SUMMARY of PhD thesis

Study of molecular mechanisms underlying *Leishmania* spp. survival within the phagocytes of the mammalian host

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Leishmaniasis, a major anthropozoonotic disease, is caused by protozoan parasites of the genus *Leishmania* when transmitted to the mammalian host by the bite of the insect intermediate vector (sand flies of the genus *Phlebotomus* or *Lutzomyia*). Given that the chemotherapeutic treatment of the disease causes serious side effects, it is expensive and there is a lack of vaccines and emergence of resistance to some of the mostly commonly used anti-leishmanial drugs, there is an urgent need for the discovery of more effective non-toxic drugs and vaccines. To this end, research on the cellular and molecular mechanisms that take place in the mammalian host is of great importance as it is expected to lead to the identification of new parasitic virulence factors and putative drug targets for effective control of Leishmaniasis.

The protozoan parasites of the *Leishmania* genus survive in the phagolysosomal compartment of the mammalian host macrophages by developing strategies subverting its parasitocidal properties. One strategy believed to contribute to the intra-phagosomal survival of the *Leishmania* promastigote form is the transfer of its major surface glycoconjugate, lipophosphoglycan (LPG), to the inner leaflet of the phagosomal membrane. Insertion of LPG alters the biophysical properties of the membrane and disturbs its lipid microdomains [71, 180]. This could result to a redistribution of specific phosphoinositides (PIs) in the parasitophorous phagosome membrane leading to subversion of the phagolysosome biogenesis, already proven to take place in infections by intracellular bacterial pathogens [142, 144]. The molecular mechanism underlying this process has not been confirmed and clarified for the *Leishmania* parasites.

The present PhD research project, aimed to contribute to a) the exploration of specific features of the biogenesis of *L. donovani* bearing phagosomes during the initial stages of phagocytosis by macrophages and b) the understanding of the molecular mechanisms underlying the parasite's survival in the phagolysosome.

Our study focused on the spatial-temporal distribution of specific molecular markers of phagosome maturation as F-actin, Rab7 and LAMP1 as well as the PIs PtdIns(4,5)P_2,

PtdIns(3,4,5)P_3 and PI3P for which no relevant study relating them to parasitophorous phagosomes has been performed to date. Furthermore, we performed preliminary experiments concerning the possible role of LPG in the subversion of the spatio-temporal distribution of the three PIs in the membrane of *L. donovani* bearing phagosomes. For these studies, we set up an *in vitro* cellular system using transiently or stably transfected RAW264.7 cells expressing GFP or YFP fusions of certain PI-binding domains that we infected with transgenic red fluorescent *Leishmania*-mRFP parasites. Experiments with opsonised inert particles were performed in parallel to detect differences in the temporal or spatial distribution of the specific PIs that could indicate subversion in PI metabolism by *Leishmania* during its phagocytic uptake by macrophages. The analysis was performed by confocal microscopy in fixed samples.

Overall, our results demonstrated a time deflection in the presence of the three molecular markers of phagosome maturation and the PIs on the parasitophorous phagosome membrane as compared with those bearing control beads. The observed differences in the temporal and spatial distribution of these molecules, suggest a delay of biogenesis of the parasitophorous phagosome, as already reported in the literature for *L. donovani* [71, 108, 206] and previously shown for the also obligatory intracellular pathogen *Mycobacterium tuberculosis* [144, 242]. The above described observed phenomenon could be due to a) the action of parasite's molecules in the macrophage's PIs metabolism [166]; b) the role of the different receptor type (e.g. FcγR, CR3, Mannose Receptor) involved in the phagocytosis of *Leishmania* as compared to opsonised beads [246]; c) the role that differences in the size and shape of *Leishmania* promastigotes as compared to spherical beads could play in a delay in parasite's engulfment and maturation of the parasitophorous phagosome [250]; and d) the role that the parasite's and flagellum's motility could play in the sealing of the newly formed phagosome [73] and an additional mechanical retardation of the phagosome maturation to phagolysosome [203].

Additionally, we studied a putative *L. donovani* virulence factor, the membrane acid phosphatase *Ld*MAcP. Because of its predicted extracellular orientation at the parasitic plasma membrane, *Ld*MAcP could be implicated in a direct interaction of the parasite with the host cell. Moreover, a previous study had shown that semi-purified membrane acid phosphatase(s) from *L. donovani* dephosphorylate PtdIns(4.5)P_2 and PtdIns(3.4.5)P_3 [166]. Despite the fact that acid ecto-phosphatase activity was identified in *Leishmania* more than 30 years ago [164], its precise localization at the parasitic cell and the role of this activity in parasite infectivity and virulence still remains to be elucidated. In the

current study we wished to elucidate the localization and structural/ functional properties of *Ld*MAcP by studying the endogenous enzyme and the recombinant r*Ld*MAcP-mRFP1 and r*Ld*MAcP-His chimeras in *L. donovani* promastigotes and mammalian cells.

The original identification of the *Ld*MAcP protein [164] was followed by a study indicating that its sequence is conserved amongst all pathogenic *Leishmania* spp. [163]. We conducted a search for *Ld*MAcP homologues in the available genome sequences of several *Leishmania* spp. that contrary to the earlier claims, *Ld*MAcP, as a membrane bound ecto-enzyme, seems to be present only in the *L. donovani* complex in which it additionally presents strain polymorphisms. This new piece of information suggests that *Ld*MAcP could become a useful tool for the diagnosis of Visceral Leishmaniasis.

Additionally, in the *L. donovani* genome were found at least three genes, besides that of *Ld*MAcP, coding for predicted HAcPs with membrane proteins type I topology, and extracellular orientation of their predicted catalytic domain. This indicated that other membrane HAcPs, may also contribute to the acid ecto-phosphatase activity on the *L. donovani* parasite surface.

Finally, the high sequence identity of *Ld*MAcP with the human hPAP (~24% for the region with resolved crystal structure (residues 33-374), allowed the construction of reliable 3D structure models for the *Ld*MAcP (AIF32067) (residues 26–313) and its homologous secreted *Ld*SAcPs (AAC79513) (residues 26-392) [163, 227]. The *in silico* structural analysis of *Ld*MAcP's and *Ld*SAcPs' sequences revealed structural differences suggesting differences in the substrate specificity and functionality of the two enzymes, hypothesis that deserves further investigation.

The predicted localization of *Ld*MAcP on the parasite surface membrane [164] on which it could act as acid ecto-phosphatase, was confirmed both by confocal microscopy and biochemically. The localization of *Ld*MAcP was followed in wild type promastigotes (endogenous form) by indirect immunofluorescence with the anti-*Ld*MAcP mouse mAb or pAb generated in this study or in transgenic promastigotes episomally expressing a recombinant C-terminally tagged *Ld*MAcP-mRFP1 chimera, by direct visualization of the mRFP1 fluorescence. Subsequently, the expression of recombinant and endogenous *Ld*MAcP polypeptides was detected biochemically in membrane fractions from transgenic *L. donovani*-r*Ld*MAcP-mRFP1 and wt *L. donovani* promastigotes. Cumulatively, we conclude that the full length *Ld*MAcP shows typical localization for type I membrane proteins following the secretory pathway. Additionally, we confirmed that *Ld*MAcP is N-glycosylated, a post-translation modification that could regulate its transport to the plasma

membrane and/or its stability. As far as the enzymatic activity of *Ld*MAcP is concerned, we showed that it confers to the acid ecto-phosphatase activity of the *L. donovani* promastigotes and that is tartrate resistant. It would be extremely interesting, to investigate the *Ld*MAcP activity in *L. donovani* amastigotes that reside and multiply within the acidic host cell phagolysosome.

The *Ld*MAcP localization and enzymatic activity were also confirmed upon its transient expression as r*Ld*MAcP-His in mammalian cells. In this case, the recombinant protein was N-glycosylated and successfully targeted by the HeLa secretory system to the cell plasma membrane with an active catalytic domain facing the extracellular milieu.

All the above results raise the intriguing possibility that the presence of the acid ecto-phosphatase activity in intact *L. donovani* promastigotes may be part of the pathogen's mechanism to manipulate a signal recognition system of the host macrophages in order to gain access and survive in its intracellular niche. To investigate this hypothesis we set up an *in vitro* macrophage cell culture infection system to examine whether the *L. donovani*-r*Ld*MAcP-mRFP1 presented an infectivity advantage over a mock transfected *L. donovani* population. Overall, these experiments indicated that overexpression of r*Ld*MAcP-mRFP1 improves the ability of the transgenic parasites to survive within macrophages in culture at least in the first 48 to72 h post-infection, suggesting a possible similar role for the endogenous *Ld*MAcP. This finding supports earlier reports linking the tartrate resistant acid phosphatase activity to *Leishmania* infectivity and virulence [159, 160]. It worth's mentioning that the tartrate sensitive secreted acid phosphatase activity has also been linked to *Leishmania* infectivity [251, 295]. Finally, the presence of *Ld*MAcP epitopes in infected macrophages with *Leishmania* parasites favors further investigation on the possible functional relationship between this ecto-phosphatase and macrophage molecules, which could serve as candidate substrates/partners.

Overall, this study contributes important information in the field of histidine acid phosphatases of the *Leishmania* parasite. The interesting results obtained from the study of *Ld*MAcP, encourage the continuation of research on the possible roles of other members of the very interesting family of acid phosphatases, in order to identify new virulence factors of the parasite and thus, new drug targets to control the disease.

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