The role of unique *Leishmania* respiratory enzymes in mice infections

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Mitochondria are multifaceted organelles with a crucial role in energy production through the oxidative phosphorylation pathway. NADH and FADH2 electrons enter the respiratory chain (RC) and are transferred to oxygen in a process coupled to the translocation of protons into the mitochondrial intermembrane space that fuels ATP production. As in most eukaryotic cells, *Leishmania* RC is made up of complexes I through IV. Aside from these enzymes, *Leishmania* contains at least three extra and unique enzymes that cause RC bifurcations: i) type II NADH dehydrogenase (NDH2), that bypasses complex I and oxidizes NADH without coupled proton pump; ii) fumarate reductase (FRD) that, together with complex II, allows electrons from NADH to enter the RC; and iii) cytochrome c peroxidase (CCP) that bypasses complex IV and transfers electrons to H2O2. The relative contribution of these enzymes for in vivo parasite survival is yet unclear.

Expression of the different enzymes was evaluated in both *Leishmania infantum* (a visceralizing species, Li) and *L. majo*r (a cutaneous species, Lm) through western blot analysis and oxygen consumption assays with intact parasites. Subcellular localization was addressed by immunofluorescence studies and western blot analysis. Gene deletion was generated by CRISPR Cas9 techniques and mutants' ability to prosper in animal models of infection was evaluated in mice.

NDH2 protein is expressed in both the promastigote and amastigote stages of *L. infantum*, while complex I activity was not detected. Overexpression of *Li*NDH2 was found to increase basal oxygen consumption of intact parasites, confirming the enzyme as a respiratory chain component. Moreover, we found that *Li*NDH2 is essential in *L. infantum*, including in the disease-causing stage. In fact, i) deletion of both *ndh2* alleles is only possible upon complementation with an episomal copy of the gene, ii) knockout promastigotes and amastigotes do not lose the *Lindh2* episome after multiple passages in culture in absence of drug pressure, in contrast to a control episome that is lost after few cycles of parasite replication, and iii) single knockout ndh2$^{+/-}$ parasites are less virulent than the wild type in mice. Furthermore, NDH2 is also essential in *L. major* a species expressing active complex I. FRD is expressed in promastigotes *L. major* displaying higher levels than *L. infantum*. Attempts to delete the *Lifrd* gene reveal its non-essential character even for in vivo infections. Mutant Lifrd$^{-/-}$ parasites are, however, less virulent than wild type in mice. CCP is highly upregulated in amastigotes of both *L. infantum* and *L. major* although it is not essential for either parasite survival. In fact, both Li and Lm ccp knockouts are not only able to infect mice but give rise to higher parasite burden in the liver (for Li) and increased footpad swelling (for Lm, Pal et al., 2010), when compared to wild type infections, suggesting that CCP controls parasite proliferation. Based on these results, our hypothesis is that the enzymatic activity of CCP is central for Leishmania to become persistent. Our working model predicts that, by acting as a complex IV competitor, CCP can lower the efficiency of the oxidative phosphorylation, and, consequently, reduce parasite numbers. This respiratory chain modulation promotes long-term parasite persistence and, hence, adaptation to host defensive strategies both immune and drug-mediated.

In short, NDH2 is essential for parasite survival *in vivo*, regardless of the presence of a functional complex I. FRD and CCP are non-essential genes that decrease or increase parasite virulence, respectively, when deleted. The current focus is on understanding the involvement of these proteins in persistence of both species.

Keywords: *Leishmania*; mitochondria; respiratory chain enzymes; infection

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