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Rapid Communication

Leishmania infantum nicotinamidase is required for late-stage development in its natural sand fly vector, *Phlebotomus perniciosus*

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ABSTRACT

Leishmania infantum nicotinamidase, encoded by the *Lipnc1* gene, converts nicotinamide into nicotinic acid to ensure Nicotinamide–Adenine–Dinucleotide (NAD^+) biosynthesis. We were curious to explore the role of this enzyme during *L. infantum* development in its natural sand fly vector, *Phlebotomus perniciosus* (Diptera, Phlebotominae), using null mutants with a deleted *Lipnc1* gene. The null mutants developed as well as the wild type *L. infantum* at the early time points post their ingestion within the blood meal. In contrast, once the blood meal digestion was completed, the null mutants were unable to develop further and establish late-stage infections. Data highlight the importance of the nicotinamide degradation pathway for *Leishmania* development in sand flies. They indicate that the endogenous nicotinamidase is essential for *Leishmania* development in the sand fly after the blood meal has been digested and the remnants defecated.

Female phlebotomine sand flies are haematophagous dipterans that are able to support *Leishmania* development and transmit this parasite to vertebrate hosts. *Leishmania* are responsible for various forms of disease, ranging from cutaneous to visceral leishmaniasis. The parasite exists in two main morphological forms: non-motile amastigotes that actively divide within mononuclear phagocytes of the mammalian host and motile flagellated promastigotes within the sand fly midgut. Amastigotes, which are ingested during the blood meal, transform into procyclic promastigotes that multiply within the blood meal, surrounded by the peritrophic matrix in the midgut of the sand fly. After completion of the blood meal digestion, the promastigotes can escape through the broken peritrophic matrix (Sádlová and Volf, 2009) and attach to the midgut epithelium. They then undergo a complex series of transformations that leads to the production of mammal-infective metacyclic stages that are located in the anterior part of the gut (reviewed in Bates and Rogers, 2004; Bates, 2007).

During its developmental life cycle, *Leishmania* must adapt to various environments that differ in their available nutritional resources. Interestingly, analysis of the *Leishmania* genome revealed the lack of numerous enzymes involved in the de novo synthesis of several essential metabolites (Berriman et al., 2005), supporting the hypothesis of a correlation between the nutritional requirements of *Leishmania* parasites and the cellular compartments in which they reside (McConville et al., 2007). Although the metabolic interactions of amastigotes within phagolysosomes have been well documented, little is known about the nutritional factors that support *Leishmania* development within the sand fly midgut.

We recently demonstrated that *Leishmania* is auxotroph for the cofactor Nicotinamide Adenine Dinucleotide (NAD^+) (Gazanion et al., 2011), meaning that its biosynthesis relies solely on the presence of external precursors, such as vitamin B3 nicotinamide (NAm) or its acid derivative nicotinic acid (Nac). In *Leishmania*, the conversion of both precursors into NAD^+ involved a shared three-step pathway with a supplementary step for NAm that is first converted into Nac by a nicotinamidase (LiPNC1) (Fig. 1). This enzyme controls the major part of NAD^+ production and, in turn, parasite growth and pathogenesis (Gazanion et al., 2011). The LiPNC1 enzyme thus represents a valuable candidate for rational drug design (Michels and Avilán, 2011). Additionally, previous studies have demonstrated that nicotinamidase activity is essential for several NAD^+-auxotroph pathogens, not only during the infection of mammals (Purser et al., 2003; Kim et al., 2004; Ma et al., 2007) but also for survival and replication in their arthropod vectors such as the spirochaete, *Borrelia burgdorferi*, in the tick midgut (Grimm et al., 2005). In this study, we investigated the importance of *Lipnc1* nicotinamidase activity for the intravectorial development of *Leishmania*. To this aim, we followed the development of

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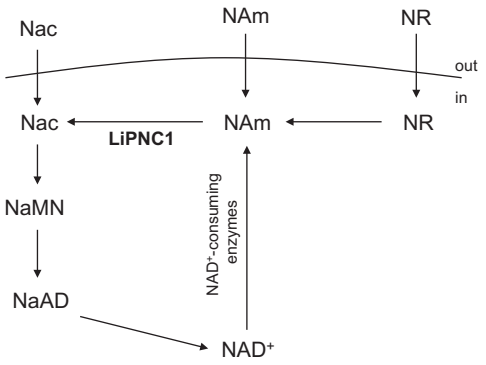

Fig. 1. Nicotinamide–Adenine–Dinucleotide (NAD^+) biosynthesis pathway in *Leishmania infantum* parasites. Targeted-gene replacement was used to delete the *Lipnc1* gene, which renders the parasites dependent on nicotinic acid (Nac) to produce NAD^+ by a set of three enzymatic reactions. NAm: nicotinamide; NR: nicotinamide riboside; NaMN: nicotinic acid mononucleotide; NaAD: nicotinic acid adenine dinucleotide.

Lipnc1−/− *L. infantum* parasites in their natural vector *Phlebotomus perniciosus*. We observed that mutants that are defective for the nicotinamidase gene are unable to grow and survive after blood meal digestion and are therefore unable to complete their intravectorial life cycle.

Four lines of *L. infantum* promastigotes (MHOM/MA/67/ITMAP-263) were used: a wild-type (WT) strain, a *Lipnc1* double-knockout parasite strain (*Lipnc1*−/−) that was generated by targeted gene replacement (ΔLipnc1::NEO/ΔLipnc1::HYG), and two complemented strains that were generated by re-expressing either the native LiPNC1 protein, namely *Lipnc1*−/− + LiPNC1(ΔLipnc1::NEO/ΔLipnc1::HYG[pSPαBLAα-LiPNC1]), or an inactive form, namely *Lipnc1*−/− + LiPNC1C_{161}A. The inactive form was obtained by a substitution of the catalytic cysteine in position 161 with an alanine, which results in the abolition of enzyme activity. All of these mutant lines have been previously characterised (Gazanion et al., 2011). The parasites were maintained on SDM-79 medium (Brun and Schönenberger, 1979) that was supplemented with 10% heat-inactivated FCS (Lonza, France), 100 IU of penicillin/ml, 100 μg of streptomycin/ml and 5 mg/l of hemin. Laboratory colonies of *P. perniciosus* (Spain) were maintained at 26 °C under the conditions described by Volf and Volfová (2011).

For each experiment, approximately 150 female sand flies were fed through a chick-skin membrane with a suspension of 5-day-old promastigotes at a density of 10^6 cells/ml mixed with heat-inactivated rabbit blood. Blood-fed females were separated, kept at 26 °C and allowed to feed on a 50% sucrose solution. On days 2, 6, 8–9 and 13–14 post *L. infantum*-containing blood meal (PBM), the females were dissected and their guts were checked for the presence and localisation of parasites. The infection intensity was estimated in situ using a light microscope by scoring the infections according to defined criteria (Cihaková and Volf, 1997), i.e. weak (<100 parasites/gut), moderate (100–1000 parasites/gut) and heavy (>1000 parasites/gut) infections in the gut lumen. The experiment was repeated twice and the data were analysed using a χ^2 test and Prism software (GraphPad software, version 5).

In additional experiments, *P. perniciosus* females infected with *Lipnc1* double-knockout parasites were divided into three groups with free access either to a 50% sucrose meal or 50% sucrose supplemented with 3 μM of Nac or with 30 μM of NAm. Females were dissected on days 4 and 8–9 PBM.

As the microscopic determination of parasite density is only semi-quantitative, quantitative PCR (qPCR) was used to accurately determine the number of parasites in the sand fly midguts on day 12 PBM (Myskova et al., 2008). Briefly, for each parasite strain, 20 midguts of fed females were placed into 200 μl of lysis tissue buffer and stored at −20 °C until use. The total DNA was isolated by using a High Pure PCR Template Preparation Kit (Roche, Czech Republic) and used as a template for qPCR amplification with the primers described by Mary et al. (2004).

The capacity of *L. infantum* WT and *Lipnc1*-deficient parasites to complete their intravectorial development in *P. perniciosus* is summarised in Fig. 2. On day 2 PBM, during blood meal digestion and before defecation, *Leishmania* developed similarly regardless of the parasite strain (Fig. 2A). Living parasites were observed in the abdominal midguts in contact with partially digested blood that was located inside the endoperitrophic space. After defecation (day 6 PBM), significant differences were observed between the *Lipnc1*−/− and WT strains ($P < 0.001$) as only one of 15 sand flies examined was still weakly infected with *Lipnc1*−/− parasites. In contrast, female sand flies that were infected with the WT strain showed a high rate of infection (73%), of which 67% had high-intensity infections (Fig. 2A). On days 8 and 14 PBM, *Lipnc1*−/− parasites were detected in sand fly midguts in only 33% and 26% of dissected females, respectively, and at very low densities (Fig. 2A), demonstrating the inability of these parasites to grow and to establish mature infections in the midgut after defecation.

The introduction of an episome carrying a WT copy of the *Lipnc1* gene in null mutant parasites led to the restoration of normal parasite development in the sand fly midgut (Fig. 2A), as demonstrated by an infection rate and an intensity of infection similar to those of the control after blood meal defecation ($P = 0.67$ and $P = 0.63$, respectively). From days 8 to 14 PBM, no significant difference was observed between WT and *Lipnc1*−/− + LiPNC1 parasites with respect to their development rate ($P = 0.23$) and parasite density ($P = 0.25$) (Fig. 2A). Both strains migrated to the thoracic midgut and reached the cardia (anterior part of the thoracic midgut near the stomodeal valve), where they accumulated as elongated nectomonads. The colonisation of the stomodeal valve was observed in 23.6% (7/31) of WT-infected females and in 58.3% (7/12) of *Lipnc1*−/− + LiPNC1-infected females. Occasionally, the haptomonads were attached to the stomodeal valve. *Lipnc1* double-knockout parasites carrying an episomal copy of the *Lipnc1* gene in which the cysteine residue in position 161 was substituted with alanine (leading to an inactive nicotinamidase enzyme) were unable to complete the intravectorial cycle, similar to the *Lipnc1*−/− parasites. Therefore, the abortive development observed in sand flies infected with *Lipnc1* null mutants is clearly linked to the absence of nicotinamidase activity. These observations strongly support the notion that active nicotinamidase activity is required to ensure the complete development of *Leishmania* within its vector.

The capacity of *L. infantum* to colonise and to develop within sand flies was confirmed by qPCR analysis performed on midguts of infected females on day 12 PBM. The results revealed no significant difference in parasite loads between the WT and the complemented strains (Fig. 2B), whereas the *Lipnc1*−/− parasite development was significantly less successful (Fig. 2B).

The incapacity of *Lipnc1*-deficient parasites to establish late-stage infections could be due to deficient growth during an early stage of development, producing an insufficient amount of parasites able to colonise sand fly midgut after blood meal defecation. To test this hypothesis, we increased the inoculum of *Lipnc1*−/− + LiPNC1C_{161}A parasites and followed parasite development in the sand fly midgut. A fivefold increase of inoculum (5×10^6 parasites/ml) did not restore the development of parasites in sand flies at days 9 and 13 PBM (Fig. 2C). Thus it seems likely that NAm degradation by the parasitic nicotinamidase is important during blood meal digestion and especially for establishing mature infection after blood meal defecation.

Finally, we tried to restore *Lipnc1*−/− development by adding Nac to sand fly sugar meal. Since NAm is also able to restore normal growth of *Lipnc1*−/− in in vitro-cultured promastigotes

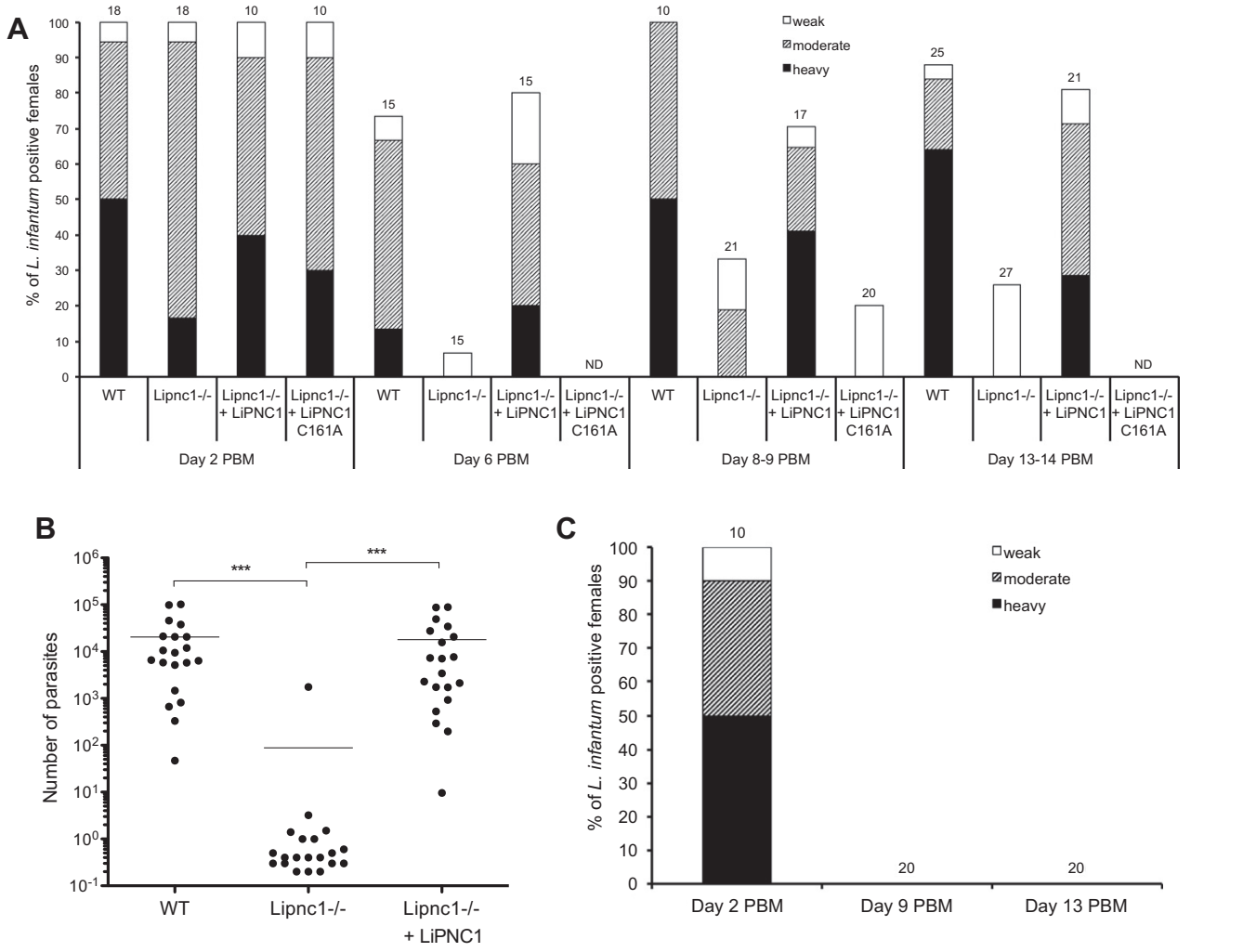

Fig. 2. Development of the *Leishmania infantum* wild type (WT) and *Lipnc1* mutant lines in *Phlebotomus perniciosus*. (A) Profile of *L. infantum*-positive *P. perniciosus* fed on rabbit blood containing either WT or mutant *L. infantum* lines (1×10^6 cells/ml). *Lipnc1*−/−: *Lipnc1* double knockout parasites; *Lipnc1*−/− + LiPNC1: complemented strain expressing a native LiPNC1 protein; *Lipnc1*−/− + LiPNC1C161A: complemented strain expressing an inactive form of LiPNC1. The rates and intensities of infection (weak, moderate, heavy) were determined on days 2, 6, 8–9 and 13–14 post *L. infantum*-containing blood meal (PBM) using light microscopy. The total number of dissected sand flies is indicated above the bars. ND: not determined. (B) Analysis of the parasite loads by quantitative PCR (qPCR) in *P. perniciosus* fed on rabbit blood containing either WT or mutant *L. infantum* lines (day 12 PBM). Twenty midguts were analysed for each line. The results were analysed using the Mann–Whitney test. $^{***}P < 0.001$. (C) Abortive development of the *L. infantum Lipnc1*−/− + LiPNC1C_{161}A strain, even after a blood meal containing a higher number of parasites (5×10^6 cells/ml). The rates and densities of infection were determined on days 2, 9 and 13 PBM using light microscopy.

but at a concentration 10-fold higher than Nac (Gazanion et al., 2011), we also included this point in our experiment. The results obtained with *Lipnc1* double-knockouts revealed that Nac and high levels of NAm supplement added into the sand fly sugar meal significantly ($P < 0.05$) improved *Leishmania* development during the early stage of infection. On day 4 PBM (soon after defecation), females with access to sugar supplemented with NAm or Nac showed high infection rates (92% and 67%, respectively) and high parasite loads (moderate and heavy infection intensities prevailed). In contrast, females with access to non-supplemented sugar had lower infection rates (42%) and low parasite loads (only weak infections occurred). In the late stage infection (days 8–9 PBM), however, the difference between groups was not significant ($P > 0.05$) as the infection rates were 29%, 40% and 49% for the control, NAm and Nac groups, respectively.

In this study, we provide the biological evidence that the LiPNC1 nicotinamidase enzyme that converts NAm into Nac, the first step in the NAD^+ synthesis pathway from a NAm precursor in *Leishmania*, is essential for the completion of the intravectorial development of *L. infantum* in its natural vector *P. perniciosus*. Whereas the WT strain developed well and colonised the stomodeal valve, *Lipnc1*−/− parasites were not able to produce mature infection in the thoracic midgut after defecation.

Currently, the nutritive view of the *Leishmania*/sand fly relationship remains largely unexplored and little is known about the nutritional factors that support *Leishmania* development within the sand fly midgut. It is predicted that the main extracellular source of nutrients available during the early stage of *Leishmania* development is provided by the blood meal, whereas the sugar meal furnishes the energy sources necessary for *Leishmania* growth in the late stages (Gontijo et al., 1996; Schlein and Jacobson, 1996; Jacobson and Schlein, 2001). In this view, glucose uptake is important, but not essential, for *Leishmania mexicana* infection of the vector host (Burchmore et al., 2003), and the genetic deletion of three glucose-transporter isoforms leads to a delayed growth of *Leishmania* in *Lutzomyia longipalpis*.

The cofactor NAD^+ is a central molecule that participates in regulation of the intracellular redox state and many biological processes such as longevity, DNA repair and transcriptional regulation (Lin et al., 2003). We previously demonstrated that *Lipnc1* gene

deletion induces large fluctuations in the NAD^+ intracellular pool that are deleterious for parasite growth and pathogenesis (Gazanion et al., 2011). In this study, we observed the unexpected growth arrest of *Lipnc1*−/− parasites in sand flies, indicating that NAm might be an important nutritional factor for *L. infantum*. These results strongly support the hypothesis that NAD^+ metabolism controls host–*Leishmania* interactions and that NAm is the primary precursor available to ensure NAD^+ biosynthesis during *Leishmania* development in both mammalian and arthropod hosts.

We demonstrated that supplementation with NAm and Nac in the sand fly sugar meal positively affected the development of *Lipnc1*−/− parasites. In early stage infection, when females took the sugar meal after defecation of blood meal remnants, the effect of NAm and Nac was pronounced. In late stage infections, when females took the sugar meal irregularly, the effect of the supplement was diminished as the NAm and Nac quantity ingested was probably not high enough to salvage the parasites. In the wild, it is known that sand flies feed on aphid honeydew, which is an important source of carbohydrate for sand flies (Moore et al., 1987) and a complex mixture of nutrients including B-vitamins (Way, 1963). However, the presence and amount of nicotinic acid and nicotinamide in aphids and in honeydew have not been defined.

Within its mammalian host, the primary NAD^+ precursor available for *Leishmania* is NAm (Gazanion et al., 2011). In humans, NAm is absorbed from the diet and circulates in the blood plasma at concentrations ranging from 0.3 μM to 2.3 mM, depending on the ingested dose (Bernier et al., 1998; Catz et al., 2005), whereas the Nac concentration is much lower (Catz et al., 2005). Differences in the blood concentrations of NAm and Nac in different mammalian hosts have been reported. Mice and rabbits have higher plasma levels of both precursors than dogs and humans (Catz et al., 2005). In the sand fly, *Leishmania* can gain access to NAD^+ precursors that are present in the blood meal only during their early developmental stage, after which the parasite must obtain these precursors from the environment present in the gut of the sand fly. Our results strongly suggest that the amount of Nac available in the blood meal is sufficient to support *Lipnc1*−/− growth, whilst subsequently NAm is the sole NAD^+ precursor present in the sand fly midgut. Whether the blood meal source and the available amount of NAm or Nac may substantially influence the outcome of the intravectorial development of *Leishmania* needs to be investigated.

In conclusion, our findings clearly highlight a new metabolic aspect of *Leishmania* promastigote development within the complex and dynamic milieu of the midgut by revealing the crucial role of the LiPNC1 nicotinamidase enzyme, which catalyses the first step of NAm assimilation into NAD^+. The apparent requirement of both promastigote and amastigote forms for a functional nicotinamidase to ensure a complete development cycle reinforces the therapeutic value of LiPNC1 and will help to delineate new transmission-blocking strategies.

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