cytosolic 2-Cys PRX overexpressing cell lines to assess *L. infantum* infection patterns in animal models.

1. Introduction

The protozoan parasites of the genus *Leishmania* are responsible for a complex of diseases named leishmaniasis. Different species of *Leishmania* exist in several geographic areas causing distinct clinical manifestations in animals and humans. In southern Europe, *L. infantum* is the agent responsible for visceral leishmaniasis, the most dangerous and potentially deadly form of the disease (Desjeux, 2004). The parasite presents a digenetic life cycle, alternating between an insect vector and a vertebrate host. Extracellular flagellated promastigotes grow and replicate in the sandfly gut and are introduced into the dermis of a mammalian host during the insect blood meal (Sacks and Kamhawi, 2001). Once in a new host, parasites are promptly taken up by resident macrophages, dendritic cells and neutrophils (Awasthi et al., 2004). Macrophages are their final host cells and it is inside their phagolysosomes that promastigotes differentiate into replicative non-motile amastigotes (McConville et al., 2007).

Macrophages are pivotal cells of the innate immune system, responsible for the detection and elimination of pathogens. Among their diverse microbicidal activities is the production of reactive oxygen (ROS) and nitrogen species (RNS) that derive from two highly reactive molecules, superoxide anion and nitric oxide radicals (reviewed in Forman and Thomas, 1986; MacMicking et al., 1997). NADPH oxidase 2 (NOX2) is the enzyme responsible for the high fluxes of superoxide ($O_2^{\bullet-}$) registered during the macrophage oxidative burst. Its activation depends on chemotactic factors or phagocytic stimuli and leads to the assemblage of all membrane and cytosolic subunits, in order to allow passage of electrons for reduction of molecular oxygen to form $O_2^{\bullet-}$ (for a review see Bedard and Krause, 2007). Superoxide anion can dismutate to hydrogen peroxide (H_2O_2) either spontaneously, if at low pH, or through a reaction catalyzed by superoxide dismutase (SOD). Several studies have demonstrated that ROS, such as H_2O_2, are toxic to

Leishmania both in vitro (Zarley et al., 1991; Castro et al., 2002a) and in vivo (Murray, 1981; Haidaris and Bonventre, 1982; Blos et al., 2003). Nitric oxide ($^{\bullet}NO$) generation in macrophages depends on inducible nitric oxide synthase (iNOS). The enzyme catalyzes the conversion of L-arginine to N^{ω}-OH-L-arginine (NOHA), which is then further oxidized to form $^{\bullet}NO$ and L-citrulline. The inductive agents might vary but, classically, IFN-γ and LPS are used to activate iNOS in vitro (reviewed in MacMicking et al., 1997). Nitric oxide might directly affect a number of molecular targets on the parasite (Vespa et al., 1994; Salvati et al., 2001), however, most of its toxicity relies on its derivatives. A powerful RNS is peroxynitrite ($ONOO^-$) (for a review see Beckman and Koppenol, 1996; Radi et al., 2001), which can be generated in macrophages by the concomitant production of $O_2^{\bullet-}$ and $^{\bullet}NO$ (Alvarez et al., 2002). It is relevant to mention that the reported second order rate constants for the combination of $O_2^{\bullet-}$ and $^{\bullet}NO$ are around 10^{10} M^{-1} s^{-1} (reviewed in Radi et al., 2001), thus approximately 5 times faster than $O_2^{\bullet-}$ interaction with SOD, for which the reaction constant was estimated to be 2 x 10^9 M^{-1} s^{-1} (Fielden et al., 1974). This suggests that, if $^{\bullet}NO$ is produced in the same compartment as $O_2^{\bullet-}$, $ONOO^-$ formation will be favored in relation to H_2O_2. Peroxynitrite was described to be a cytotoxic molecule for *Trypanosoma* and *Leishmania* in vitro (Denicola et al., 1993; Gatti et al., 1995) and, later on, macrophage-derived $ONOO^-$ was also shown capable of killing *Leishmania amazonensis* (Linares et al., 2001) and *Trypanosoma cruzi* (Alvarez et al., 2004). Since there are strong suggestions that activation of the iNOS pathway is crucial for the control of *Leishmania* infections in general (Cunha et al., 1993; Diefenbach et al., 1998; Murray and Nathan, 1999), many authors argue that this directly results from the formation of lethal $^{\bullet}NO$-derived reactive species. In view of this, to establish a successful infection, *Leishmania* species should be able to escape the effects of ROS and/or RNS.

In the last years the relevance of ROS and/or RNS in the infectious process have been however questioned. Some authors suggested that the deleterious consequences to the parasites of iNOS activation would not be the oxidation of pathogen molecules via $^{\bullet}$NO-derivatives but, instead, a deficiency in essential nutrients in the phagosome. For instances, NOHA, the intermediate compound in the synthesis of $^{\bullet}$NO, acts as a strong inhibitor of the enzyme arginase, which is essential for polyamine synthesis. Consequently, iNOS activation may deplete the parasites from indispensable polyamines and eventually limit their proliferation in the host cell (Iniesta et al., 2001). It is also possible that activation of iNOS channels most of the macrophage L-arginine to the production of $^{\bullet}$NO, compromising the parasites' accessibility to this essential amino acid (Chaturvedi et al., 2007; Wanasen and Soong, 2008). In addition to this debate, some *Leishmania* species and stages have been reported to modulate the production of $O_2^{\bullet-}$ and $^{\bullet}$NO, raising doubts about their relevance in vivo (Descoteaux et al., 1991; Nandan and Reiner, 1995; Proudfoot et al., 1996; Pham et al., 2005). Moreover, the importance of ROS and especially of RNS as microbicidal agents might differ among host species. For example, the output of $^{\bullet}$NO produced by human macrophages is considered to be much lower than in murine cells (Albina, 1995). In fact, the production of $^{\bullet}$NO as a general antimicrobial mechanism of human macrophages is far from gathering general consensus (Schneemann and Schoedon, 2002).

Leishmania possess a number of low molecular weight compounds and of enzymes displaying antioxidant function. Among these, 2-cysteine peroxiredoxins (2-Cys PRXs) present a key role in hydroperoxide elimination (reviewed in Castro and Tomás, 2008). This type of enzymes exists in a variety of organisms (bacteria, yeast, protozoa, plants and animals) and are capable of reducing H_2O_2, organic hydroperoxides and, at least in some cases, $ONOO^-$ (Bryk et al., 2000; Jaeger et al., 2004; Trujillo et al., 2004; Nickel et al.,

2005; Ogusucu et al., 2007). Previous work from our group (Castro et al., 2002a; Castro et al., 2004) described two active 2-Cys PRXs in the *L. infantum* cytosol, *Li*TXNPx1 and *Li*TXNPx2, and one in the mitochondrion, *Li*mTXNPx. Transformed parasites overexpressing one of the cytosolic 2-Cys PRXs were shown to present an increased resistance to exogenously added H_2O_2 and *tert*-butyl hydroperoxide (t-bOOH) (Castro et al., 2002a). However, the capacity of *L. infantum* 2-Cys PRXs to reduce $ONOO^-$ is, to date, not clearly established.

In this report, we addressed the capacity of *L. infantum Li*TXNPx2 to display peroxynitrite reductase activity in vitro and in the parasite context. In addition, we analyzed the infectivity patterns of *Li*TXNPx2 overexpressing parasites in macrophages producing H_2O_2 and $ONOO^-$. Our main objectives were i) to determine if H_2O_2 and/or $ONOO^-$ can exert leishmanicidal activity when produced by murine and human macrophages and ii) to evaluate if parasites transformed to overexpress cytosolic 2-Cys PRX are suitable models for studying the impact of H_2O_2 and $ONOO^-$ upon an in vivo infection. Our results show that both *L. infantum* cytosolic 2-Cys PRX reduce $ONOO^-$ at similar rate constants and suggest that H_2O_2, as well as $ONOO^-$, present leishmanicidal capacity when produced by macrophages. More importantly, characterization of the *Li*TXNPx2 overexpressers supports their use as a tool to dissect the significance of H_2O_2/$ONOO^-$ in controlling *Leishmania* infections in vivo.

2. Materials and methods

2.1. Parasite culture

Leishmania infantum promastigotes (strain MHOM/MA/67/ITMAP-263) were cultured in RPMI 1640 medium with Glutamax supplemented with 10% heat inactivated fetal bovine serum (iFBS), 50 $U.ml^{-1}$ penicillin, 50 $\mu g.ml^{-1}$ streptomycin and 20 mM Hepes sodium salt pH 7.4 (complete RPMI medium) at 25ºC.

2.2. Vector construction and parasite transfection

A strategy similar to the one previously described to generate pTEXTXNPx1 transformed parasites (Castro et al., 2002a) was used to produce *L. infantum* overexpressing *Li*TXNPx2. Primers used to amplify the open reading frame were 5'-cgc**ggatcc**ATGTCCTGCGGTGACGCC-3' and 5'-caccg**ctcgag**TTACTTATTGTGATCGAG-3' (restriction sites in bold).

2.4. Expression and purification of LiTXNPx2 in E. coli

*Li*TXNPx2 was produced as a recombinant protein in *E. coli*, as previously described (Castro et al., 2004), using the pET28a expression vector (Novagen) and oligonucleotide 5'-ccgcgca**catATG**TCCTGCGGTGACGC-3' as sense primer and 5'-caccg**ctcgag**TTACTTATTGTGATCGAG-3' as antisense primer.

2.5. Antibody production

Anti-*Li*TXNPx2 polyclonal antibody was raised in Wistar rats (Charles River, France) housed under specific pathogen-free conditions at the Instituto de Biologia Molecular e Celular animal facility (Porto, Portugal). Briefly, animals were immunized subcutaneously with 100 μg of recombinant *Li*TXNPx2 in Freund's complete adjuvant. Four weeks later, 50 μg of *Li*TXNPx2 in Freund's incomplete adjuvant were again administrated by the same inoculation route. Animal experiments were approved by the IBMC ethics committee.

2.6. Peroxynitrite synthesis

Peroxynitrite was synthesized in a quenched flow reactor from sodium nitrite and hydrogen peroxide (H_2O_2) under acidic conditions and quantified as described previously (Radi et al., 1991). H_2O_2 remaining from synthesis was eliminated by treating the peroxynitrite stock solution with granular manganese dioxide. Nitrite (NO_2^-) resulting from natural peroxynitrite decomposition was typically < 30% of peroxynitrite concentration.

2.7. Protein thiol reduction

*Li*TXNPx1, *Li*TXNPx2 and *Li*mTXNPx were reduced by the addition of over a 10-fold excess of dithiothreitol (DTT) for 1 h. Excess reductant was removed, immediately before protein use, by high-pressure liquid chromatography (Hitrap column, GE Healthcare). Proteins were collected manually in rubber-capped tubes with UV-visible detector at 280

nm. Elution buffer was 100 mM potassium phosphate pH 7.0, plus 100 μM diethylene triamine pentaacetic acid (DTPA) and was extensively degassed before use. Once collected, samples were bubbled for 2 to 5 min with argon at 4 °C. Protein concentration was measured by the Bradford method.

2.8. Kinetic studies by stopped-flow spectrophotometry

The kinetics of peroxynitrite decomposition was studied in a stopped flow spectrophotometer (SF17MV, Applied Photophysics) with a mixing time of > 2 ms. Peroxynitrite decay was followed at 310 nm using a split time mode which allowed obtaining 200 experimental points during the initial 20 ms of the reaction, while other 200 points were obtained from 20 ms to 20 s. The reaction was monitored at 310 nm and not at the typical 302 nm (Hughes and Nicklin, 1968) in order to avoid interference by background protein absorption. An initial rate approach was used in this study (Alvarez et al., 1999). Reported values are the mean of at least 3 independent determinations. All reactions were performed at 25ºC.

2.9. Hydrogen peroxide and peroxynitrite sensitivity assays

The growth inhibitory effect of H_2O_2 on *L. infantum* promastigotes was analyzed as previously described (Castro et al., 2002a). Briefly, exponentially grown cultures were seeded at 10^6 ml^{-1} in 2 ml complete RPMI medium in 24-well plates in the absence of selective drugs and incubated for 24 h at 25ºC. Different H_2O_2 concentrations were then added to each well. Four to five days later parasite growth was measured by absorbance reading at 600 nm. To assess the effect of peroxynitrite on *L. infantum* promastigote growth, ascending concentrations of peroxynitrite were added to aliquots of logarithmic-phase cells (~1.5 x 10^8) under strong vortexing. After an incubation period of 15 min at 25ºC, 6 x 10^6 parasites of each aliquot were seeded in 2 ml complete RPMI medium in 24-well plates and incubated at 25ºC. Three to four days later parasite growth was measured by absorbance reading at 600 nm.

2.10. Western blotting

Leishmania infantum protein extracts were obtained as described elsewhere (Castro et al., 2004), resolved by SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk and probed against the adequate primary antibodies. Secondary antibodies labeled with horseradish peroxidase (HRP) were used

and signal detection was achieved using ECL detection reagents (GE Healthcare). For nitrotyrosine detection, blots were incubated with anti-nitrotyrosine (NO_2Y) primary antibody in 5% BSA, 0.6% Tween-20 and subsequent washings were performed in 150 mM NaCl, 10 mM Tris-HCl pH 7.6, 0.6% Tween-20.

2.11. Indirect immunofluorescence assays (IFATs)

Immunofluorescence assays were performed as described elsewhere (Castro et al., 2002a). Briefly, cells were fixed with 4% paraformaldehyde (w/v) in 0.27 M NaCl, 3.6 mM KCl, 4.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4 pH 7.2 (PBS), permeabilized with 0.1% (v/v) TritonX-100, blocked with 1% BSA and incubated first with primary antibody for 1 h at room temperature and then with adequate secondary antibodies (Alexa Fluor 488). Polyclonal antibody against inducible nitric oxide synthase isoform was purchased from Abcam (ref. ab3523). All samples were mounted in 50% (v/v) glycerol in PBS, analyzed with an AxioImager Z1 fluorescence microscope and photographed with an Axiocam MR3.0 using the Axiovision 4.6 software (Zeiss).

2.12. Isolation of murine and human macrophages

Murine macrophages, obtained from BALB/c mice (Charles River, France) by peritoneal lavage, were seeded at 4 x 10^5 cells per well in 24-well plates with round glass coverslips and allowed to adhere for 3 h at 37ºC in 5% CO_2 atmosphere in DMEM Glutamax culture medium complemented with 20% iFBS, 50 $U.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin (DMEM complete medium). Some experiments were performed with the murine macrophage cell line J744A.1 (American Type Culture Collection TIB-67) cultured in DMEM complete medium at 37ºC, 5% CO_2. Human peripheral blood mononuclear cells (PBMCs) were isolated from blood of apparently healthy donors by density sedimentation using Histopaque-1077 (Sigma). For this, erythrocytes were lysed with 10 mM Tris-HCl, 150 mM NH_4Cl pH 7.4 and PBMCs were then seeded at 1.2 x 10^7 cells per well in 24-well plates with round glass coverslips. Monocytes were separated by adherence and cultured in DMEM Glutamax complemented with 10% iFBS, 10% equine adult serum, 1% non-essential amino acids, 50 $U.ml^{-1}$ penicillin and 50 $\mu g.ml^{-1}$ streptomycin at 37ºC, 5% CO_2 for 6 days. Five to 10% of total PBMCs were determined to be monocytes by cell counting.

2.13. Macrophage monolayer infections

Murine macrophages were activated with 300 $U.ml^{-1}$ recombinant murine IFN-γ and 5 $\mu g.ml^{-1}$ LPS 24 h previous to infection. Human macrophages received 500 $U.ml^{-1}$ recombinant human IFN-γ and 5 $\mu g.ml^{-1}$ LPS 48 h before infection. In both cases macrophages were incubated with stationary-phase serum-opsonized promastigotes at a parasite:macrophage ratio of 10:1, for 3 h at 37ºC, 5% CO_2. Non-internalized parasites were removed by gentle washing with pre-warmed Hanks Balanced Salt Solution (HBSS) and macrophages were further cultured for 24 h and 72 h in DMEM complete medium with IFN-γ and LPS. Control infections were performed in the absence of IFN-γ and LPS or upon addition of 2.5 mM aminoguanidine (AG) to activated cells 5 h previous to infection. All conditions were performed in triplicate. Murine cells were fixed with methanol, stained with Diff Quick, and the number of infected cells and amastigotes per cell determined by light microscopy, counting a minimum of 400 and 100 macrophages, respectively. Human macrophage monolayers were processed for IFAT, as described before, using a mixture of anti-TSA and anti-mTXNPx primary antibodies, Alexa Fluor 488 anti-rabbit IgG (as secondary antibodies to detect parasites) and covered with a solution of DAPI or propidium iodide (to evidence macrophages). Cells were then visualized in an AxioImager Z1 fluorescence microscope and photographed with an Axiocam MR3.0 using the Axiovision 4.6 software (Zeiss). The number of infected cells and amastigotes per cell were counted manually in each photograph using a purposely designed experimental software (to be published elsewhere). An average of ~800 macrophages per coverslip were counted.

2.14. Detection of superoxide anion by nitroblue tetrazolium staining (NBT)

Intracellular production of superoxide anion was estimated by the reduction of NBT to dark-colored insoluble formazan, based on the method described by Baehner and Nathan (1968). For this, 2 x 10^5 macrophages seeded in 8-well chamber slides were infected with opsonized parasites (parasites:macrophage ratio 10:1) and incubated with 1 $mg.ml^{-1}$ NBT solution freshly prepared in Dulbecco's PBS (dPBS: 0.9 mM $CaCl_2$, 0.5 mM MgCl, 2.7 mM KCl, 1.45 mM KH_2PO_4, 137 mM NaCl, 8.1 mM Na_2HPO_4, 5.5 mM Glucose pH 7.4) for 30 min at 37ºC. Cells were washed with HBSS and fixed with methanol. Formazan deposits were evaluated microscopically and parasite presence was confirmed by propidium iodide staining. Negative controls without parasite infection were performed in

parallel. All conditions were carried out in triplicate. Cells were analyzed with an Axioskop microscope.

2.15. Detection of nitric oxide production by the Griess assay

Nitric oxide production was estimated by determination of the NO_2^- concentration in macrophage culture supernatants, following the Griess assay (Green et al., 1982). Briefly, 100 μl of cell supernatant (from about 1 x 10^5 macrophages) were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthalene-ethylenediamine dihydrochloride, 2.5% H_3PO_4). After 10 min at room temperature, product formation was determined spetrophotometrically at 550 nm. Increasing quantities of $NaNO_2$ were used as standards.

2.16. Detection of nitric oxide production by the DAF-2 DA fluorescent probe

Production of nitric oxide ($^{\bullet}NO$) by macrophages in real-time was assessed with the cell permeable fluorescent probe diaminofluorescein-2-diacetate (DAF-2 DA) (Kojima et al., 1998). For this, macrophage monolayers (4 x 10^5 cells per well in 24-well plates) were cultured in DMEM complete medium with or without IFN-γ and LPS. After different incubation periods, macrophages were loaded with 10 μM DAF-2 DA in dPBS supplemented with 1 mM L-arginine for 30 to 45 min at 37ºC, washed twice in dPBS and analyzed at 37ºC in a SpectraMax Gemini XS fluorimeter (Molecular Devices) for 120 min (Ex. 488 nm, Em. 515 nm).

2.17. Statistical analysis

Data sets were compared by independent-samples *t*-test using SPSS software version 16.0.

3. Results

3.1. The two Leishmania infantum cytosolic 2-cysteine peroxiredoxins interact with peroxynitrite in vitro

In this study we aimed at investigating whether *L. infantum* parasites overexpressing cytosolic 2-Cys PRXs could present increased resistance to H_2O_2 and $ONOO^-$ generated by macrophages and, thus, serve as a tool to dissect the relevance of

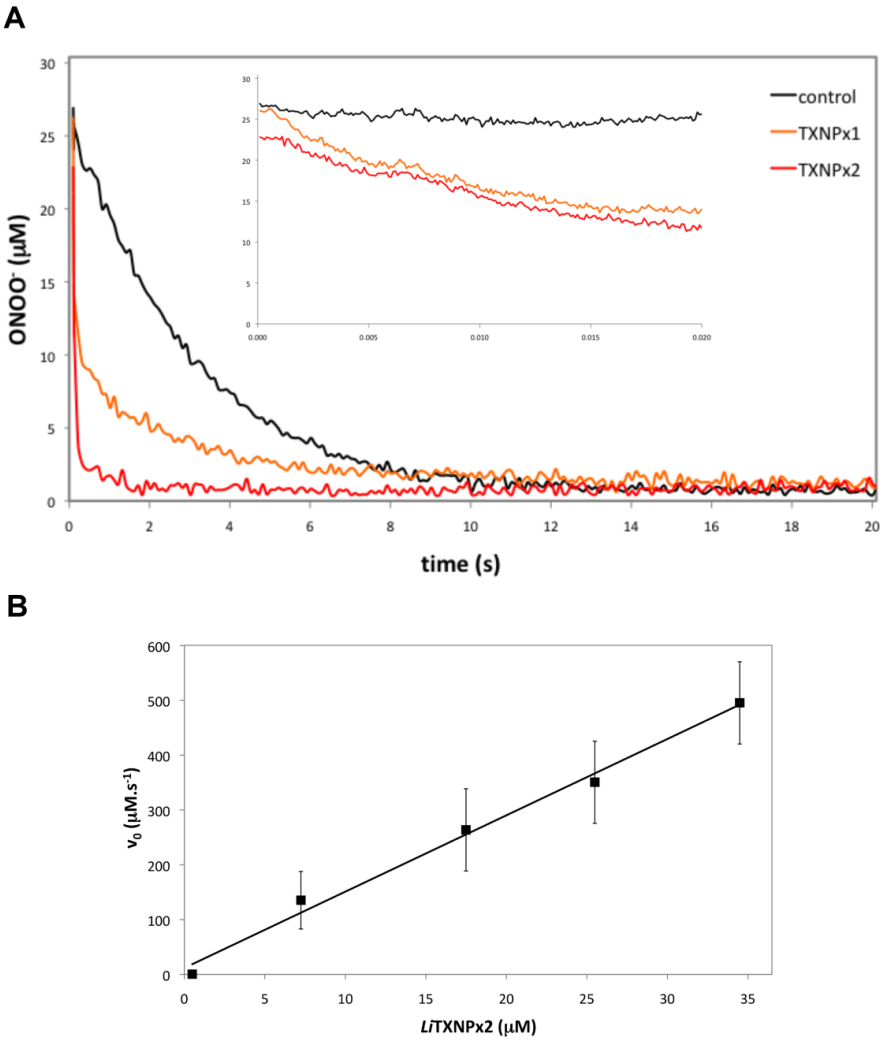


Fig. 1. Peroxynitrite reduction by *Leishmania infantum* 2-cysteine peroxiredoxins. (A) Primary plot showing 25 μM peroxynitrite decay in 50 mM potassium phosphate buffer pH 7.4, 0.1 mM DTPA in the absence (black curve) or in the presence of reduced 40 μM *Li*TXNPx1 (orange curve), 43 μM *Li*TXNPx2 (red curve). The reaction was followed at 310 nm in a stopped flow spectrophotometer at 25ºC. The inset shows the first 20 ms of the reaction. (B) Initial rate of peroxynitrite reduction in the presence of increasing concentrations of *Li*TXNPx2 at 25ºC and pH 7.4 (2 ms to 20 ms). The rate constant for the reaction between peroxynitrite and reduced *Li*TXNPx2 was determined by dividing the slope of this plot by the initial concentration of peroxynitrite used in the assay (16.5 μM). The same experiment was performed with increasing concentrations of *Li*TXNPx1 (not shown). Reported values are the mean of at least 3 independent determinations.

these compounds in the context of a *Leishmania* infection. Previous results by Barr and Gedamu (2003) in *L. chagasi* suggested that only one of the two cytosolic 2-Cys PRXs present in this parasite was capable of reducing $ONOO^-$. We thus analyzed if the capacity of both *L. infantum* cytosolic 2-Cys PRXs to interact with $ONOO^-$ was dissimilar. For this, $ONOO^-$ decomposition at pH 7.4 in the absence or in the presence of *Li*TXNPx1 and

*Li*TXNPx2 was followed in stopped flow experiments. As depicted in Fig. 1A the rate of $ONOO^-$ decay is accelerated in the presence of the reduced form of both 2-Cys PRXs, suggesting that the two enzymes have $ONOO^-$ reductase activity. By following the effect of increasing concentrations of 2-Cys PRXs on the initial rate of $ONOO^-$ decomposition (Fig. 1B), second order rate constants for the reaction were determined. Contrary to what was previously reported when characterizing the *L. chagasi* enzymes (Barr and Gedamu, 2003), the rate constants determined for the reaction of *Li*TXNPx1 and *Li*TXNPx2 with $ONOO^-$ were identical (1.0 x 10^6 M^{-1} s^{-1}, 8.5 ± 0.5 x 10^5 M^{-1} s^{-1}, respectively). The *L. infantum* parasitic line overexpressing *Li*TXNPx2 was then selected to proceed with the study of H_2O_2/$ONOO^-$ effects on *L. infantum* survival inside the host cell. First, however, we demonstrated that their H_2O_2 and $ONOO^-$ reducing capacity was maintained in the context of the parasite.

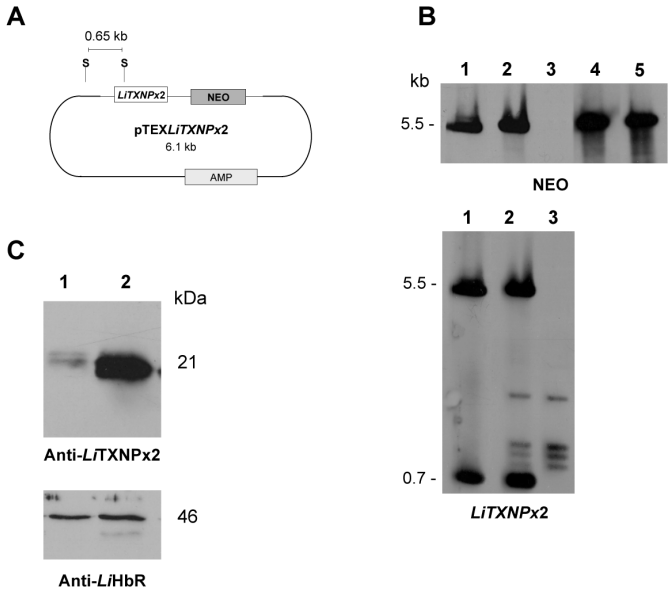


Fig. 2. *Li*TXNPx2 overexpression in transformed parasites. (A) Map of the pTEXTXNPx2 episome. The neomycin resistance gene (NEO) and *Sac*I (S) restriction sites are indicated. (B) Southern blot of *Sac*I digested DNA from pTEXTXNPx2 (lane 1) and pTEX vectors (lane 5) and of *Sac*I digested genomic DNA from pTEXTXNPx2 (lane 2), wild-type (lane 3) and pTEX (lane 4) *L. infantum* promastigotes. The blot was successively hybridized with the NEO and *LiTXN*Px2 probes as indicated. Transformed pTEXTXNPx2 and pTEX control parasites present a specific band of ~5.5 kb when hybridized with the NEO probe, which correspond to the expression vector. pTEXTXNPx2 transformed parasites present two bands of ~5.5 kb and ~0.65 kb that correspond to the *LiTXNPx*2 episomal copy and 4 others corresponding to endogenous *Li*TXNPx. (C) Western blot analysis of 25 μg total protein extract from pTEX (lane 1) and pTEXTXNPx2 transformed parasites (lane 2). The blot was successively hybridized with antibodies anti-*Li*TXNPx2 and anti-*Li*HbR as loading control (Carvalho et al., 2009).

3.2. Overexpression of LiTXNPx2 protects the parasites against exogenously added hydrogen peroxide and peroxynitrite

Previous work from our group demonstrated that overexpression of *Li*TXNPx1 increased promastigote resistance to exogenously added H_2O_2 and t-bOOH (Castro et al., 2002a). To assess whether *Li*TXNPx2 can also provide protection from hydroperoxides and $ONOO^-$, parasites overexpressing the protein were produced. For this, the *LiTXNPx*2 gene was cloned into the trypanosomatid expression vector pTEX (Kelly et al., 1992) and the resulting plasmid introduced into wild-type (WT) *L. infantum* promastigotes. Neomycin resistant clones were analyzed by Southern blotting, using the NEO and *LiTXNPx*2 genes as probes (Fig. 2A and B). As depicted in Fig. 2B, empty pTEX control parasites and pTEXTXNPx2 transformed parasites contained the episome without evidence for any genetic rearrangements. Overexpression of *Li*TXNPx2 in pTEXTXNPx2 transformed parasites was confirmed by Western blotting (Fig. 2C). The two parasite cell lines were then compared in terms of resistance to H_2O_2 and $ONOO^-$. Figure 3A shows that *L. infantum* promastigotes overexpressing *Li*TXNPx2 present an increased resistance to exogenously added H_2O_2 when compared with pTEX control parasites transformed with the empty vector. Likewise, pTEXTXNPx2 transformed parasites were also more protected against exposure to a bolus of $ONOO^-$, although in this case, the difference between *Li*TXNPx2 overexpressing parasites and controls was less evident (Fig. 3B). To confirm that pTEXTXNPx2 transformed parasites were indeed more resistant to $ONOO^-$, we compared the $ONOO^-$-dependent protein tyrosine nitration of these cells with pTEX control parasites by Western blot analysis using the anti-nitrotyrosine antibody (anti-NO_2Y). Indeed, tyrosine nitration is a common modification observed in proteins after $ONOO^-$ exposure. It consists of incorporation of a nitro group ($-NO_2$) to the ortho-position of a tyrosine residue (fig. 3C and Radi, 2004). It is noteworthy that $ONOO^-$ itself does not

directly nitrate proteins, however, the process can occur through $ONOO^-$ derivatives, such as $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ (fig. 3C). As shown in Fig. 3D, protein tyrosine nitration was significantly inhibited in *Li*TXNPx2 overexpressers comparing to controls. Antibodies against *Li*TXNPx2 and *Li*mTXNPx were used as overexpression and loading controls, respectively.

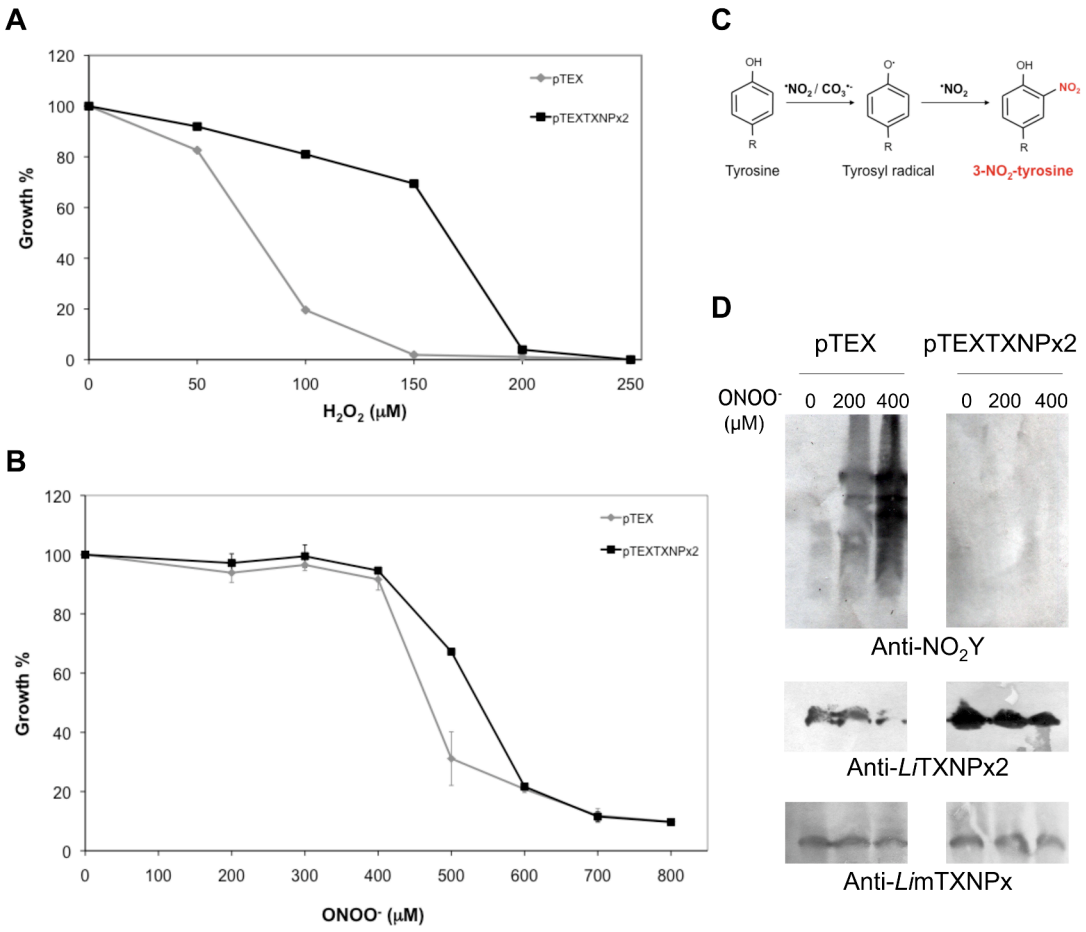


Fig. 3. Effect of hydrogen peroxide and peroxynitrite on the replication of *Leishmania infantum* promastigotes. Transformed pTEX (♦) and pTEXTXNPx2 (■) *L. infantum* promastigotes were exposed to a bolus of (A) H_2O_2 and peroxynitrite (B) at various concentrations and 5 or 4 days later, respectively, cell culture densities were assessed spectrophotometrically at 600 nm. Data are expressed as a percentage of parasite growth in cultures without peroxide. In (A) one representative experiment out of five is shown, all conditions were performed in duplicate. In (B) S.D. between triplicates are indicated by error bars and the graphic shows one representative assay out of three performed. (C) Peroxynitrite dependent 3-NO_2-tyrosine formation. Both $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ can perform a one-electron abstraction of tyrosine resulting in the tyrosyl radical. This rapidly combines with $^{\bullet}NO_2$ yielding 3-NO_2-tyrosine. Adapted from Radi et al. 2001. (D) Western blot comparing total protein extract from 5 x 10^6 pTEX and pTEXTXNPx2 transformed promastigotes exposed to 0, 200 and 400 µM peroxynitrite. Nitrated proteins were detected with the anti-nitrotyrosine (anti-NO_2Y) serum as indicated. After stripping, the blot was re-hybridized with anti-*Li*TXNPx2 (overexpression control) and anti-*Li*mTXNPx (loading control).

3.3. Establishment of the conditions for production of H_2O_2 and $ONOO^-$ in murine and human macrophages

To evaluate the toxicity to *L. infantum* of the macrophage-derived reactive species under analysis, we aimed at comparing the levels of infectivity of *Li*TXNPx2 overexpressing parasites and controls in ex vivo infections of macrophage monolayers. Two different types of host cells were used, BALB/c peritoneal macrophages and human peripheral blood monocyte-derived macrophages. In order to analyze the effects of the production of H_2O_2 and $ONOO^-$ during the infection assays, we first had to guarantee the conditions for production of the precursor molecules ($O_2^{\bullet-}$ and $^{\bullet}NO$) upon *L. infantum* infection in both types of host cells. Superoxide radical anion is generated by NADPH oxidases (NOXs) upon microbe phagocytosis (reviewed in Bedard and Krause, 2007). We thus determined the generation of $O_2^{\bullet-}$ in murine and human cells by the nitroblue tetrazolium (NBT) assay, subsequently to cell contact with *L. infantum* promastigotes and amastigotes. The NBT reagent consists on a soluble dye which when reduced, for example by $O_2^{\bullet-}$, is converted into insoluble dark blue formazan that can easily be observed by light microscopy (Fig. 4A). Figure 4B shows formazan accumulation in both macrophage types, indicating $O_2^{\bullet-}$ production upon promastigote contact. Release of $O_2^{\bullet-}$ was confirmed to be associated to the presence of *L. infantum* promastigotes, since the formazan deposits were formed in the region of macrophage engulfment of the parasites (Fig. 4C). On the contrary, no positive formazan macrophages are found after amastigote infection (Fig. 4B), suggesting that the *L. infantum* mammalian stage can impair the production of $O_2^{\bullet-}$ by macrophages. Negative controls were performed with promastigotes alone (data not shown). In order to induce $^{\bullet}NO$ generation in murine and human macrophages, cells were incubated with classical iNOS activating molecules, IFN-γ and LPS. The first method used to estimate $^{\bullet}NO$ production was the determination of nitrites (NO_2^-) in cell supernatants

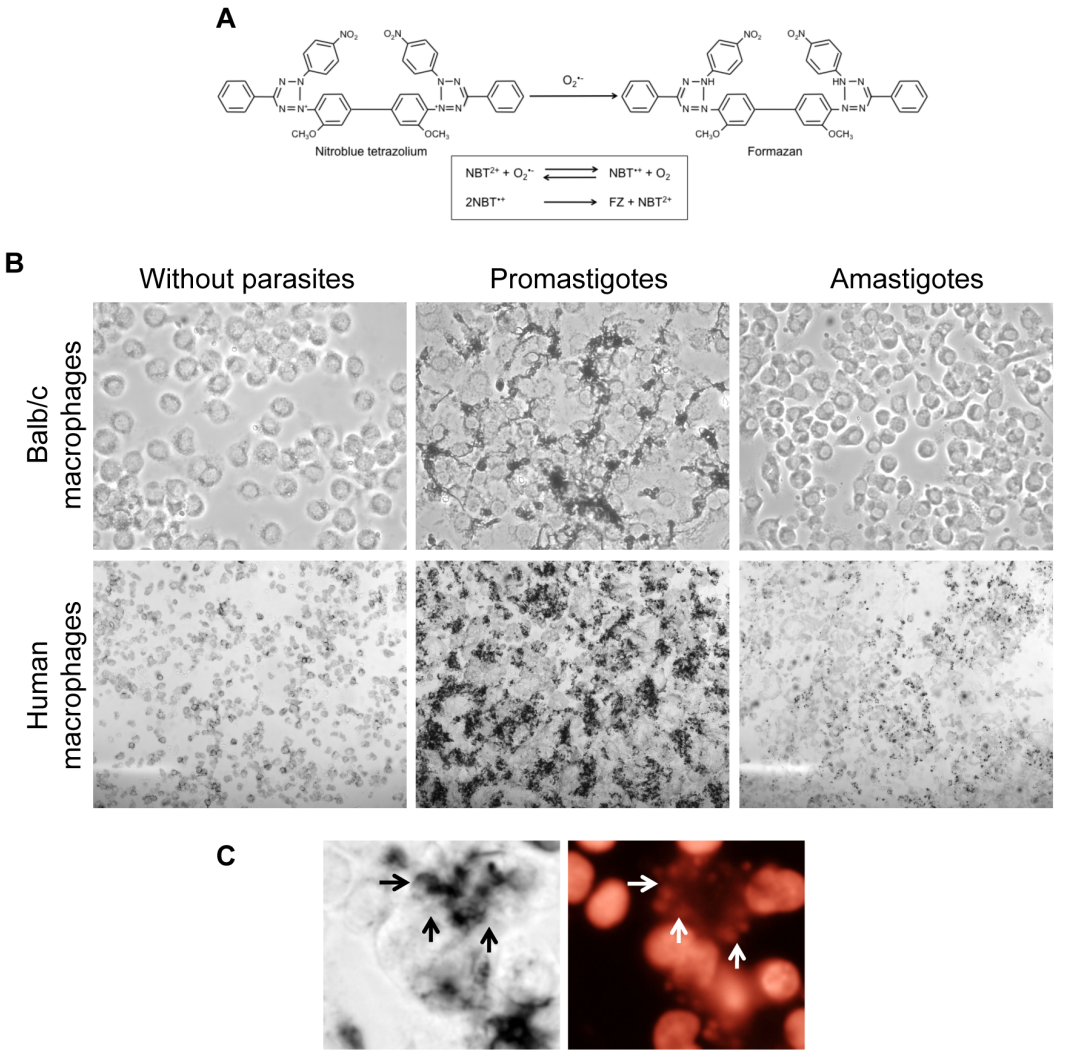


Fig. 4. Production of superoxide anion by murine and human macrophages upon contact with *Leishmania infantum* promastigotes. (A) Nitroblue tetrazolium reduction to formazan. Nitroblue tetrazolium (NBT^{2+}) can be reduced by superoxide radical anion ($O_2^{\bullet-}$) to yield a tetraazoinyl radical ($NBT^{\bullet+}$) that dismutates to insoluble dark blue formazan (FZ). (B) BALB/c and human macrophages were incubated with 1 $mg.ml^{-1}$ NBT solution in the absence or presence of pTEX transformed *L. infantum* promastigotes and amastigotes, as indicated. Dark deposits of insoluble formazan are formed only upon promastigote infection. Photographs were taken at 400 x magnification for murine cells and 100 x magnification for human cells. (C) Larger view of NBT stained BALB/c macrophages (image-left) and of cell nuclei stained in red with propidium iodide (image-right). Arrows point out the co-localization of formazan deposits with promastigotes nuclei. One representative experiment out of three is shown.

through the Griess assay (Green et al., 1982). After 24 h of simultaneous incubation with IFN-γ and LPS, murine macrophages already presented NO_2^- accumulation, which increased significantly after 48 h of macrophage activation (Fig. 5A). Contrary to this, human macrophages did not seem to respond to IFN-γ/LPS treatment, since no NO_2^- accumulation was detected in cell supernatants in any of the time points tested. This phenotype was maintained when human macrophages were incubated with *L. infantum*

promastigotes (Fig. 5A) and also when different combinations of IFN-γ/LPS were used (200 to 500 $U.ml^{-1}$ IFN-γ and 5 ng to 5 $\mu g.ml^{-1}$ LPS, data not shown). These results were confirmed with a more sensitive technique to estimate $^{\bullet}NO$ generation involving the cell permeable fluorescent probe DAF-2 DA (Fig. 5B and C), that allows real-time detection of $^{\bullet}NO$ derivatives (e.g., N_2O_3, $^{\bullet}NO_2$, N_2O_4). As expected, in this assay murine macrophages were shown capable of producing $^{\bullet}NO$ after 24 h of treatment with the induction agents (Fig. 5B). Moreover, immunofluorescence assays and Western blot analysis suggest that, in the conditions tested, iNOS is unlikely to be expressed in human peripheral blood monocyte-derived macrophages (Fig. 6). In conclusion, in our experimental model, H_2O_2 and $ONOO^-$ are expected to be produced in murine macrophages upon infection by *L. infantum* promastigotes. However, in human parasitized macrophages only H_2O_2 formation can be guaranteed.

3.4. pTEXTXNPx2 transformed parasites present higher infectivity than control parasites towards murine and human macrophages

The ability of *Li*TXNPx2 overexpressing parasites to infect and survive inside macrophage phagolysosomes was assayed ex vivo in murine and in human cells (Fig. 7). These two types of macrophages showed differences in their phagocytic capacity, with murine macrophages being 2-fold more infected than human cells. This difference is evident after 3 h of infection with both pTEX and pTEXTXNPx2 transformed parasites and might be attributed to the macrophage differentiation status, as human monocyte-derived macrophages are less differentiated than murine peritoneal cells. Nevertheless, the infection profile of *L. infantum* parasites is similar in the two macrophage types. Thus, after 24 h, both the percentage of infection and the number of intracellular amastigotes drop, increasing again at 72 h of infection.

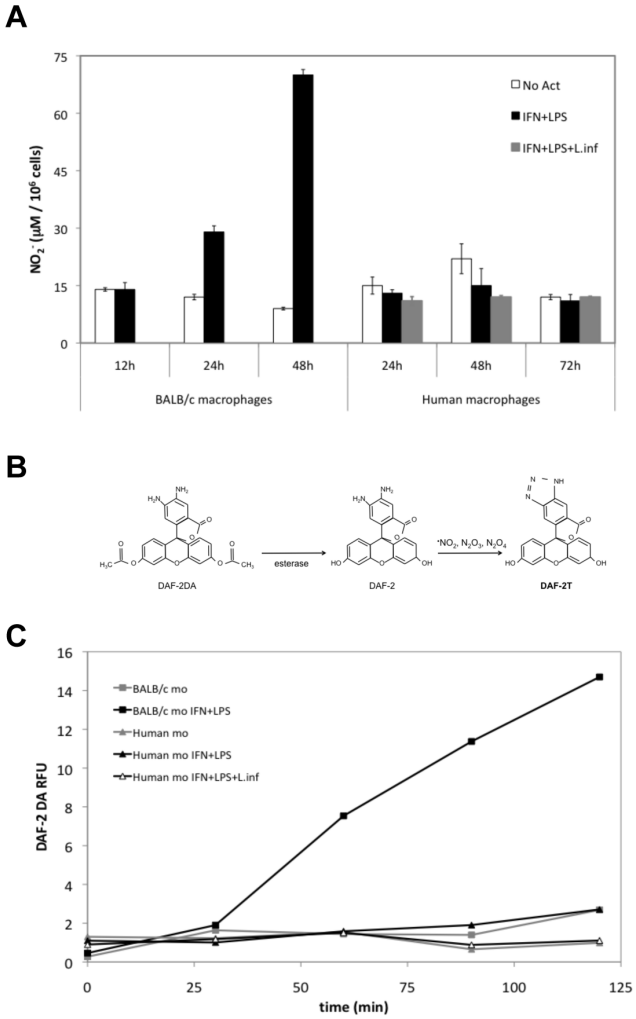


Fig. 5. Comparison of nitric oxide generation in murine and human macrophages. (A) Determination of the production of nitrite (NO_2^-) by BALB/c and human macrophages through the Griess assay. Macrophages were incubated with medium alone (white bars) or with medium containing 300 $U.ml^{-1}$ (murine) or 500 $U.ml^{-1}$ (human) IFN-γ and 5 $\mu g.ml^{-1}$ LPS (black bars). Human macrophages were also infected with wild-type *L. infantum* promastigotes (10:1 ratio) in the presence of IFN-γ and LPS (grey bars). Supernatants were collected at the indicated time points. (B) DAF-2DA oxidation via peroxynitrite derivatives. The fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2DA) enters the cell through diffusion, subsequently acetate groups are removed by cytosolic esterases yielding 4,5-diaminofluorescein (DAF-2), which is a relatively non-fluorescent compound at physiological pH and is better retained by cells than its parent compound. In the presence of $ONOO^-$ derivatives, DAF-2 is converted to the fluorescent triazole derivative (DAF-2T). (C) Detection of real-time nitric oxide production by oxidation of the DAF-2 DA probe in murine and human cells. Macrophages (mo) were incubated in medium alone (■,▲) or medium with 300 $U.ml^{-1}$ (murine) IFN-γ and 5 $\mu g.ml^{-1}$ LPS for 24 h (■) or with 500 $U.ml^{-1}$ (human) IFN-γ and 5 $\mu g.ml^{-1}$ LPS for 48 h (▲). Human macrophages were also incubated with wild-type *L. infantum* promastigotes (10:1 ratio) in the presence of IFN-γ and LPS (△). DAF-2 DA relative fluorescence units (RFU) were determined in a fluorimeter (Ex. 488 nm, Em. 515 nm) at 37ºC for 2 h. One representative experiment out of three is shown.

When comparing the infectious potential of the two *L. infantum* lines, *Li*TXNPx2 overexpressing parasites are consistently more effective at establishing infection than pTEX controls. This difference is always statistically significant at 72 h p.i., despite the

macrophage origin and the absence/presence of macrophage activating agents. Since pTEXTXNPx2 transformed parasites present an increased capacity to eliminate both exogenously added H_2O_2 and $ONOO^-$, the higher infectivity levels are likely to be due to this enhanced antioxidant power. It is, however, important to clarify whether both of these oxidants are being detoxified or if increased parasite survival is due to only one of these compounds. The specific involvement of H_2O_2 in infection can be perceived in non-activated macrophages both of murine and human origin. As observed in Fig. 7A and 7C at 72 h p.i., pTEXTXNPx2 transformed parasites show a 2-fold increase in the percentage of infection when compared to pTEX controls in both macrophage types. In agreement, at the

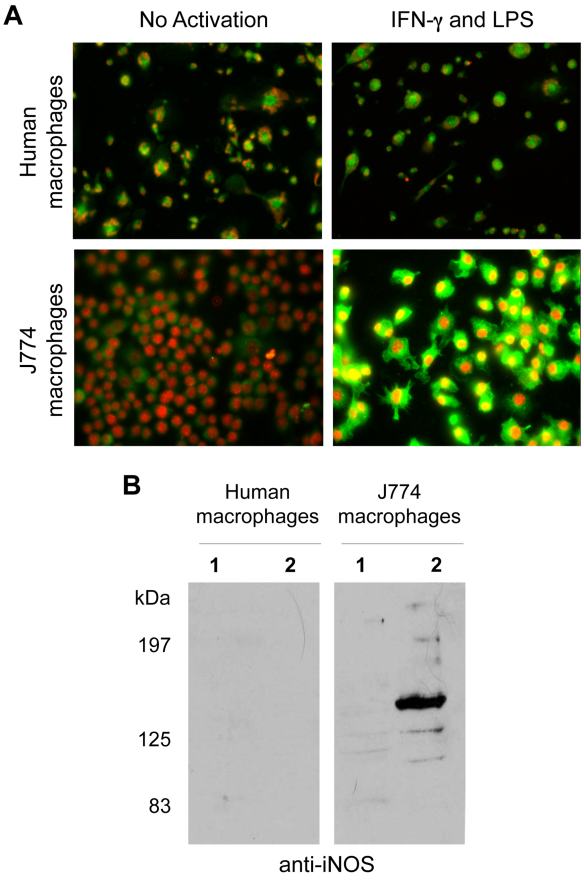


Fig. 6. Evaluation of nitric oxide synthase expression in murine and human macrophages. (A) Indirect immunofluorescence staining of human macrophages and J774 murine macrophage cell line with anti-iNOS primary antibody (green) and propidium iodide (red). Macrophages were pre-treated or not with 500 $U.ml^{-1}$ (human) or 300 $U.ml^{-1}$ (murine) IFN-γ and 5 $\mu g.ml^{-1}$ LPS and incubated for 24 h (murine cells) or 72 h (human cells). Photographs were taken at 200 x magnification in the case of human cells and 400 x magnification for murine cells. (B) Western blot comparing iNOS expression in 40 μg protein extracts of human and J774 macrophages. Human macrophages incubated for 72 h and murine macrophages for 24 h in medium alone (lane 1) or in medium with IFN-γ and LPS (lane 2). The blot was hybridized with anti-iNOS antibody, as indicated.

same time point and condition, *Li*TXNPx2 overexpressers also present twice as much intracellular amastigotes than pTEX control parasites (Fig. 7B and D). Together these results indicate that, by itself, improvement in H_2O_2 detoxification favors *L. infantum* infection of murine and human cells.

To investigate the effect of $ONOO^-$ in *L. infantum* intracellular survival we analyzed data obtained with macrophages stimulated with IFN-γ and LPS previously to infection. Since $O_2^{\bullet-}$ reacts preferably with $^{\bullet}NO$ than with SOD, formation of $ONOO^-$ in the phagosome will probably be favored in relation to H_2O_2 (reviewed in Radi et al., 2001). Figure 7A shows that, even though the infection levels decrease in activated macrophages, at 72 h p.i., pTEXTXNPx2 transformed parasites still present significantly higher infection percentages than pTEX controls. This, therefore, suggests that their enhanced $ONOO^-$ reductase capacity may reflect positively on infection of murine cells. In terms of number of intracellular parasites (Fig. 7B), pTEXTXNPx2 overexpressers maintain higher replication levels, presenting a number of amastigotes per macrophage two-fold higher than pTEX control parasites. It is noteworthy, however, that in murine activated macrophages, pTEXTXNPx2 transformed parasites cannot reach the infection levels observed in non-activated macrophages, that is, overexpression of the 2-Cys PRX does not entirely compensate for the damaging effects of iNOS induction. This suggests that IFN-γ/LPS activation of iNOS may interfere with pathogen infection/proliferation through mechanisms unrelated to the production of $ONOO^-$, as suggested previously (Iniesta et al., 2001; Chaturvedi et al., 2007; Wanasen and Soong, 2008).

Concerning activated human macrophages, the results from the ex vivo infections performed confirm that, in the conditions tested, $^{\bullet}NO$ production is impaired in these cells. Fig. 7C and 7D show that in macrophages infected with pTEX control parasites, the presence of IFN-γ and LPS does not lead to a higher degree of parasite elimination than in

non-activated macrophages. Moreover, even though the percentage of infection of *Li*TXNPx2 overexpressers in activated human macrophages registers a slight decrease, in comparison to non-activated macrophages, this is not reverted by the addition of the iNOS inhibitor aminoguanidine (AG). Again, these data suggest that in our experiments activation of iNOS was ineffective in human cells.

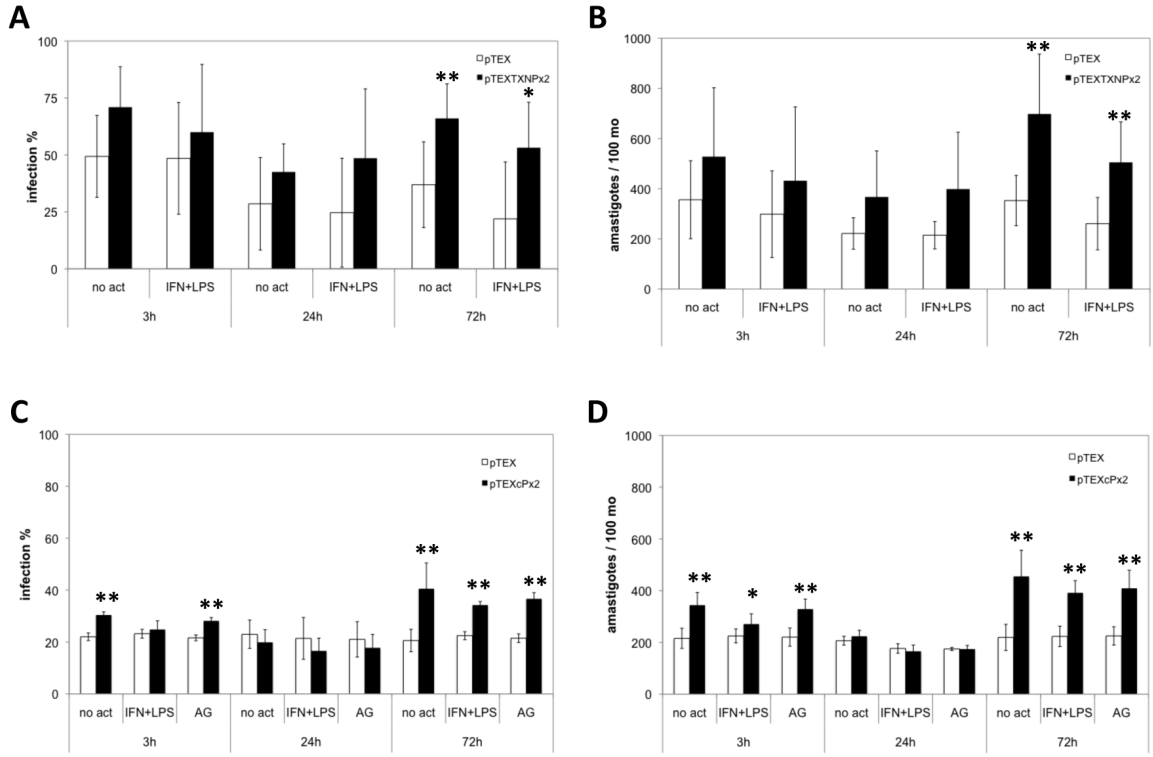


Fig. 7. Infection of murine and human macrophage monolayers. BALB/c (A and B) and human macrophages (C and D) were incubated with opsonized stationary-phase pTEX control parasites (white bars) or pTEXTXNPx2 promastigotes (black bars) at a ratio of 10 parasites per macrophage. At 3, 24 and 72 h after infection cells were stained with Diff Quick (2 murine macrophage assays) or by IFAT (1 murine macrophage assay and 3 human macrophage assays) and the percentage of infected macrophages (A and C) and the total number of amastigotes within 100 infected macrophages (B and D) was assessed microscopically. Macrophages were pre-treated (IFN+LPS) or not (no act) with IFN-γ and LPS and with IFN-γ, LPS and aminoguanidine (AG), as indicated (for details see Materials and methods). Results are the mean of the values obtained in three independent experiments ± S.D. Each experiment was performed in triplicate. Statistical significance between pTEX control parasites and pTEXTXNPx2 transformed parasites $*P < 0.05$ and $**P < 0.01$.

4. Discussion

The dual capacity of macrophages for being long-term hosts and effector cells for pathogen clearance has been an intriguing issue in *Leishmania* infection research. One of the macrophage microbicidal mechanisms is the generation of oxidative and nitrosative species, which are main agents in the so-called oxidative stress process. Amongst these reactive species, H_2O_2 and $ONOO^-$ are considered the most biologically relevant oxidants (reviewed in Rosen et al., 1995; Nathan and Shiloh, 2000; Radi et al., 2001). Both were proven toxic to *Leishmania* parasites in vitro (Zarley et al., 1991; Gatti et al., 1995) and the same was suggested for some parasite species using in vivo models (Linares et al., 2001; Blos et al., 2003). Nevertheless, the importance of ROS/RNS in infection is a controversial subject, namely if different parasite stages or host cells are considered (Murray and Nathan, 1999; Schneemann and Schoedon, 2002; Pham et al., 2005). In this paper we addressed the effects of H_2O_2/$ONOO^-$ toxicity on parasite intracellular survival, showing that, when produced by macrophages, both compounds present leishmanicidal capacity against *L. infantum* parasites. Moreover, our findings support the use of *L. infantum* lines overexpressing cytosolic 2-Cys PRXs as a suitable tool to assess the contribution of H_2O_2/$ONOO^-$ in animal infections.

Typical 2-Cys PRXs are key elements on *Leishmania* antioxidant defense by participating in hydroperoxide elimination cascades (reviewed in Castro and Tomás, 2008). These enzymes are obligate homodimers and their catalytic mechanism depends on two active cysteine residues. The peroxidatic cysteine directly interacts with the oxidant, forming a sulfenic acid derivative, while the resolving cysteine forms an intermolecular disulfide bridge with the oxidized peroxidatic cysteine (from a different subunit), later resolved by intervention of a specific oxidoreductase. Different types of organisms present 2-Cys PRXs and these were shown to be able to reduce H_2O_2, organic hydroperoxides and

$ONOO^-$ (Bryk et al., 2000; Jaeger et al., 2004; Trujillo et al., 2004; Nickel et al., 2005; Ogusucu et al., 2007). *L. infantum* possesses at least three active 2-Cys PRXs, two cytosolic, *Li*TXNPx1 and *Li*TXNPx2, and one mitochondrial, *Li*mTXNPx (Castro et al., 2002a; Castro et al., 2004). These enzymes were also shown capable of reducing H_2O_2 and organic hydroperoxides both in vitro and in the parasite context (Castro et al., 2002a; Castro et al., 2002b; Castro et al., 2004). In addition, we showed here that, contrary to what was reported for *L. chagasi* enzymes (Barr and Gedamu, 2003), both *L. infantum* cytosolic 2-Cys PRXs catalyze the reduction of $ONOO^-$ to form nitrite and water. Moreover, determination of second order rate constants for the reaction of the peroxiredoxins with $ONOO^-$ indicate that i) *L. infantum* cytosolic 2-Cys PRXs do not present significant kinetic differences between them and ii) these were similar to the ones previously obtained for *T. brucei* and *T. cruzi* enzymes (Trujillo et al., 2004).

Complementary to previous work, which determined that *Li*TXNPx1 and *Li*mTXNPx maintained peroxidase activity in the context of the parasite (Castro et al., 2002a), we clearly demonstrated that *Li*TXNPx2 overexpression confers resistance to both exogenously added H_2O_2 and $ONOO^-$ in *Leishmania* promastigotes. In fact, in relation to controls, *Li*TXNPx2 overexpressing cells presented an increased parasitic growth upon oxidant challenge elicited by H_2O_2 and $ONOO^-$ and showed a much lower level of tyrosine nitration. Our findings contradict the conclusion of Iyer and coworkers (2008), which suggested that $ONOO^-$ is not an effector molecule against *L. donovani* promastigotes in vitro. A possible explanation for the contrasting results is the misinterpretation of the in vitro model applied. Thus, in the above-mentioned study, *L. donovani* was challenged with H_2O_2 and $^{\bullet}NO$, which was incorrectly deduced to generate $ONOO^-$. The data presented herein, obtained by direct parasite exposure to H_2O_2 and $ONOO^-$ solutions, unequivocally document the enhanced antioxidant potential of *Li*TXNPx2 overexpressers specifically

against these compounds. These results supported the use of the transformed parasites as a means to dissect the effects on intracellular parasite killing of the presumed two most relevant macrophage-derived microbicidal oxidants (H_2O_2 and $ONOO^-$).

It was previously suggested that *Leishmania* peroxiredoxins overexpressers are better outfitted to detoxifying ROS and RNS (Barr and Gedamu, 2003; Iyer et al., 2008). However, none of these studies performed ex vivo infections with activated macrophages, therefore, iNOS induction was not achieved and the importance of macrophage-derived $ONOO^-$ to parasite viability could not be addressed. In a paper from Piacenza and coworkers (2008) *T. cruzi* viability was assessed after exposure to macrophage-derived $ONOO^-$. Nevertheless, in this case, parasites were co-cultured with murine macrophages, but not allowed to be internalized, and $ONOO^-$ cytotoxicity was evaluated only shortly after exposure. In this report we aimed at determining if H_2O_2 and $ONOO^-$ can act as leishmanicidal agents, when they are produced directly by macrophages. For this, we took advantage of the increased peroxide elimination capacity of *Li*TXNPx2 overexpressing parasites. Formation of H_2O_2 and $ONOO^-$ upon *L. infantum* infection of murine and human macrophages was ascertained through the production of their precursor molecules, $O_2^{\bullet-}$ and $^{\bullet}NO$.

Both macrophage types were shown capable of ingesting *L. infantum* promastigotes with coincident production of $O_2^{\bullet-}$. Consequently, H_2O_2 is an oxidant molecule very likely to be formed during parasite phagocytosis. From the consistent trend observed towards a higher infectivity of *Li*TXNPx2 overexpressing parasites, we may, therefore, conclude that parasite improved elimination of H_2O_2 may contribute *per se* to an increased intracellular resistance in both murine and human host cells. Thus, upon promastigote infection in vivo, H_2O_2 should be an important anti-leishmanial agent of both mice and humans. Nevertheless, and in agreement with previous reports in *L. donovani* and

L. pifanoi (Pham et al., 2005; Lodge and Descoteaux, 2006), *L. infantum* amastigotes were also shown to abrogate the macrophage oxidative burst. This modulation of the oxidative response by *Leishmania* amastigotes rises the question whether $O_2^{\bullet-}$ and its derivatives (e.g. H_2O_2) do play any role in later stages of the infection, where only amastigotes contact with host phagocytes. Arguing in favor of anti-leishmanial activity of ROS is the study by Blos et al. (2003) showing that although NOX knockout mice ($gp91^{phox\ -/-}$) are able to cope with *L. major* in an early stage of infection, NOX activity is nonetheless crucial for parasite killing during the chronic phase of infection.

The effect of $ONOO^-$ on *L. infantum* infections is less straightforward to assess. Under the experimental conditions performed, effective expression of iNOS was only achieved in murine macrophages, with consequent formation of $^{\bullet}NO$ derivatives upon 24 h of IFN-γ/LPS induction. In human macrophages it was not possible to stimulate production of $^{\bullet}NO$ nor to induce iNOS expression. Ex vivo infections with murine cells suggest that, in addition to H_2O_2, $ONOO^-$ can also limit the intracellular proliferation of *L. infantum*. This is supported by the observation that, in IFN-γ/LPS activated cells, *Li*TXNPx2 overexpressing parasites are also more infective than controls. Although H_2O_2 will surely play a role, phagosome produced $O_2^{\bullet-}$ will react preferably with $^{\bullet}NO$, present in the same compartment, instead of with SOD, thus, $ONOO^-$ should be the major reactive species formed in activated macrophages. The increased survival of *Li*TXNPx2 overexpressers in activated murine cells in relation to controls is, therefore, due to upregulation of the peroxiredoxin and consequent increase in $ONOO^-$ elimination. However, our data also suggest that in activated cells H_2O_2/$ONOO^-$-independent mechanisms also mediate parasite killing. This is suggested by the finding that *Li*TXNPx2 overexpression is not able to fully compensate the negative effects of iNOS expression. Although we have not dissected which mechanism(s) was responsible for *Leishmania* killing, such alternative iNOS-

dependent microbicidal mechanisms might be associated to toxicity to the parasite of the $^{\bullet}NO$ molecule itself or of its derivatives (Vespa et al., 1994; Byun et al., 1999; Salvati et al., 2001). Alternatively it could be associated to L-arginine metabolism (Iniesta et al., 2001; Wanasen and Soong, 2008) or to iron homeostasis (Fritsche et al., 2007; review in Huynh and Andrews, 2008).

Contrary to what was observed in murine cells, in our experimental model IFN-γ/LPS treatment of human macrophages did not significantly contribute to parasite killing. In fact, non-activated and activated cells presented the same level of infection. Also, although we have registered a slight decrease in the percentage of infection of *Li*TXNPx2 overexpressing parasites, this was not compensated by addition of an iNOS inhibitor, suggesting that it is not due to $^{\bullet}NO$. Other authors faced similar difficulties to show $^{\bullet}NO$ release by human macrophages. In some cases this was attributed to a very low level of $^{\bullet}NO$ production in human cells (Murray and Teitelbaum, 1992; De Maria et al., 1994; Albina, 1995; Vouldoukis et al., 1995; Weinberg, 1998; Gantt et al., 2001). Arguing in favor of this hypothesis is, for instances, a study by Gantt and coworkers (2001), in which IFN-γ incubation of human macrophages significantly increased parasite killing, although without registering any NO_2^- accumulation in cell supernatants. The authors, therefore, suggested that although very low levels of $^{\bullet}NO$ were being formed in human cells, they were enough to cause toxicity. In summary, the results obtained with *L. infantum* ex vivo infections suggest the possibility of a link between production of $ONOO^-$ and mice anti-leishmanial strategies but do not allow us to anticipate a similar conclusion for humans.

Several lines of evidence have highlighted the importance of ROS/RNS as effective microbicidal mechanisms in vivo. First, infection of NOX and iNOS deficient mice with *Leishmania* results in a phenotype of host susceptibility (Murray and Nathan, 1999; Blos et al., 2003). Thus, expression of both enzymes seems to be important for optimal control of

infection. Moreover, the observation that *L. donovani* survived better in macrophages from patients with chronic granulomatous disease (NOX deficient), also suggests a correlation between the lack of an appropriate oxidative response and vulnerability to visceral leishmaniasis (Pearson and Steigbigel, 1981; Pearson et al., 1983; Asensi et al., 2000). Still another evidence favoring the relevance of ROS/RNS in control of infection are the reports that correlate severity of the disease and an enhanced antioxidant defense of the parasite (Acestor et al., 2006; Walker et al., 2006; Giudice et al., 2007). Upregulation of 2-Cys PRXs might, thus, bring to the parasites an improved capacity to deal with macrophage-derived H_2O_2/$ONOO^-$ challenge in vivo. The data presented in this report support the usefulness of the cytosolic 2-Cys PRX overexpressing parasites to clarify the role of H_2O_2/$ONOO^-$ in control of infection in animals.

In conclusion, we have demonstrated that *L. infantum Li*TXNPx2 apart from interacting with H_2O_2 can also present $ONOO^-$ reductase activity in vitro and in the context of the parasite. More importantly, we have showed that macrophage-derived H_2O_2 and $ONOO^-$ are effective leishmanicidal agents against *L. infantum*. Our data also support the use of *L. infantum* lines overexpressing cytosolic 2-Cys PRX as an instrument for assessing the actual contribution of H_2O_2/$ONOO^-$ in vivo.

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Chapter 5

Final considerations

Life would not be possible without oxidation reactions, which paradoxically can also be damaging to cells, particularly in an aerobic environment. Most of the harmful effects result from common by-products of mainstream chemical reactions, such as superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), nitric oxide radical ($^{\bullet}NO$), peroxynitrite anion ($ONOO^-$) and others. Consequently, all living organisms developed auto-protective antioxidant systems. Amongst the most important and widespread antioxidant molecules are glutathione (GSH), glutathione-dependent peroxidases, superoxide dismutase (SOD), catalase and thioredoxin-dependent peroxiredoxins (Meister and Anderson, 1983; Bowler et al., 1992; Michiels et al., 1994; Flohé and Harris, 2007). *Leishmania* parasites, as well as other trypanosomatids, lack catalase, selenocysteine-containing glutathione peroxidases and classical heme peroxidases and are generally more sensitive to reactive species than their hosts (Ferret et al., 2002; Müller et al., 2003; Krauth-Siegel et al., 2007). In addition, trypanosomatid antioxidant defense system involves unusual features, which are exclusive of this group of organisms. One of these peculiarities is the use of the dithiol trypanothione [$T(SH)_2$] as the single reducing agent of a series of compounds and enzymes (Fairlamb and Cerami, 1992; Krauth-Siegel et al., 2005; Castro and Tomás, 2008). Tryparedoxin (TXN), an oxidoreductase belonging to the thioredoxin superfamily, is one of the enzymes whose redox regeneration depends on $T(SH)_2$ (Gommel et al., 1997) and since most of the $T(SH)_2$-mediated reactions are accelerated by TXNs, the $T(SH)_2$/TXN couple naturally presents special physiological relevance in terms of antioxidant defense (Krauth-Siegel et al., 2007).

The study of Nogoceke and coworkers (1997) was the first to describe an antioxidant cascade mediated by $T(SH)_2$ and TXN, in which the reduction of hydroperoxides proceeds in independent catalytic events: i) $T(SH)_2$ is reduced by the

NADPH-dependent flavoenzyme trypanothione reductase, ii) $T(SH)_2$ transfers the reducing equivalents to TXN and iii) TXN reduces a 2-cysteine peroxiredoxin (2-Cys PRX). This cascade was shown to operate in several trypanosomatid species, including *T. brucei* (Tetaud et al., 2001), *T. cruzi* (Guerrero et al., 2000; Lopez et al., 2000; Wilkinson et al., 2000) and *Leishmania* spp. (Levick et al., 1998; Flohé et al., 2002; Castro et al., 2004) and some of their components were proved to be essential for parasite survival and infectivity (Tovar et al., 1998; Ariyanayagam et al., 2005; Comini et al., 2007).

The research project presented here was focused on *L. infantum*, the prevailing leishmaniasis agent in the Mediterranean region, and more specifically on the $T(SH)_2$/TXN/2-Cys PRX antioxidant cascade. We have set different objectives, which reflect the subject approach from three distinct perspectives: drug development, biological/evolutionary characterization and parasite-host interaction.

Leishmania infantum tryparedoxins: biology, evolution and drug targeting

Isolation of a TXN molecule from the trypanosomatid *C. fasciculata* marked the discovery of a new member of the thioredoxin superfamily (Nogoceke et al., 1997). From here on, redox active TXNs were characterized in *T. brucei* (Lüdemann et al., 1998), *T. cruzi* (Lopez et al., 2000), *L. infantum* (Castro et al., 2004), *L. major* (König and Fairlamb, 2007), *L. amazonensis* (Hsu et al., 2008) and assumed to be common to all trypanosomatids. Despite their dependence on $T(SH)_2$, TXNs were shown to be functionally equivalent to thioredoxins and glutaredoxins (Krauth-Siegel et al., 2007). Accordingly, participation in peroxide elimination cascades is regarded as one of the crucial functions of TXNs (Nogoceke et al., 1997; Tetaud et al., 2001; reviewed in Castro and Tomás, 2008). In *L. infantum*, previous work from our group isolated a cytosolic and a

mitochondrial TXN (*Li*TXN1 and *Li*TXN2, respectively) and demonstrated that both could function as physiological reductants of the parasite 2-Cys PRXs (Castro et al., 2004). Also, ongoing studies in our laboratory indicate that *Li*TXN1 and *Li*TXN2 can reduce non-selenium glutathione peroxidase-like enzymes (nsGPX) in vitro, further implicating these enzymes in redox protection (H. Castro and A.M. Tomás, unpublished results). In addition, and similar to what happens with several thiol-disulfide oxidoreductases (Arnér and Holmgren, 2000; Berndt et al., 2008), TXNs have the potential to contribute to other biological pathways. In fact, the participation of TXNs in cell replication processes was suggested by studies in which *T. brucei* and *C. fasciculata* enzymes were found capable of reducing ribonucleotide reductase and the universal minicircle sequence binding protein (Dormeyer et al., 2001; Onn et al., 2004; Milman et al., 2007).

Advances in the trypanosomatids genome projects evidenced the presence of several oxidoreductase-like sequences containing the CXXC motif. Thus, there are two different TXN types (type I and type II), a thioredoxin-like and also a glutaredoxin-like sequence, however, the link between them is still unclear. While studying a *T. brucei* thioredoxin, Schmidt and Krauth-Siegel (2003) concluded that it presented lower redox activity and that it was much less abundant when compared to the cytosolic TXN. Thus, the authors suggested that this enzyme probably could not substitute for the cytosolic TXN in *T. brucei*. In addition, the same research group demonstrated that *T. brucei* thioredoxin is dispensable for parasite survival (Schmidt et al., 2002). On the other hand, characterization of a glutaredoxin from *T. brucei* revealed that this could interact with glutathione but not with trypanothione (Melchers et al., 2007), however, further studies are required to establish its specific role in the parasites. During our study we characterized the type II TXN molecules present in *L. infantum* (*Li*TXN3 and *Li*TXN5) and determined that they were very unlikely to be alternative molecules to type I TXNs, either *Li*TXN1 or

*Li*TXN2 (Chapter 3). Definitive experimental evidence for the irreplaceability of TXN1 ultimately came up with the observation that knockdown of *T. brucei* cytosolic TXN dramatically affected parasite survival (Comini et al., 2007). In agreement to this, we showed that in *L. infantum* TXN1 is essential to both insect and mammalian stages of the parasite, thus accomplishing the first objective of this thesis, which was to genetically validate the enzyme as a drug target (Chapter 2).

Being essential to parasite survival and different from host thioredoxins, cytosolic TXN fulfils the basic criteria for drug targeting. However, in the context of rational drug design, additional factors must be considered. For example, it is important to analyze if TXN molecules are amenable to inactivation by specific compounds. The structural conformation of TXNs and, in particular, the position of their active site, exposed at the surface of the protein rather than in an hydrophobic groove, have been referred as a serious obstacle to the inhibition of these proteins. Nevertheless, pharmacological inhibition of human thioredoxin, which presents the same structural characteristics as TXNs, was proved feasible (Pallis et al., 2003). Thus it is possible to affirm that design of specific TXN-inhibitors although challenging is not unattainable. Another aspect to be analyzed when considering TXN's drugability is to what extent they need to be inhibited in order to affect parasite survival. We estimated *Li*TXN1 cellular concentration to be around 1 to 2.5 μM in promastigotes and 40 μM in amastigotes (Chapter 2), values which are inferior to the ones reported previously for *L. major* (König and Fairlamb, 2007) as well as for other trypanosomatids (Comini et al., 2007). Still, in amastigotes it can be considered a relatively abundant protein. More importantly, *Leishmania* parasites seem to be able to adjust TXN levels according to their needs. For instance, *L. infantum TXN*1 single knockout parasites can upregulate *Li*TXN1 expression to 75% of wild-type levels (Chapter 2). Similarly, *L. amazonensis* exposure to reactive oxygen species was reported to trigger an increase in

TXN expression (Hsu et al., 2008). These fluctuations in the intracellular levels of TXN might represent a drawback to the application of TXN-specific drugs. A couple of studies also analyzed the minimum degree of TXN activity compatible with normal parasitic growth. In the case of *T. brucei*, parasite viability was only impaired when the cytosolic TXN activity was depleted in more than 95% (Comini et al., 2007). Nevertheless, a 60% reduction rendered parasites more susceptible to H_2O_2 challenge (Comini et al., 2007). In our experiments, a reduction in only 25% of normal *Li*TXN1 levels was sufficient to affect amastigote survival in macrophage host cells (Chapter 2), suggesting that even partial inactivation of the enzyme may be sufficient to attain effective inhibition of *Leishmania* intracellular growth. Since the $T(SH)_2$/TXN couple is involved in redox regulation, we can speculate that the decreased infectivity observed in *LiTXN*1 single knockout parasites may be due to failure in peroxide detoxification systems. Still, further studies are required to clarify the precise biological process(es) in which the enzyme is essential and to identify the mechanisms responsible for controlling TXN expression.

In addition to an hydroperoxide metabolizing system in the cytosol, *Leishmania* spp. are thought to possess an analogous system in mitochondria. A *L. infantum* TXN, *Li*TXN2, was previously found to be restricted to mitochondria (Castro et al., 2004). Since, *Li*TXN2 preferentially reacts with the mitochondrial 2-Cys PRX (*Li*mTXNPx) in vitro, it was considered that these enzymes were functionally linked, protecting the parasites against reactive species generated within the mitochondrion (Castro et al., 2004). We were, however, surprised with recent findings of our group showing that *Li*mTXNPx is essential for amastigote survival but *Li*TXN2, its presumable physiological reductant, is not (H. Castro and A.M. Tomás, manuscript in preparation). This suggests that the mitochondrial 2-Cys PRX activity does not depend on *Li*TXN2 either because *L. infantum* hold alternative reducing molecules in this organelle or because the crucial function of

*Li*mTXNPx in amastigotes is redox independent. After characterization of two *L. infantum* TXN-like gene sequences (*LiTXN*3 and *LiTXN*5) we concluded that none of these could substitute *Li*TXN2 in reduction of *Li*mTXNPx (Chapter 3). We demonstrated that *Li*TXN3 and *Li*TXN5 are not typical TXNs and that they cannot participate in the classical hydroperoxide elimination cascade. *Li*TXN3 is a mitochondrial membrane-anchored protein but very likely oriented towards the cytosol. Thus, in spite of its mitochondrial localization, *Li*TXN3 is not expected to interact with inner mitochondrial proteins, which also invalidates its involvement in kinetoplast replication through reduction of the universal minicircle sequence binding protein (Onn et al., 2004; Milman et al., 2007). Concerning *Li*TXN5, this was shown to be an endoplasmic reticulum membrane protein without measurable oxidoreductase activity. All together, these findings indicate that most likely neither *Li*TXN3 nor *Li*TXN5 act as typical TXN molecules in *L. infantum*. Therefore, we have achieved the second goal proposed for this research project by providing the first data on an up to now uncharacterized set of *Leishmania* TXN-like molecules (Chapter 3).

With the characterization of the *L. infantum* TXN3 and TXN5 proteins additional questions arose, namely, why have these proteins evolved differently from classical TXNs? Two distinct explanations might be suggested. First, it is possible that atypical TXNs have progressively lost function, their sequences being at present in the process of becoming pseudogenes. On the other hand, these proteins might have evolved specific functions in the cell. The first hypothesis can be supported by the observation that *Leishmania* keep in their genomes several pseudogenes and coding sequences for inactive proteins (Ghedin et al., 2004; Parsons et al., 2005; Eschenlauer et al., 2006). In fact, selective pressure for deletion of pseudogenes in eukaryotes is not as significant as it is for prokaryotes and, more importantly, maintenance of inactive gene copies might represent an essential

reservoir for genetic diversity (Harrison and Gerstein, 2002). In addition, it is important to denote that trypanosomatid genes are arranged into large polycistronic clusters, which promote cotranscription of several genes in the same precursor mRNA (Stiles et al., 1999) and might, therefore, prevent the loss of superfluous genes. At this stage we cannot, however, discard the possibility that the atypical TXN proteins characterized perform a particular role in the parasite cell. Indeed, it is equally reasonable to assume that TXN duplication could have favored gene divergence and possibly the acquisition of new protein functions, as it is the case for other duplicated genes (Field and Field, 1997; Harrison and Gerstein, 2002). This possibility introduces a second question, which refers to the actual functions performed by atypical *Leishmania* TXNs in the cell. These may be either redox-dependent or independent activities. Although *Li*TXN3 did not reduce *L. infantum* peroxidases and even though it is probably a poorly active oxidoreductase, we cannot exclude its participation in protein reduction in the cell (Chapter 3). It is conceivable that *Li*TXN3 performs some of the redox-sensitive functions displayed by other eukaryotic dithiol oxidoreductases such as, for example, chaperone activity, cell signaling or DNA synthesis (Arnér and Holmgren, 2000; Vlamis-Gardikas, 2008). Report of redox-independent functions for thioredoxin-like molecules are far more rare to find. Nevertheless, a redox-inactive thioredoxin of animal cells was suggested to play a role as an escort protein in the secretory pathway (Mkrtchian and Sandalova, 2006) and, more recently, an yeast mitochondrial thioredoxin was shown to participate in arginine biosynthesis, without the need of thiol-exchange mechanisms (Song et al., 2008). Since no reductase activity was observed in *Li*TXN5, but yet the protein was found to be expressed in wild-type parasites, the possibility that it is implicated in redox-independent pathways should not be overlooked.

When comparing all known trypanosomatids TXN sequences, it is noticeable that they can be sorted into two different groups. Interestingly, one of these groups includes enzymes proved to be kinetically active in the hydroperoxide elimination cascade, i.e., classical TXNs (*Trypanosoma* spp. TXN1 and *Leishmania* spp. TXN1 and TXN2 – type I TXNs). While the other comprises uncharacterized proteins (*Trypanosoma* spp. TXN2, *Leishmania* spp. TXN3, TXN4 and TXN5 – type II TXNs). These last molecules contrast with typical TXNs, namely by presenting a C-terminal hydrophobic extension. However, since thus far only *Li*TXN3 and *Li*TXN5 were analyzed, it is not possible to anticipate a common function for all these molecules. Nevertheless, the observation that there are two types of TXNs permits some assumptions. Firstly, the primitive trypanosomatid organism should already possess the two distinct types of TXN genes, corresponding to what currently occurs in the *T. cruzi TXN* locus (Chapter 3). Next, we can observe that *Leishmania* mitochondrial TXN2 does not belong to the same group as *Trypanosoma*'s TXN2. In fact, *Trypanosoma*'s TXN2 are more related to *Leishmania* atypical TXNs than to classical TXNs. Indirect evidence suggested that *Tb*TXN2 might be an active mitochondrial enzyme (Motyka et al., 2006). However, similar to what was observed for *Li*TXN3 (Chapter 3), the protein hydrophobic C-terminal can be anchoring them to the membrane and further studies are required to draw definitive conclusions about its membrane orientation and probable molecular partners. Hence, the second *Trypanosoma* TXN might actually not participate in the mitochondrial hydroperoxide elimination cascade.

***Leishmania infantum* 2-cysteine peroxiredoxins: biology and interaction with the host cell**

The first peroxidases described to receive electrons from the $T(SH)_2$/TXN couple were 2-Cys PRXs (Nogoceke et al., 1997). For this reason, they were designated as tryparedoxin peroxidases (TXNPx). Originally typical eukaryotic 2-Cys PRXs were exclusively associated to oxidative stress response, more recently these enzymes were proposed to have broader functions, such as cell signaling or chaperone activity (Flohé and Harris, 2007). In trypanosomatids, 2-Cys PRXs have been suggested to be key players in peroxide removal (reviewed in Krauth-Siegel et al., 2007; Castro and Tomás, 2008), being present in the cytosol and mitochondrial compartments. Furthermore, 2-Cys PRXs are presumed to be the trypanosomatids' most abundant peroxidases (Nogoceke et al., 1997; Comini et al., 2007; König and Fairlamb, 2007), which is suggestive of a significant role in parasite metabolism. In *T. brucei* a cytosolic 2-Cys PRX was shown to be essential for bloodstream forms of the parasite, while downregulation of the mitochondrial enzyme did not lead to a significant phenotype alteration (Wilkinson et al., 2003). In *L. amazonensis*, partial depletion of cytosolic and mitochondrial 2-Cys PRXs by antisense RNA enhanced parasite susceptibility to oxidants (Lin et al., 2005). In addition, although generation of *L. infantum* null mutants for mitochondrial 2-Cys PRX did not cause any defects on promastigote growth or antioxidant capacity, it significantly impaired amastigote survival (H. Castro and A.M. Tomás, manuscript in preparation). Depletion of *Leishmania* cytosolic 2-Cys PRXs through classical gene knockout may be difficult to accomplish since they are part of a multicopy locus, in which their genes are interspaced with different ORFs. Thus, confirmation of the essential role of cytosolic 2-Cys PRXs in *Leishmania* cells remains to be elucidated.

When considering the interaction between pathogens and host phagocytes, one of the most studied events is the oxidative attack that the latter can perpetrate against parasitic cells. Perhaps for this reason, the deleterious action of ROS and RNS is generally considered to be the main macrophage microbicidal mechanism. However, in the case of *Leishmania* infections, the relevance of reactive oxygen and nitrogen species (ROS and RNS) in impairment of parasitic growth has been questioned (Descoteaux et al., 1991; Murray and Nathan, 1999; Iniesta et al., 2001; Iniesta et al., 2002; Pham et al., 2005). By showing that overexpression of a *L. infantum* cytosolic 2-Cys PRX (*Li*TXNPx2) confers resistance to exogenously added H_2O_2 and $ONOO^-$, our study provided evidence for the importance of PRXs to *Leishmania* antioxidant defense (Chapter 4). More importantly, we demonstrated that, in contrast with control parasites, *L. infantum* TXNPx2 overexpressers present higher infectivity rates in host macrophages. These findings are in line with results from other groups (Wilkinson et al., 2000; Castro et al., 2002; Barr and Gedamu, 2003; Lin et al., 2005; Piacenza et al., 2008) and may indicate that production of those oxidants participates in control of a *Leishmania* infection. Moreover, we showed that *Li*TXNPx2 overexpression was equally advantageous in murine and human macrophages, indicating that both cell types mount an oxidative attack as a means to control pathogen proliferation. By connecting cytosolic 2-Cys PRXs to survival within the host cell, we have fulfilled the third and last aim of this project (Chapter 4).

It is worth noting that all the assumptions based on the infectivity phenotype of 2-Cys PRX overexpressers can only be related to H_2O_2 and/or $ONOO^-$ elimination. Other anti-parasitic mechanisms triggered by host cell invasion or activation, such as, toxicity of other reactive molecules (e.g., $O_2^{\bullet-}$, $^{\bullet}NO$, $^{\bullet}NO_2$, $CO_3^{\bullet-}$) (Vespa et al., 1994; Byun et al., 1999; Gantt et al., 2001; Salvati et al., 2001; Bonini et al., 2004; Palazzolo-Ballance et al., 2007), alterations in L-arginine metabolism (Iniesta et al., 2001; Wanasen and Soong,

2008) or in iron homeostasis (Fritsche et al., 2007; Huynh and Andrews, 2008), cannot be influenced by peroxidase activity and, thus, were not investigated. Nevertheless, the observation that *Li*TXNPx2 overexpression does not fully compensate the deleterious consequences of macrophage cytokine-activation indicates that, indeed, parasite killing in murine cells is not exclusively dependent on H_2O_2 and $ONOO^-$ (Chapter 4). In conclusion, the higher infectivity of the cytosolic 2-Cys PRX transformed parasites is a strong indication that H_2O_2/$ONOO^-$ detoxification is crucial for parasite survival. This strengthens the relevance of the oxidative and/or nitrosative process as a leishmanicidal mechanism.

In this study, a comparative analysis between the oxidative response of murine and human macrophages against *Leishmania* was performed. Induction of nitrosative damage by human cells has been persistently debated (Weinberg, 1998; Fang and Vazquez-Torres, 2002; Schneemann and Schoedon, 2002). In our experimental settings we could stimulate inducible nitric oxide synthase (iNOS) expression in murine macrophages but not in human cells (Chapter 4). This might be explained by the fact that murine and human iNOS genes present different promoter regions, which may respond distinctly to the same stimuli (Taylor and Geller, 2000). In fact, the factors that consistently activate iNOS in human macrophages are yet to be identified and hence, further studies are required to better understand human iNOS transcriptional regulation. Nevertheless, reports on the human immune response to infection revealed expression of iNOS at inflammation sites as well as an increase in $^{\bullet}NO$ concentration in the plasma of patients with chronic infections (Bachmaier et al., 1997; Facchetti et al., 1999; Fang and Vazquez-Torres, 2002; Reade and Young, 2003). These observations support the implication of $^{\bullet}NO$ in human infections, although its production might not be as significant as it is in murine cells (Albina, 1995; Taylor and Geller, 2000; Reade and Young, 2003). Finally, it can also be possible that $^{\bullet}NO$

formation is replaced by other microbicidal mechanism(s) in humans, therefore, justifying why •NO is not as relevant in humans as in mice.

In conclusion, our work indicated that *L. infantum* cytosolic *Li*TXN1 is essential for parasite survival and revealed that additional TXN molecules (*Li*TXN3 and *Li*TXN5) are unlikely to act as classical TXN enzymes. In addition, we showed that both *L. infantum* cytosolic 2-Cys PRXs display peroxynitrite reductase activity and that their overexpression enhances the parasites' antioxidant capacity. Using *Li*TXNPx2 overexpressers we established a direct link between *L. infantum* oxidative defense and infectivity towards both murine and human cells.

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Abbreviations:

3'-Race	Rapid Amplification of cDNA 3'-ends
APX	Ascorbate-dependent hemeperoxidase
bp	Base pair
BSA	Bovine serum albumine
C-terminal	Carboxy-terminal
cDNA	Complementary DNA
Cf	*Chritidia fasciculata*
Chr	Chromosome
CL	Cutaneous leishmaniasis
Cys	Cysteine
Da	Dalton
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dPBS	Dulbecco's PBS
DTT	Dithiothreitol
EDTA	Ethylene-diamide-tetra acetic acid
eEF1B	Eukaryotic elongation factor 1B
ER	Endoplasmic reticulum
G418	Neomycin
GR	Glutathione reductase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HBSS	Hanks Balanced Salt Solution
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFAT	Indirect fluorescence antibody test
iFBS	Inactivated fetal bovine serum
IFN-γ	Interferon-gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IPTG	Isopropyl-β-D-thiogalactopyranoside
Lb	*Leishmania braziliensis*
Li	*Leishmania infantum*
Lm	*Leishmania major*
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
MAA	Medium for Axenic Amastigotes
MCL	Mucocutaneous leishmaniasis
min	Minute
mRNA	Messenger ribonucleic acid

mTXNPx	Mitochondrial Tryparedoxin Peroxidase
MW	Molecular weight
My	Million years
N-terminal	Amino-terminal
NADPH	Nicotinamide adenine dinocleotide phosphate, reduced state
NOHA	N^{ω}-OH-L-arginine
NOS	Nitric oxide synthase
NOX	NADPH oxidase
nsGPX	Non-selenium glutathione peroxidase
ON	Over-night
$ONOO^-$	Peroxynitrite
ORF	Open Reading Frame
ox	Oxidized
PAGE	Polyacrilamide gel electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PRX	Peroxidase
red	Reduced
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcription PCR
s	Second
SDS	Sodium dodecyl sulfate
syn.	Synonymous with
t-bOOH	tert-butyl hydroperoxide
$T(SH)_2$	Trypanothione, reduced state
Tb	*Trypanosoma brucei*
Tc	*Trypanosoma cruzi*
Tco	*Trypanosoma congolensis*
TR	Trypanothione reductase
Tv	*Trypanosoma vivax*
TXN	Tryparedoxin
TXNPx	Tryparedoxin Peroxidase
U	Unit
UTR	Untranslated region
VL	Visceral leishmaniasis
WHO	World Health Organization
WT	Wild-type

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Appendix 1

Publications and communications in scientific meetings

1- Thesis-related work

Publications:

Romao, S., Castro, H., Sousa, C., Carvalho, S., Tomás, A. M., 2009. The cytosolic tryparedoxin of *Leishmania infantum* is essential for parasite survival. Int. J. Parasitol. 39, 703-711.

Romao, S., Castro, H., Sousa, C., Carvalho, S., Tomás, A. M. (in preparation). *Leishmania infantum* encodes two membrane-bound tryparedoxins without typical enzymatic activity.

Romao S., Teixeira F., Castro H., Piacenza L., Alvarez M. N., Trujillo M., Radi R., Tomás A. M. (in preparation). Pverexpression of a *Leishmania infantum* cytosolic 2-cysteine peroxiredoxin increases parasite resistance to oxidant challenge elicited by murine and human macrophages.

Communications in scientific meetings:

Romao S., Castro H., Piacenza L., Alvarez M. N., Trujillo M., Radi R., Tomás A. M. (2007), "Peroxynitrite detoxification by *Leishmania infantum* tryparedoxin peroxidases: implications for parasite infectivity" (oral communication). Free Radicals in Montevideo, V Meeting of SFRBM – South American group, V International Conference on Peroxynitrite and Reactive Nitrogen Species, September 2-6, Montevideo, Uruguay

Romao S., Castro H., Piacenza L., Alvarez M. N., Trujillo M., Radi R., Tomás A. M. (2007), "Peroxynitrite detoxification by *Leishmania infantum* tryparedoxin peroxidases: implications for parasite infectivity" (oral communication). Society for Free Radical Research Europe 2007 Meeting, October 10-13, Vilamoura, Portugal

Romao S., Teixeira F., Castro H., Piacenza L., Alvarez M. N., Trujillo M., Radi R., Tomás A. M. (2008), "Peroxynitrite detoxification by *Leishmania infantum* tryparedoxin peroxidases: implications for parasite infectivity in mouse and human cells" (oral communication). SFRR-Europe 2008 Meeting, July 5-9, Berlin, Germany

Romao S., Castro H., Sousa C., Carvalho S. and Tomás A. M. (2007). "The cytosolic tryparedoxin is essential for *Leishmania infantum* survival" (poster). 10th IUBMB conference and 36th annual meeting of SBBq, 21-25 May 2007, Salvador, Bahia, Brazil

Romao S., Amorim C., Castro H., Sousa C., Carvalho S., Tomás A. M. (2006), The Several Tryparedoxin Sequences of Leishmania infantum: Is the cytosolic TXN really a drug target? (poster). 11th International Congress on Parasitology, 6th to 11th August, Glasgow, Scotland, UK

Romao S., Amorim C., Castro H., Sousa C., Carvalho S. and Tomás A. M. (2006) The Tryparedoxin Sequences of *Leishmania infantum* (poster). ISOP and IWOP-9 joint meeting, June 20-24, Lisbon, Portugal

Romao S., Amorim C., Castro H., Sousa C., Carvalho S. and Tomás A. M. (2006) The Tryparedoxin Sequences of *Leishmania infantum* (poster). Gordon conference: Biology of host-parasite interactions, June 25-30, Newport, Rhode Island, USA

Romao S., Castro H., Sousa C., Tomás A. M., (2005) The Tryparedoxins of *Leishmania infantum*: Characterization of *Li*TXN5 a tryparedoxin-like protein (poster). Kinetoplastid Molecular Cell Biology Meeting, April 12-15, Woods Hole,Massachussetts, USA

Awards:

The Theodore L. Jahn and Eugene C. Bovee Award (2006), INTERNATIONAL SOCIETY OF PROTISTOLOGISTS

Young Investigator Award (2007), SOCIETY FOR FREE RADICAL BIOLOGY AND MEDICINE - South America

Young Investigator Award (2007), SOCIETY FOR FREE RADICAL BIOLOGY AND MEDICINE – Europe (prize shared with Helena Castro)

2- Collaborative work

Publications:

Castro, H., **Romao, S.**, Gadelha, F. R., Tomás, A. M. (2008) *Leishmania infantum*: Provision of reducing equivalents to the mitochondrial tryparedoxin/tryparedoxin peroxidase system. Exp. Parasitol. 120, 421-423

Silva M. S., Barata L., Ferreira A. E., **Romao S.**, Tomás A. M., Freire A. P., Cordeiro C. (2008) Catalysis and structural properties of *Leishmania infantum* glyoxalase II: trypanothione specificity and phylogeny. Biochemistry 47, 195-204

Communications in scientific meetings:

Teixeira F., **Romao S**., Carvalho S., Tomás A. M. (2008) Analysis of the mechanisms involved in intramacrophagic elimination of *Leishmania* (poster) SFRR-Europe 2008 Meeting, July 5-9, Berlin, Germany

Castro H., **Romao S**., Sousa C., Trujillo M., Radi R., Tomás A. M. (2007) Leishmania infantum mitochondrial peroxiredoxin is not essential for parasite survival (poster) SFRR Europe, October 10-13, Vilamoura, Portugal

Castro H., **Romao S.**, Tomás A. M. (2007). Abrogation of *Leishmania infantum* mitochondrial peroxiredoxin impairs parasite infectivity. Function(s) for this enzyme? (poster) Kinetoplastid Molecular Cell Biology Meeting, 23-26 April, Woods Hole, USA

Castro H., **Romao S.**, Irigoin F., Radi R., Tomás A. M. (2007). Consequences of mitochondrial peroxiredoxin abrogation in *Leishmania infantum* infectivity: is the enzyme related to apoptosis regulation? (poster) Society for Free Radical Biology and Medicine 14th Annual Meeting, 14-18 November, Washington DC , USA

Castro H., **Romao S.**, Sousa C., Trujillo M., Radi R., Tomás A. M. (2006) Leishmania infantum mitochondrial peroxiredoxin is not essential for parasite survival (poster) 11th International Congress on Parasitology, 6th to 11th August, Glasgow, Scotland, UK

Other scientific work

1- Science public promotion

Seminars to students of secondary schools:

Romao S. and Osório, N. (April 2004), Leishmaniasis and *Leishmania* Research in Portugal. Secondary School Condes de Resende, Canelas, Portugal.

Romao S. and Osório, N. (April 2004), Leishmaniasis and *Leishmania* Research in Portugal. Secondary School Gago Coutinho, Alverca, Portugal.

School Visits:

Research on *Leishmania* group: Carvalho S., Castro H., Cruz T., **Romao S.**, Teixeira F., Tomás A. M. (2007). BioBigBrother: Espreitar a vida de um parasita. IBMC, Porto, Portugal.

Participation in a student's book:

Tomás, A.M., **Romao, S.**, 2009. Biologia do Parasita. In: Santos-Gomes, G., Fonseca, I. (Eds.), Leishmaniose Canina, Merial Portuguesa, Chaves Ferreira editora, Lisboa.

Interviews:

Romao S., (2008). Sorry did you say leish…what? A reportage on Leishmaniasis. (Appendix 2).

Appendix 2

Sorry, did you say leish…what?!

❖ A reportage on leishmaniasis ❖



Photo by Carlos Costa

Leishmaniasis is an unknown disease to the general public but it is on top of the list for neglected diseases of the World Health Organization. What is it after all? Is it relevant to humans? Should we worry? Physicians, veterinarians, researchers and patients were interviewed to get different scopes for the same infirmity.

By Susana Romao

Leishmaniasis in Portugal

Impact of human and canine leishmaniasis. Treatment and control.

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Leishmaniasis in India and Brazil

Human leishmaniasis numbers and treatment.

Page 8

The scientists progress and responsibility

Scientific contribution and educative campaigns.

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Leishmaniasis, an unpronounceable disease

Last May, twenty-one year old Jorge Silva (fictitious name) went to the São Teotónio Hospital, in Viseu, because of a persistent fever, tiredness and lack of appetite that was afflicting him for a few weeks. After some tests, his doctor informed him that he had an infectious disease called leishmaniasis. He had never heard about it, but the word infectious gave him the chills. Fortunately after this scary sentence came another, much more reassuring, saying that treatment was available and that he would be fine after a few weeks. And indeed, one week later, Jorge went home, with the feeling that he was cured.



Lenea Campino

"I though that everything would be explained with the genome sequence conclusion, but I was wrong."

Lenea Campino, director of the IHMT Leishmaniasis Unit in Lisbon.

Most people, just like Jorge, have never heard about leishmaniasis, but the truth is that not all stories have the same happy ending. The disease is spread in 88 countries with a human impact of 51.000 deaths a year and 350 million people at risk to be infected (World Health Report, 2004). Lenea Campino, head researcher for the Leishmaniasis Unit at the Instituto de Higiene e Medicina Tropical (IHMT) in Lisbon, describes the disease as a parasitosis essentially from animals in the case of Europe and South America. She explains that the agent for leishmaniasis is a protozoan parasite, belonging to the *Leishmania* genus that can be transmitted to humans or animals by the bite of an insect vector, the sandfly. In the Mediterranean areas, the dog represents the most important reservoir for the disease, meaning that, the canine population maintains the parasite on the environment and might occasionally transmit it to humans. For this reason it is called a zoonosis. But there are places, like in India, for example, where the transmission is essentially from man to man without an animal intermediate and, in that case, the disease is considered to be an anthroponosis.

Leishmaniasis has different types of clinical manifestations, which usually are divided into three main groups: cutaneous, mucocutaneous and visceral forms. These distinct pathologies depend on the *Leishmania* species responsible for infection but also on the immune system status of the infected host. The most severe form of the disease is visceral leishmaniasis, the only form responsible for fatalities.

For more information about leishmaniasis please consult table 1, references 1-4 and additionally the World Health Organization (WHO) websites:

www.who.int/topics/leishmaniasis

www.who.int/tdr/diseases/leish

Leishmaniasis in Portugal

According to the director of the IHMT Leishmaniasis Unit, "We have always had leishmaniasis in our country. The official discovery of a Portuguese case of

leishmaniasis was made in 1910, which was also the first year of epidemiologic records in Portugal". The country has some well-known endemic areas, *i.e.*, regions where the infection is prevalent and maintained over time. Vila Real and the metropolitan area of Lisbon are two of the most important ones. Leishmaniasis was for long considered a childhood illness, but nowadays we face a new scenario, with a higher prevalence of *Leishmania* infection in HIV positive people. Still, pediatricians of endemic regions see an average of 1 to 2 children with leishmaniasis every year. The country total numbers are around 2 cases *per* million habitants *per* year. These official numbers are available because, since 1951, visceral leishmaniasis is a disease

Table 1 – Summary of leishmaniasis distribution and treatment*

Leishmaniasis	Distribution	Clinical signs	Treatment	Clinical efficacy	Observations
Cutaneous	- Asia and Africa (*L. major*) - South America (*L. amazonensis*)	Skin ulcers on exposed parts of the body (face, arms and legs), usually self-heal within a few months.	- Antimony compounds (Glucantime®;Pentostam®) - Allopurinol (Zyloprim®;Zyloric®)	90-100% cure rates in areas w/o resistance	High toxicity, fatigue, nausea, muscle and joint pain, cardiotoxicity, hepatic and renal dysfunction.
			- Pentamidin (NebuPent®)	~90% cure rate	Frequent hypotension, hypoglycemia, renal damage and nausea.
Mucocutaneous	- South America (*L. braziliensis*)	Lesions on mucous membranes (nose, mouth, throat).	- Antimony compounds (Pentostam®)	Dependent on disease severity	Same as for cutaneous leishmaniasis
Visceral	- Asia and Africa (*L. donovani*) - Europe and South America (*L. infantum*)	Fever (can be irregular), weight loss, anemia, spleen and liver swelling.	- Antimony compounds (Glucantime®;Pentostam®)	~90% cure rates in areas w/o resistance	High toxicity, fatigue, nausea, muscle and joint pain, cardiotoxicity, hepatic and renal dysfunction. Painful IM injections.
			- Amphotericin B (Fungilin®; Fungizone®), liposomal formula (Abelcet®; Ambisome™)	>90% cure rates	Simple formulation presents extreme toxicity, generalized pain, convulsions, renal damage, etc. Liposomal version much less toxic. Less painful IV administration.
			- Pentamidin (NebuPent®)		Frequent hypotension, hypoglycemia, renal damage and nausea. Painful IM injections.
			- Miltefosine (Impavido™)	>90% cure rates	Nausea and vomiting. Teratogenicity. Easy oral administration.

*Since there are more than 20 *Leishmania* species, causing diverse forms of the disease, with a very wide variety of symptoms, only the most common features were listed. Sources: www.who.int/leishmaniasis, www.who.int/tdr and references 1-4.

of obligatory notification. However, in Portugal notification fails in about 50% of the cases, meaning that we have approximately twice the number of leishmaniasis cases than the official numbers report. In the case of HIV co-infection the official declarations are even less precise, since leishmaniasis will only be notified if the parasitosis is identified together with the first diagnose of HIV. It is worth noting that for each sick person there are 10 others that are infected but not ill, which can exponentially increase the number of cases in immunosuppressed patients.

José Arêlo Manso, director of the Pediatric Service of the Centro Hospitalar de Trás-os-Montes e Alto Douro, in Vila Real, has been following the infection in children since 1988, "In our hospital, in the last 20 years, we had 115 cases, of which 70% were in the first 6 years. The situation is gradually improving". Out of the 115 cases mentioned by Arêlo Manso, 2 were from children that died, also in the first years of the study. The reduction in the number of infected children can be related to a general improvement in life conditions. Today families live better, children have a healthier nutrition and access to the health care system is facilitated. Consequently, parents take their child to the hospital in an earlier phase of the disease than they did 15 years ago, which renders treatment easier and more efficient. In Vila Real, the first-line drug used against leishmaniasis is Glucantime® (an antimony compound). "We have a good response to the old drugs. We don't disregard the side effects that can occur but we know how to prevent them", says Arêlo Manso. The antimony efficacy is around 90%, with the rest of the cases considered to be treatment failures. In Europe, no cases of resistance to this particular drug have been reported, contrary to what happens in India. The Vila Real pediatrician follows a different therapeutic scheme from the one recommended by the WHO (20 mg/kg/day during 28 days), he uses 28 mg/kg/day for 14 days. This way, he says, "We've been having good results and it also reduces the time one has to stay in hospital. Obviously it is also better for the kids, since they only have to do 14 days of intramuscular injections, instead of the classical 28. We have also used liposomal amphotericin B, but it was in a very serious case, of a 4-month baby with only 4 kg! In ordinary, non-complicated, situations we apply antimony salts… they are obviously more toxic, but we have to be aware of other things". The price is one of them, since



Centro Hospitalar Trás-os-Montes e Alto Douro in Vila Real

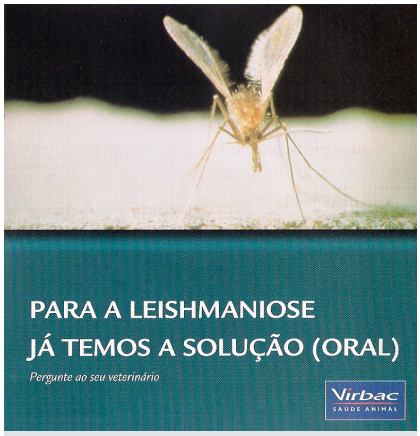
"In our hospital, in the last 20 years, we had 115 cases, of which 70% were in the first 6 years. The situation is gradually improving."

José Arêlo Manso, director of the Pediatric Service of the Centro Hospitalar de Trás-os-Montes e Alto Douro.

Drawing at the left made by Mafalda Costa, 6 years old, during her stay at the hospital, copied from "Criatividade Infantil" - Associação Transmontana de Saúde Infantil.

antimony salts are 25 times cheaper than the liposomal formulated amphotericin B. Luís Varandas, pediatrician at the Hospital Dona Estefânia, in Lisbon, has a different opinion, "4 or 5 years ago, we started using only liposomal amphotericin B in leishmaniasis treatment, because it is more efficient and much simpler. I don't think we should be subjecting a child to a series of painful intramuscular injections, when we can do a short-term treatment of intravenous injections of amphotericin B". In either case, after the beginning of the treatment, children often feel better at the 3rd or 4th day. This quick health improvement is very reassuring to parents, since they can get a little bit confused with the explanation of their children infirmity, "Parents tend to be worried about the rest of the family too, they ask how you can catch it and, obviously, if the sick child will be ok", says Luís Varandas.

Usually parents, even the ones living in endemic areas, arrive at the hospital with no suspicion about what is afflicting their children, but "Medical teams are very alerted to these cases and today we also have easier and more rapid serological diagnosis techniques", says Arêlo Manso. The physician also calls our attention to the fact that not everything that is written in textbooks is 100% correct and one should know that every case is a different case, with its own specificity. For example, a classical perspective of infantile leishmaniasis delimits the disease from the age of 1 to 3 years. However, in the pediatrician's personal experience, 45% of the cases are diagnosed before the first year of age. Another example is the frequency of fever. Whilst it is one of the most common symptoms in leishmaniasis, it is possible that a patient manifests diverse symptoms of the disease but may never present fever.



Portuguese advertisement for canine leishmaniasis oral treatment.

Apart from infantile visceral leishmaniasis or adult infections, the later generally in immunocompromised people, "Portugal also has cutaneous leishmaniasis", emphasizes Lenea Campino. This form of the disease is not of obligatory notification. Therefore, no exact numbers for cutaneous leishmaniasis prevalence are available. The researcher explains, "In Portugal the cutaneous form is caused by the same species that originates visceral leishmaniasis, that is, *Leishmania infantum*" but because these cutaneous lesions are usually self-healing, treatment is not frequently applied. The difficulty in these cases is to correctly diagnose a skin ulcer, which could be due to many other factors.

As mentioned above, dogs are the main reservoir for leishmaniasis in Europe. Luís Cardoso, veterinarian and professor of parasitology at the Universidade de Trás-os-Montes e Alto Douro, points out that it is possible to detect other animals, like horses, chickens or cats infected with *Leishmania*, "but to say they are reservoirs is a bit excessive". Some Portuguese research groups have been devoted to the study of *Leishmania*

infections in the canine population. Such studies are important in three different ways. Firstly, leishmaniasis is a disease that directly affects dogs. Secondly, dogs can transmit the disease to humans, via insect vector, turning leishmaniasis into a public health issue. Finally, canine leishmaniasis can be used as a model of the disease in imunocompromised humans.

Various serological surveys performed in Portugal revealed a high overall prevalence of canine infection, with about 20% registered in Lisbon, Peso da Régua and Alijó. This number would be even greater if the surveys were carried out in terms of polymerase chain reaction (PCR) analysis, as explains Luís Cardoso, "In a 2001 report by Solano-Gallego and colleagues they have found more than 60% of infected dogs by PCR, but only around 25% of seroprevalence". These percentages, however, are already more than enough to maintain leishmaniasis as an endemic disease, given that 2.5% of prevalence is sufficient to keep endemic conditions.

It is interesting to notice that not all infected dogs can be considered sick dogs. In this context a relevant question arises: can asymptomatic *Leishmania* infected dogs serve as reservoirs and consequently transmit the disease to other animals or human beings? According to Luís Cardoso they can, "Both symptomatic and asymptomatic animals can transmit the parasite, but normally the sick dog will infect more, because it has a higher number of parasites or higher parasitaemia". If that is the case, "I wonder why we don't have more cases of human leishmaniasis in Lisbon, for example", comments Lenea Campino. Unfortunately, there are no studies on the direct relationship between the percentages of canine infection and human infection in the same specific area. Luís Cardoso explains "We probably have more infected dogs than we think we do. Actually canine leishmaniasis is increasing, contrary to human leishmaniasis, which is apparently stabilized. A possible explanation includes the fact that we have more veterinary clinics. We see more animals and, consequently, we diagnose more infections. But the reality is that the impact of the disease on dogs is more significant than on humans. I would say, that regarding leishmaniasis, the dog is the weakest link. Why there aren't more humans infected? Probably because humans are more resistant to infection. It would be interesting to carry out some correlations studies in the field between canine and human prevalences, to see what comes out". The reality is that these type of studies are quite complex to perform. One of the reasons is that getting permission to take blood samples from dogs is


Photo by Luís Cardoso

"Besides the dog, there are other animals infected with *Leishmania*: cats, horses, chickens… But to say that they are reservoirs is a bit excessive."

Luís Cardoso, veterinarian and professor of parasitology at the UTAD in Vila Real. At the photo a dog infected with leishmaniasis.

not easy, but testing dog owners is even more difficult.

The WHO guidelines on leishmaniasis treatment and control recommend the elimination of all *Leishmania*-positive dogs. Here pediatricians and veterinarians take opposite sides. The first ones have no doubts that an infected dog should be sacrificed, because of the danger it represents to children and to the general population. The veterinarians prefer a second option: to treat the dog. Nuno Nazário, veterinarian at the Hospital Veterinário da Figueira da Foz, receives some cases of leishmaniasis and it is with some disappointment that sometimes he has to follow the owner's will and euthanize a dog that, in his opinion, could be well after treatment. He explains, "In face of leishmaniasis people reveal some concerns. We explain them what it is and that the animal can be treated, but sometimes they just don't want to take any risks and prefer to euthanize the dog". The economic issue is another possible explanation for not following the treatment option, since leishmanicidal drugs for veterinary use are very expensive, with an average cost of 150 to 200€ *per* treatment.

Contrary to what is recommended by WHO, dogs receive the same drugs as humans, namely Glucantime®, Zyloric® (allopurinol), amphotericin B and Milteforan® (miltefosine). The problem of using the same drugs in animals as in humans is the potential of favoring parasite resistance on a larger scale, but until now, European countries do not seem to be concerned with this fact.

Vaccination against leishmaniasis is another possible way to control the disease. Currently, only three vaccines are licensed for leishmaniasis: a live vaccine for humans in Uzbekistan, a killed vaccine for humans and a vaccine for dog prophylaxis, both in Brazil. The latter, Leishmune®, is still at experimental phase, but revealing promising results in preventing disease development and transmission. However, the Brazilian Ministry of Agriculture still recommends the elimination of infected dogs as a way to prevent human disease instead of the use of canine treatment. One of the arguments for this is that it is not possible to vaccinate the entire canine population, since there are always abandoned dogs or other situations where animals will not be submitted to vaccination. In the same way, one can argue that is also not guaranteed that all infected animals will be eliminated. According to Luís Cardoso, another problem of Leishmune® is the fact that vaccinated dogs become seropositive for *Leishmania* and this poses an additional difficulty on the detection of truly infected dogs. "Even with a vaccine", he added, "it will be very



Carlos Costa

"Understanding the pathology of leishmaniasis can lead to an extended comprehension of a larger range of diseases."

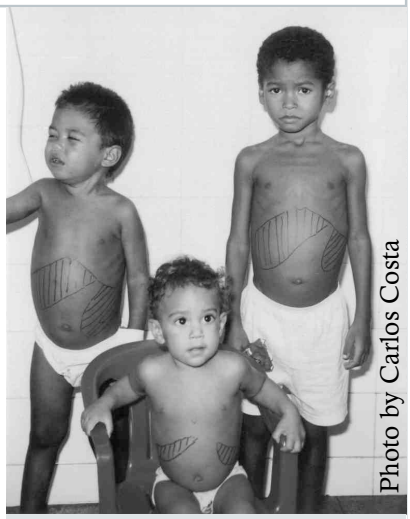
Carlos Costa, director of the Instituto de Doenças Tropicais Natan Portella in Teresina, Piauí.

difficult to eradicate leishmaniasis."

For human leishmaniasis look up for references 5-10 and for canine leishmaniasis look up references 11-17. Reference 18 reviews vaccines for leishmaniasis.

Leishmaniasis in India and Brazil

Unlike in Europe, visceral leishmaniasis is a considerable problem for human populations in India and, to a lesser extent, in Brazil. According to Kalpana Pai, a researcher formerly at the Kala Azar Medical Research Center at Banaras Hindu University, in Varanasi, the cases in India may range between 100.000 and 250.000 a year, however these numbers are most likely underestimated. "The standard therapeutic scheme followed uses antimony compounds. However, over the last 10 to 15 years, patients from endemic regions have been showing refractoriness to these compounds and several studies have reported parasitic resistance to antimonials. So, keeping in mind the patient medical history and the region, physicians apply different regimens", explains Kalpana Pai. Thus, patients from non-endemic regions receive antimony treatment and patients from endemic regions are given amphotericin B or miltefosine, a new oral drug. The researcher thinks that the present therapy is suitable enough, since patients tolerate the drugs quite well. Still, she reinforces the fact that drug suitability will always depend on medical discrimination on the patient detailed history and on the region of origin. Carlos Costa, director of the Instituto de Doenças Tropicais Natan Portella in Teresina, Piauí, and a medical doctor since 1976, sees around 200 to 300 leishmaniasis patients every year. In 90% of the cases the treatment is efficient. The others, he explains, "About 3% need a second treatment regimen and the remaining die. The total national numbers for Brazil are around 250 fatalities a year due to leishmaniasis". As in India, the first-line treatment is based on antimonial compounds, with the difference that in Brazil no cases of drug resistance were reported. In the case of patients with more complicated conditions, such as pregnant women and older people, the chemotherapeutical preference is amphotericin B, both simple and in liposomal form. Carlos Costa adds that "Patients are never parasite-free, and if one day they get imunosuppressed the disease might reappear".



Children with leishmaniasis at the Piauí Hospital. The spleen and liver swelling is evidenced by a marker.

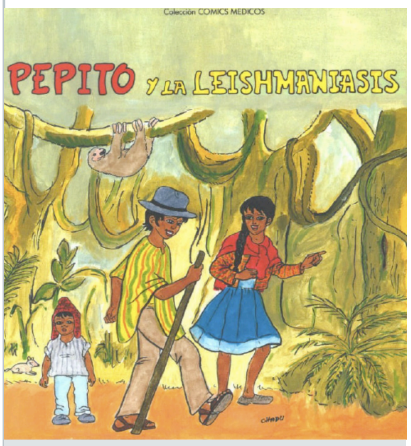
Social and economic considerations

The impact of leismaniasis is not the same in the different countries affected, not even along the territory of one same country. India, Bangladesh, Nepal, Sudan and Brazil account for 90% of all cases of visceral leishmaniasis, with very well delimited areas of high endemicity, such as, the Bihar state in India. But, despite the well-known endemic regions and the considerable high number of infected people around the world,

leishmaniasis is still considered a neglected disease. Why is that so? "Leishmaniasis is not on TV, it is not on the spotlights like AIDS or cancer. So people know that AIDS or cancer can kill, but they do not have any information about leishmaniasis", says Carlos Costa. The researcher Lenea Campino also agrees that this is a forgotten disease. In Portugal, for example, "Most of the medical doctors have never seen a case and the media refer only to canine leishmaniasis, disregarding that human leishmaniasis also exists in our country and that it can be fatal if left untreated". On the other hand, Kalpana Pai argues that not many pharmaceutical companies are ready to invest in R&D, not even when scientists have promising ideas. The reason for this reluctance may be the target population, since it is mainly an infirmity from developing countries, "It is a poor man's disease", says the Indian researcher. But even in Europe, the use of a new compound against leishmaniasis is not always promptly accepted. The fear of the unknown and established old habits are some of the obstacles to change. More importantly, the costs of new compounds are generally excessive. One example is liposomal amphotericin B that is much less toxic than the classical antimonial treatment, but also 25 times more expensive. This makes it unaffordable even in the so-called developed countries.

In addition to the investment in more effective and in less toxic drugs and better diagnosis techniques, epidemiological studies are also important to control this type of vector-borne diseases. Epidemiological analysis of leishmaniasis might clarify the disease distribution and the causes of disease frequency in human populations. However, it has been almost impossible to obtain financial support to study the epidemiology of the disease. "If we think that Europe does not have malaria cases in years, but still people are receiving money to check the hypothesis of malaria reappearance in the next century… What about leishmaniasis? It already exists here, but no one cares!", says Lenea Campino. Also in Brazil, where the disease has a larger impact than in Europe, the national health system does not always treat patients on due time. Carlos Costa is aware of the difficulties in dealing with a neglected disease. Before arriving at his hospital, the reference hospital for infectious diseases in Piauí, the patients pass through 3 or 4 consultations until they are correctly diagnosed with leishmaniasis. In Carlos Costa's opinion, this delay in public health services response penalizes the patients and even the country, since "The longest we take to correctly diagnose the infection, the higher the probability of death. And we should not forget that each taken life is more than an economic loss".

For further information on this topic look up references 19-20.



Educative comic book from 1999. Available at:

http://www.who.int/leishmaniasis/resources/en/

The scientist's progress and responsibility

In a quick search over PubMed, a free digital archive of biomedical and life science journals, the entry leishmaniasis gives a total of around 15.000 publications. It is less than a third of what is published for malaria and about 10 times less what is available for AIDS. This is obviously a consequence of the impact of the disease on humans. Malaria and AIDS kill more than 2 million people each year, 40 times more than leishmaniasis. But should a disease be neglected only because it is not on top of the list of the most deadly infections? Lenea Campino alerts for the problem of neglecting a potentially deadly disease because of the dangerous consequences that can arise from a possible imbalance between the sandfly vector, the parasite, the host and the environment. Some of the factors that may trigger this disequilibrium are global warming, because of the influence it can exert over the vector population, and long-distance tourism, travel or commercial trades, due to the possibility of transcontinental transport of microorganisms. Nevertheless, "Scientific progress has been achieved in the last years, mostly because of technical improvements", states the researcher, "Unfortunately, until now, scientists have failed to develop a less invasive and more accurate diagnostic method". There are a number of studies on new diagnostic techniques, some done in collaboration with hospitals and medical teams. But it seems that what is accomplished on the bench does not directly translate into practical use. Arêlo Manso participated in a few of these studies, namely with the Faculdade de Farmácia da Universidade do Porto. During that study the patient's samples were very rapidly and efficiently processed. However, when the grant period ended, the project was interrupted and no actual improvements reflected on the everyday life of the physicians dealing with leishmaniasis.

Concerning parasite biology and immunology, a lot has been discovered in the last decades. It is worth noting that some of that acquired knowledge has a wide spectrum of applications beyond *Leishmania* parasites or leishmaniasis. For example, of major importance was the discovery of the Th1/Th2 dichotomy in *Leishmania major in vivo* murine infections. Studies on *Leishmania* have also been useful to understand the mechanisms underlying the persistence of various intracellular pathogens after clinical cure. Additionally, since *Leishmania* parasites are unicellular eukaryotic organisms with most organelles in single number, they have provided excellent clues about organelle biogenesis. But if we focus on scientific research clearly directed to leishmanicidal mechanisms, drug targets or vaccine candidates, the truth is that the majority of these have no realistic potential to be used in the clinics. In fact, antimonial drugs, the first compounds to be applied against visceral leishmaniasis back in 1940's, are still the most commonly used, regardless of the risky side effects and despite the new drugs discovered meanwhile. Carlos Costa thinks that scientific progress on leishmaniasis has been limited and is very far away from the advances needed, "Science has much to contribute to leishmaniasis, with more rigorous studies, with sharper analysis and with more well-financed projects".

In agreement, Kalpana Pai sadly notes that, "Still lots of work need to be done on immunopathogenesis aspects. In parallel, one should also conduct research on alternative medicines and strategies for effective elimination of the parasite and the sandfly vector. In summary, a multidisciplinary approach is needed".

Should we blame this unsatisfactory scientific progress on the lack of funding or on the public unawareness of the disease? Isn't the scientific community one important opinion and policy maker of our society? In fact, most of the time scientists do not have a social intervention, as they should, either because they do not have the initiative to interact with the non-scientific community or because they simply do not know how to communicate science to the general public. Probably, if scientists would be more actively integrated in political decision committees and also more involved in educational campaigns, people would be more familiarized with leishmaniasis, with its research progresses and difficulties and with prevention actions and control measures. Moreover, if the scientists were capable of engaging the society in their research areas, funding support would probably arrive naturally and with it, better control strategies and more suitable treatments.

A few good examples of bridging society and research information on leishmaniasis exist. One of them is the LeishRisk project, a platform that aims at linking several partners working on *Leishmania* to promote knowledge interchange, its final goal being control of leishmaniasis worldwide (www.leishrisk.net). Another interesting campaign, directed to some South American rural populations, is the didactic BD *Pepito y la Leishmaniasis*. This booklet tells a story of a family that is affected by leishmaniasis, explaining in a simple way the general characteristics of the disease and how to prevent it.

It is a general opinion that the scientific community should be more proactive and more present in people's everyday life. However, in Carlos Costa's point of view, regarding leishmaniasis control, "Not even the scientists know how to control it, either because it is a too complex problem or because we simply don't know enough!" ❖

Acknowledgments

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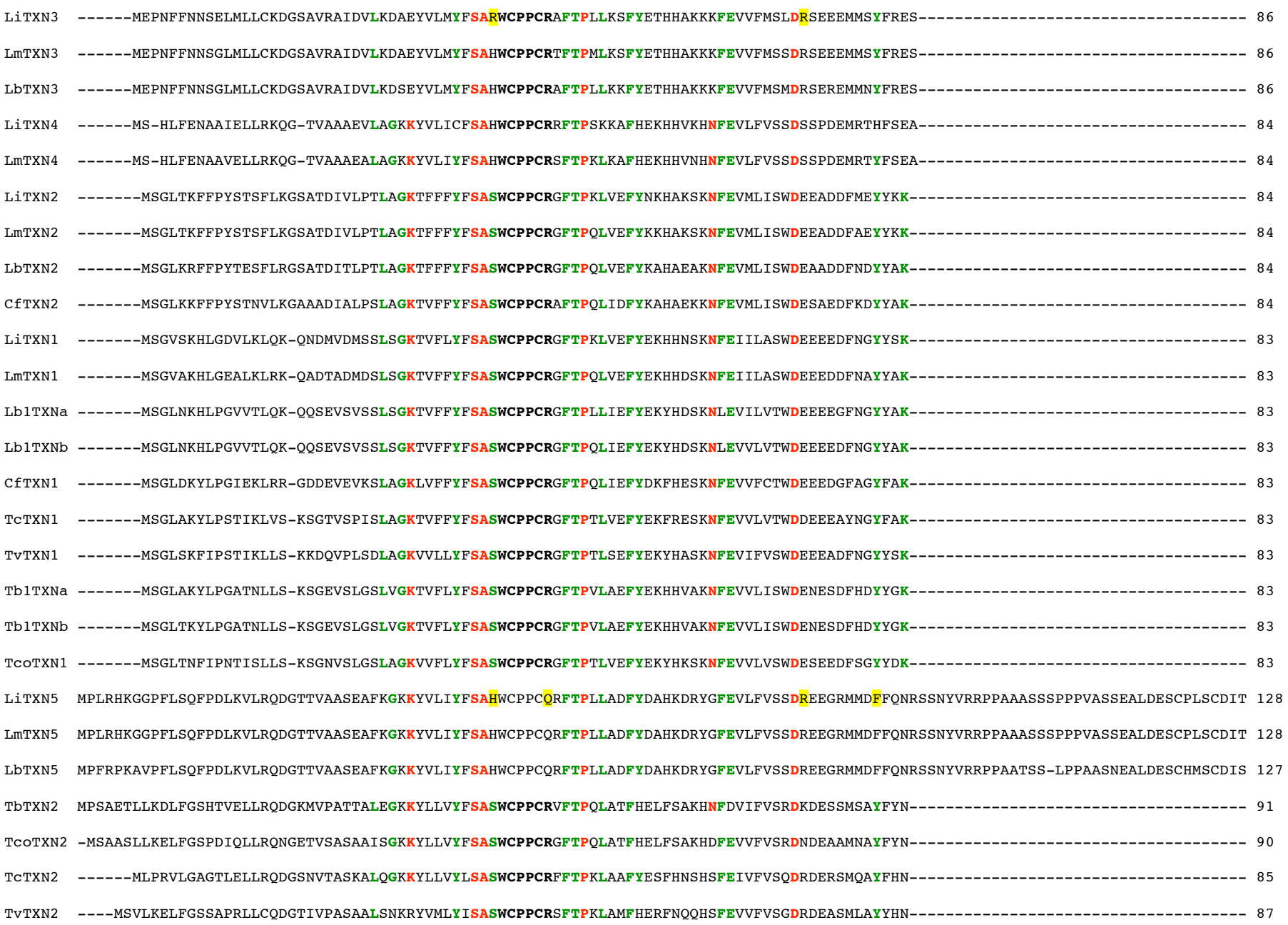
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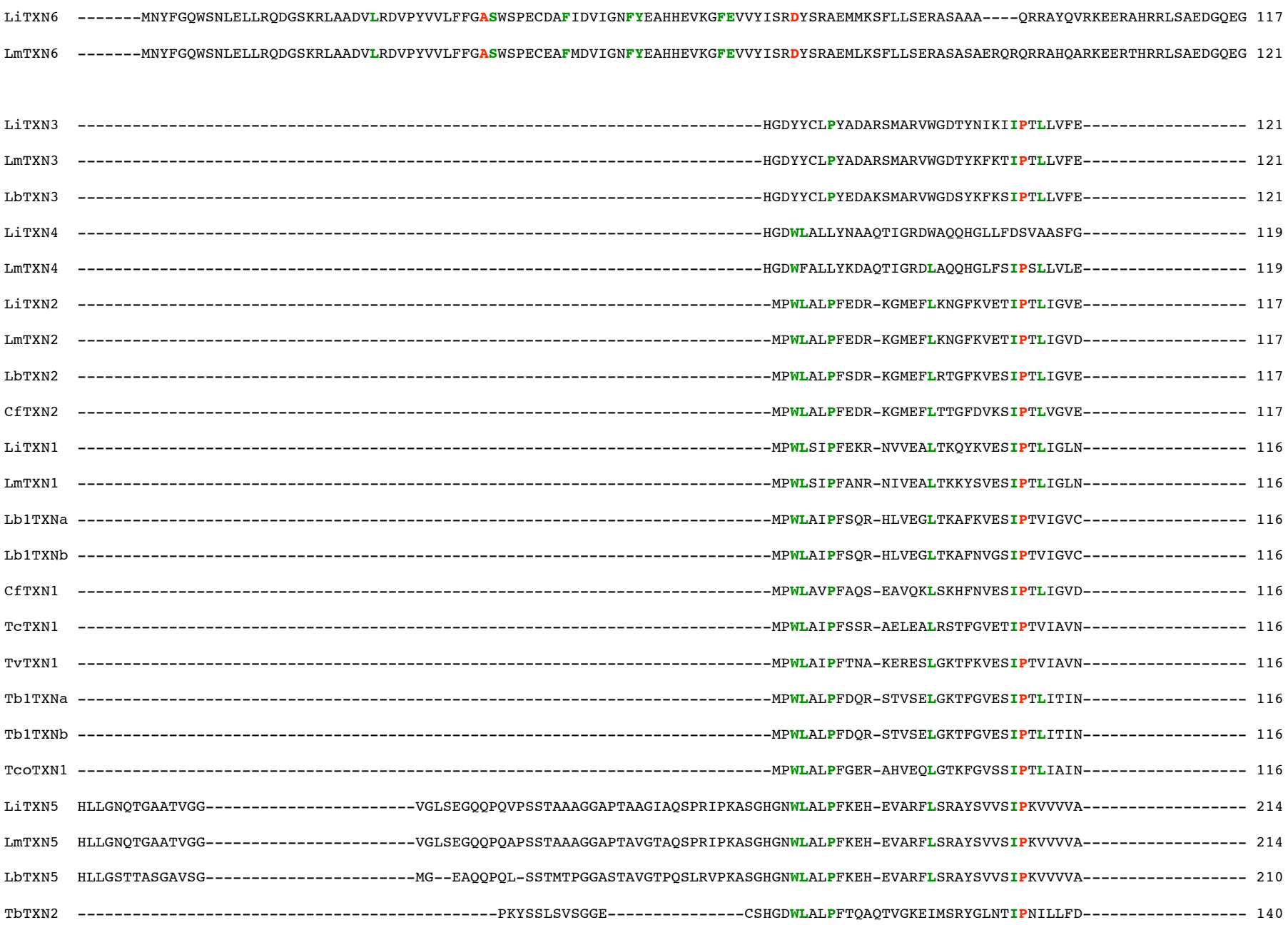
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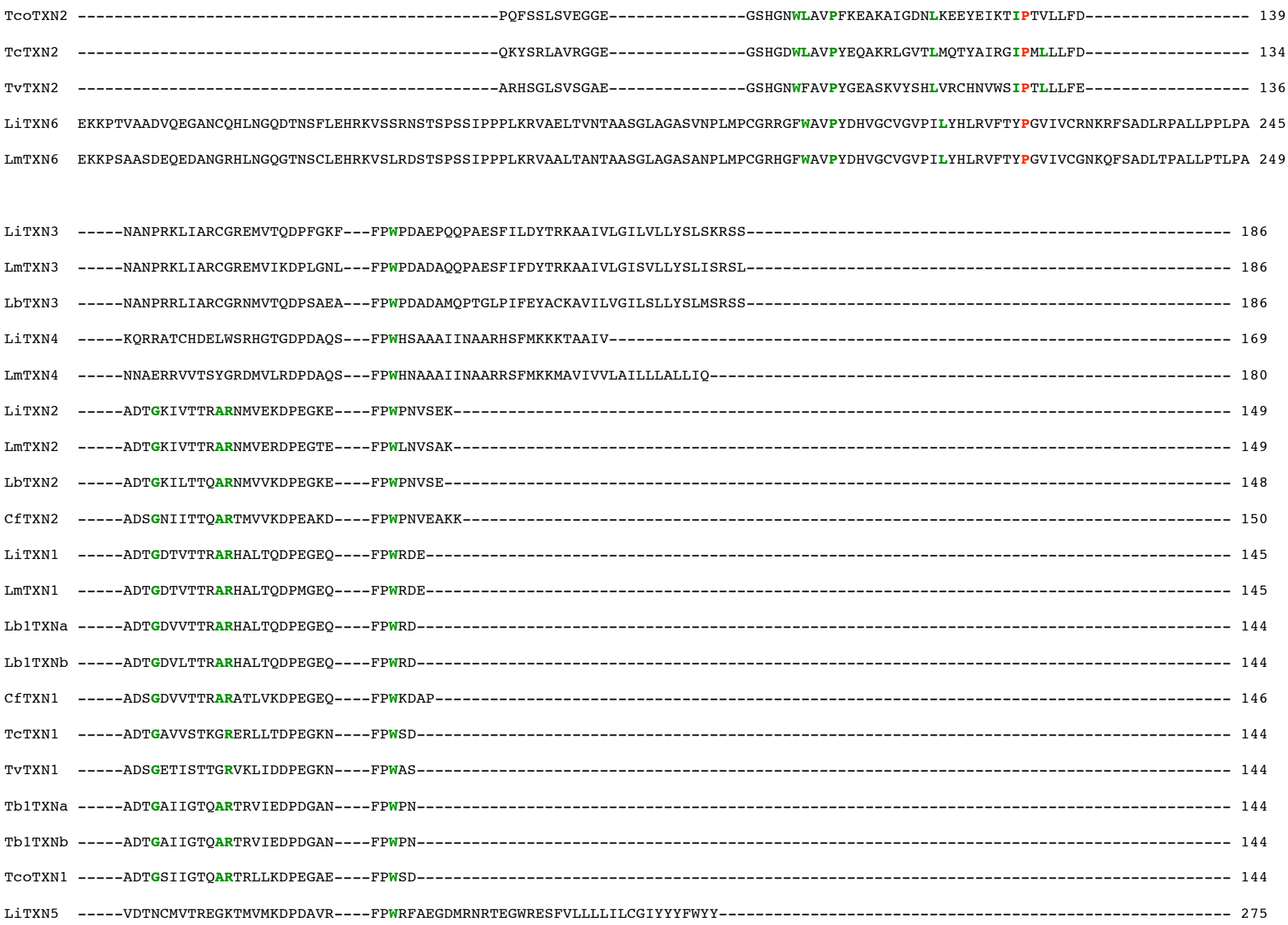
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August 2008

Appendix 3


LiTXN3 ------MEPNFFNNSELMLLCKDGSAVRAIDVLKDAEYVLMYFSARWCPPCRAFTPLLKSFYETHHAKKKFEVVFMSLDRSEEEMMSYFRES---------- 86
LmTXN3 ------MEPNFFNNSGLMLLCKDGSAVRAIDVLKDAEYVLMYFSAHWCPPCRTFTPMLKSFYETHHAKKKFEVVFMSSDRSEEEMMSYFRES---------- 86
LbTXN3 ------MEPNFFNNSGLMLLCKDGSAVRAIDVLKDSEYVLMYFSAHWCPPCRAFTPLLKKFYETHHAKKKFEVVFMSMDRSEREMMNYFRES---------- 86
LiTXN4 ------MS-HLFENAAIELLRKQG-TVAAAEVLAGKKYVLICFSAHWCPPCRRFTPSKKAFHEKHHVKHNFEVLFVSSDSSPDEMRTHFSEA---------- 84
LmTXN4 ------MS-HLFENAAVELLRKQG-TVAAAEALAGKKYVLIYFSAHWCPPCRSFTPKLKAFHEKHHVNHNFEVLFVSSDSSPDEMRTYFSEA---------- 84
LiTXN2 -------MSGLTKFFPYSTSFLKGSATDIVLPTLAGKTFFFYFSASWCPPCRGFTPKLVEFYNKHAKSKNFEVMLISWDEEADDFMEYYKK---------- 84
LmTXN2 -------MSGLTKFFPYSTSFLKGSATDIVLPTLAGKTFFFYFSASWCPPCRGFTPQLVEFYKKHAKSKNFEVMLISWDEEADDFAEYYKK---------- 84
LbTXN2 -------MSGLKRFFPYTESFLRGSATDITLPTLAGKTFFFYFSASWCPPCRGFTPQLVEFYKAHAEAKNFEVMLISWDEAADDFNDYYAK---------- 84
CfTXN2 -------MSGLKKFFPYSTNVLKGAAADIALPSLAGKTVFFYFSASWCPPCRAFTPQLIDFYKAHAEKKNFEVMLISWDESAEDFKDYYAK---------- 84
LiTXN1 -------MSGVSKHLGDVLKLQK-QNDMVDMSSLSGKTVFLYFSASWCPPCRGFTPKLVEFYEKHHNSKNFEIILASWDEEEEDFNGYYSK---------- 83
LmTXN1 -------MSGVAKHLGEALKLRK-QADTADMDSLSGKTVFFYFSASWCPPCRGFTPQLVEFYEKHHDSKNFEIILASWDEEEDDFNAYYAK---------- 83
Lb1TXNa -------MSGLNKHLPGVVTLQK-QQSEVSVSSLSGKTVFFYFSASWCPPCRGFTPLLIEFYEKYHDSKNLEVILVTWDEEEEGFNGYYAK---------- 83
Lb1TXNb -------MSGLNKHLPGVVTLQK-QQSEVSVSSLSGKTVFFYFSASWCPPCRGFTPQLIEFYEKYHDSKNLEVVLVTWDEEEEDFNGYYAK---------- 83
CfTXN1 -------MSGLDKYLPGIEKLRR-GDDEVEVKSLAGKLVFFYFSASWCPPCRGFTPQLIEFYDKFHESKNFEVVFCTWDEEEDGFAGYFAK---------- 83
TcTXN1 -------MSGLAKYLPSTIKLVS-KSGTVSPISLAGKTVFFYFSASWCPPCRGFTPTLVEFYEKFRESKNFEVVLVTWDDEEEAYNGYFAK---------- 83
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Tb1TXNb -------MSGLTKYLPGATNLLS-KSGEVSLGSLVGKTVFLYFSASWCPPCRGFTPVLAEFYEKHHVAKNFEVVLISWDENESDFHDYYGK---------- 83
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TcoTXN2 -MSAASLLKELFGSPDIQLLRQNGETVSASAAISGKKYLLVYFSASWCPPCRGFTPQLATFHELFSAKHDFEVVFVSRDNDEAAMNAYFYN---------- 90
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TvTXN2 ----MSVLKELFGSSAPRLLCQDGTIVPASAALSNKRYVMLYISASWCPPCRSFTPKLAMFHERFNQQHSFEVVFVSGDRDEASMLAYYHN---------- 87


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LmTXN3 ----------HGDYYCLPYADARSMARVWGDTYKFKTIPTLLVFE---------- 121
LbTXN3 ----------HGDYYCLPYEDAKSMARVWGDSYKFKSIPTLLVFE---------- 121
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LmTXN2 ----------MPWLALPFEDR-KGMEFLKNGFKVETIPTLIGVD---------- 117
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LiTXN2 -----ADTGKIVTTRARNMVEKDPEGKE----FPWPNVSEK---------- 149
LmTXN2 -----ADTGKIVTTRARNMVERDPEGTE----FPWLNVSAK---------- 149
LbTXN2 -----ADTGKILTTQARNMVVKDPEGKE----FPWPNVSE---------- 148
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Lb1TXNb -----ADTGDVLTTRARHALTQDPEGEQ----FPWRD---------- 144
CfTXN1 -----ADSGDVVTTRARATLVKDPEGEQ----FPWKDAP---------- 146
TcTXN1 -----ADTGAVVSTKGRERLLTDPEGKN----FPWSD---------- 144
TvTXN1 -----ADSGETISTTGRVKLIDDPEGKN----FPWAS---------- 144
Tb1TXNa -----ADTGAIIGTQARTRVIEDPDGAN----FPWPN---------- 144
Tb1TXNb -----ADTGAIIGTQARTRVIEDPDGAN----FPWPN---------- 144
TcoTXN1 -----ADTGSIIGTQARTRLLKDPEGAE----FPWSD---------- 144
LiTXN5 -----VDTNCMVTREGKTMVMKDPDAVR----FPWRFAEGDMRNRTEGWRESFVLLLLILCGIYYYFWYY---------- 275

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LmTXN5  -----VDTNCMVTREGKTMVMKDPDAVR----FPWRFAEGDMRNRTEGWRESLVLLLFILCGIYYYFWFY---------------------------------------------------------- 275
LbTXN5  -----VDTNCMVTREGKAMVMKDPDALR----FPWRFAEGDMRNRTEGWRECIVLLLIVMCVFYYYFWYY---------------------------------------------------------- 271
TbTXN2  -----LSTEELVTSEARQLIGSNCRSAEG---FPWRGAS----APVISFQGLATVFVVFLMLYQFWQSWS---------------------------------------------------------- 198
TcoTXN2 -----LSTGNLVTQEARHNIADNFRTAEG---FPWRRSP----FAWFTPRRIIKICAAIVLLYYYWE---------------------------------------------------------- 194
TcTXN2  -----LETGELVTRNARDLVARNLDTAEG---FPWAGNTDGEGLRGSYWNQLFLVFIIASVLYQFWR---------------------------------------------------------- 193
TvTXN2  -----LETGKLVTPHARDHVIRNLHTAAG---FPWEGVD----PPAVSLQQVWKVVVVLIVLYLIHLVVAN---------------------------------------------------------- 195
LiTXN6  VHQAPAEHSLTLPPEIWPPGGSETQSAQPSLSSPIDRANAPQRRQKTPMVARPECYPDVVTIAGRF---------------------------------------------------------- 311
LmTXN6  VHQAPAERSLTLSPEIWPPEGSETESTQP---SPIDRANAPQRRQKTPMVARPECYPDVLTIAGRFMIERDPNGEDFPWDKMNAKTRWAALVFFVIVAVLTVIVLSWALPISRIKRQTALKAQAEL 372
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