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IMP Dehydrogenase Deficiency in *Leishmania donovani* Causes a Restrictive Growth Phenotype in Promastigotes but is not Essential for Infection in Mice

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Abstract

Leishmania cannot synthesize purines *de novo* and therefore must scavenge purines from its host for survival and growth. Biochemical and genomic analyses have indicated that *Leishmania* species express three potential routes for the synthesis of guanylate nucleotides: 1) a two-step pathway that converts IMP to GMP; 2) a three-step pathway that starts with the deamination of guanine to xanthine, followed by phosphoribosylation to XMP and then conversion to GMP; or 3) direct guanine phosphoribosylation by HGPRT. To determine the role of the first of these pathways to guanylate nucleotide synthesis, an *L. donovani* line deficient in IMP dehydrogenase (IMPDH), the first step in the IMP to GMP pathway, was constructed by targeted gene replacement. The Δ*impdh* lesion triggered a highly restrictive growth phenotype in promastigotes in culture but did not impact parasitemias in mice. The dispensability of IMPDH *in vivo* is the first definitive demonstration that intracellular *L. donovani* amastigotes have access to a sufficient pool of guanine, xanthine, or guanylate precursors from the host.

Keywords

Leishmania donovani; gene targeting; purine salvage; inosine-5′-monophosphate dehydrogenase; guanylate nucleotides

Leishmania donovani is the etiological agent of visceral leishmaniasis, a disease that is invariably fatal if untreated. *Leishmania* species are digenetic, existing as the extracellular promastigote within the phlebotomine sandfly vector and as the intracellular amastigote in the phagolysosome of macrophages and other cells of the reticuloendothelial system. Drug regimens for visceral leishmaniasis are far from ideal because of toxicity, cost, intrusive routes of and prolonged administrations, and resistance. Thus, there is an acute need for new drugs and new drug targets. One pathway that has triggered considerable therapeutic interest for the treatment of parasitic diseases is that for the synthesis of purine nucleotides. While

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mammals synthesize purine nucleotides from amino acids and one-carbon compounds, *Leishmania*, as well as all protozoan parasites studied to date, are effectively auxotrophic for purines and consequently must salvage purines from their hosts [1–3]. Thus, supplementation of the growth medium with purines is an absolute nutritional requirement for *Leishmania* promastigotes cultured in defined medium.

The purine salvage pathway of *Leishmania spp.* is particularly complex, consisting of four enzymes that are capable of converting preformed host purines into the parasite nucleotide pool: 1) hypoxanthine-guanine phosphoribosyltransferase (HGPRT); 2) xanthine phosphoribosyltransferase (XPRT); 3) adenine phosphoribosyltransferase; and 4) adenosine kinase [3,4]. Genetic studies have provided powerful evidence that none of the four enzymes alone is essential and that both *L. donovani* promastigotes and amastigotes funnel all host purines into substrates of either HGPRT or XPRT [5–7]. Thus, HGPRT and XPRT are vital salvage enzymes, whereas adenine phosphoribosyltransferase and adenosine kinase are functionally superfluous. These same studies have implicated certain purine nucleotide interconversion enzymes as potential “Achilles heels” for purine salvage by the parasite and imply that they could be rational therapeutic targets.

Inosine-5′-monophosphate dehydrogenase (IMPDH) (AAA29253.1) is one such nucleotide interconversion enzyme and catalyzes the irreversible conversion of IMP to XMP (Fig. 1). XMP is then converted by GMP synthetase (GMPS) to GMP, which serves as a precursor for all other guanylate nucleotides in the cell (Fig. 1). Thus, IMPDH plays a critical role in steering salvaged purines toward guanylate nucleotide synthesis and away from adenylate nucleotide synthesis, although GMP can also be reductively deaminated through GMP reductase to IMP and serve as a source of adenylate nucleotides. IMPDH is essential for mammalian cells, as well as most organisms, and has been highly touted as an antiviral, antibacterial, anticancer, and antiparasitic drug target [8]. Because *L. donovani* promastigotes can salvage hypoxanthine or adenine as the sole purine nutrient, a process that requires IMPDH, IMPDH is clearly operative in the insect vector stage of the parasite. Furthermore, mycophenolic acid and ribavarin, two potent and specific inhibitors of IMPDH enzymes [8,9], are also robust inhibitors of *L. donovani* promastigote growth when either hypoxanthine or adenine is provided as the sole purine source [10], implying that IMPDH is essential to the promastigote under these specific growth conditions. Finally, IMPDH, as well as GMP reductase, are both inhibited by metabolites of the well-characterized anti-leishmanial pyrazolopyrimidines, allopurinol, allopurinol riboside, 4-thiopurinol, and formycin B. [11,12]

The IMPDH from *L. donovani* has been characterized biochemically and is found in the glycosome [13], a peroxisome-like organelle unique to parasites of the Kinetoplastida order [14]. This organellar sequestration of IMPDH is mediated by a COOH-terminal tripeptide, Ala-Lys-Met (AKM) [13]. Biochemical and bioinformatic analyses of available *Leishmania* genomes (http://tritrypdb.org/tritrypdb), however, have revealed a second prospective pathway for guanylate nucleotide synthesis in *Leishmania*, one in which guanine is first converted to xanthine by guanine deaminase (GDA), xanthine is then phosphoribosylated to XMP via XPRT, and XMP is converted to GMP by GMPS [1–3] (see Fig. 1). This alternative route to guanylate nucleotide production is likely to be operational in *L. donovani* promastigotes since Δ*hgprt* promastigotes can grow in exogenous guanine and guanosine [6], and XPRT, the only remaining functionally active purine salvage enzyme in Δ*hgprt* parasites, recognizes guanine very inefficiently [13]. A third possible path for GMP synthesis in *Leishmania*, direct phosphoribosylation of guanine by HGPRT, does not seem robust in *L. donovani* since Δ*xprt* promastigotes cannot grow on guanine as the sole source of purine in the culture medium [6]. The functionality of the two routes for GMP synthesis that have been validated in promastigotes, the IMPDH-GMPS and the GDA-XPRT-GMPS

pathways, have not, however, been evaluated in amastigotes, the infectious and mammalian form of the parasite that resides within the phagolysosome of infected macrophages. Purine pool levels within the phagolysosome to which amastigotes have access are not known.

To test whether IMPDH is indispensable to *Leishmania* amastigotes, we generated Δ*impdh* null mutants in a strain of *L. donovani* that retains the ability to infect mice [7]. Two independent Δ*impdh* clones were derived from two separate *IMPDH/impdh* heterozygotes, *IMPDH/impdh*-1 and *IMPDH/impdh*-2, and were designated Δ*impdh*-1 and Δ*impdh*-2, respectively. The Δ*impdh*-1 and Δ*impdh*-2 clones were also complemented by integrating either a wild type *IMPDH*, or a mutant *impdh* ΔAKM copy in which the AKM glycosomal targeting signal had been removed, into the ribosomal RNA locus. These "add-back" lines were designated Δ*impdh*-1[pRP-*IMPDH*] or Δ*impdh*-2[pRP-*IMPDH*] and Δ*impdh*-2[pRP-*impdh*ΔAKM] or Δ*impdh*-2[pRP-*impdh*ΔAKM], respectively.

The Δ*impdh* lesions in the knockout lines were confirmed by Southern blot analysis of genomic DNA prepared from wild type, *IMPDH/impdh*-1, Δ*impdh*-1, *IMPDH/impdh*-2 and Δ*impdh*-2 hybridized to the *IMPDH* coding sequence (Fig. 2A). Equal loading of each lane in the Southern blot was ascertained by ethidium bromide staining (data not shown). The gene replacements at the *IMPDH* locus in the null mutants were corroborated by Western blot analysis of lysates from wild type, Δ*impdh*-1, Δ*impdh*-2 and "add-back" promastigotes (Fig. 2B) using polyclonal antisera specific for IMPDH [13]. These immunoblots confirmed the presence of IMPDH protein in wild type and "add-back" parasites and the absence of IMPDH expression in the null mutants (Fig. 2B). This *IMPDH* expression pattern was also observed when the promastigotes were converted to axenic amastigotes (data not shown). The glycosomal and cytosolic localizations, respectively, of IMPDH in the Δ*impdh*-2[pRP-*IMPDH*] and Δ*impdh*-2[pRP-*impdh*ΔAKM] "add-backs," were verified by indirect immunofluorescence (Fig. S1). Because IMPDH is a key branchpoint between synthesis of adenylate and guanylate nucleotides and pharmacologic ablation of IMPDH activity was lethal to *L. donovani* promastigotes when adenine or hypoxanthine was the sole purine source [10], the growth phenotypes conferred by the genetic lesions in the Δ*impdh* lines were tested under defined growth conditions. Whereas supplementation of the medium with xanthine, xanthosine, guanine, or guanosine was permissive for Δ*impdh*-1 and Δ*impdh*-2 promastigote proliferation, neither of the null mutant clones could grow in medium in which adenine, adenosine, hypoxanthine, or inosine was the singular purine source (Fig. 2C, Table S1). Conversely, wild type, Δ*impdh*-2[pRP-*IMPDH*] and Δ*impdh*-2[pRP-*impdh*ΔAKM] promastigotes could replicate in all eight purines tested, although growth was generally less robust in guanine or guanosine (Fig. 2C, Table S1). Growth of Δ*impdh*-1[pRP-*IMPDH*] and Δ*impdh*-1[pRP-*impdh*ΔAKM] "add-back" lines was also permissive in all purines tested (data not shown). Thus, the restrictive growth phenotype of Δ*impdh* promastigotes could be ascribed to the genetic lesion and not to some ancillary epigenetic change in the null parasites. Moreover, the ability of the Δ*impdh*-1[pRP-*impdh*ΔAKM] and Δ*impdh*-2[pRP-*impdh*ΔAKM] promastigotes to grow in adenine, adenosine, hypoxanthine, and inosine implied that glycosomal localization of IMPDH is not essential for IMPDH function in the insect vector stage of the parasite. It should be noted, however, that IMPDH expression levels are higher in the Δ*impdh*-1[pRP-*impdh*ΔAKM] and Δ*impdh*-2[pRP-*impdh*ΔAKM] "add-backs" than in wild type parasites (Fig. 2B), so a significant but incomplete functional deficit due to cytosolic mislocalization of the mutant impdh could have been masked by over-expression. The null genotype and restrictive growth phenotype of cultured Δ*impdh* parasites authenticates a single *IMPDH* encoding locus in *L. donovani*. In contrast, mammalian cells have two similar but distinct *IMPDH* genes [15].

To test whether the loss of *IMPDH* impacted the ability of *L. donovani* to establish infection in Balb/c mice, a well-established rodent model for visceral leishmaniasis in which parasite

can replicate but do not cause disease [16], groups of five mice were inoculated with either wild type, Δ*impdh*-1, Δ*impdh*-2, Δ*impdh*-2[pRP-*IMPDH*], or Δ*impdh*-2[pRP-*impdh*ΔAKM] stationary phase promastigotes via tail vein injection and parasite loads in livers and spleens determined after four weeks (Fig. 2D). Parasite burdens in livers and spleens of mice infected with each of the five lines were comparable indicating that IMPDH function is not essential to establish infection in mice. These data establish that Δ*impdh L. donovani* amastigotes can satisfy their guanylate nucleotide requirement by salvaging host purines from the phagolysosome through an IMPDH-independent route. This route presumably involves direct access to the guanylate nucleotide pool of the phagolysosome, a mechanism that would entail guanine deamination to xanthine, xanthine phosphoribosylation to XMP, and XMP conversion to GMP, or alternatively, through HGPRT-catalyzed phosphoribosylation of guanine to GMP (see Fig. 1). Whether the IMPDH-dependent route is utilized by amastigotes at all *in vivo* awaits additional genetic analysis. We conjecture at this point that the GDA-XPRT-GMPS pathway predominates over HGPRT as the major route of guanine salvage for at least three reasons: 1) Δ*xprt* parasites generated in our laboratory are unable to grow in guanine, suggesting that all guanine is deaminated to xanthine, an unusable purine for a Δ*xprt* mutant [6], since xanthine is not a substrate for HGPRT [17]; 2) we have shown by thin layer chromatography that intact *L. donovani* promastigotes fully convert 100% of exogenous [^{14}C]guanosine (57 μM) to intracellular xanthine in <5 minutes; and 3) repeated attempts to generate a Δ*gmps* cell line by targeted gene replacement have failed, even when supplied with excess amounts of guanine in the selection medium. Taken together, these investigations insinuate that GMPS, but not IMPDH, is a valid therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GDA	guanine deaminase
GMPS	GMP synthetase
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
IMPDH	IMP dehydrogenase
XPRT	xanthine phosphoribosyltransferase

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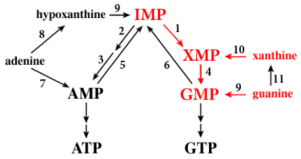

Fig. 1.

Diagram of the purine salvage pathway of *L. donovani*. Relevant enzymes depicted include: 1, IMPDH; 2, adenylosuccinate synthetase; 3, adenylosuccinate lyase; 4, GMPS; 5, AMP deaminase; 6, GMP reductase; 7, APRT; 8, adenine deaminase; 9, HGPRT; 10, XPRT; 11, GDA. The pathways central to guanylate nucleotide synthesis are depicted in color.

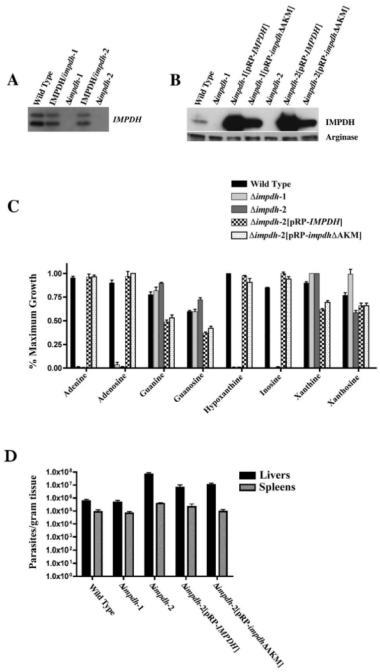

Fig. 2.

Molecular characterization of Δ*impdh* parasites and test of *IMPDH* function in infectivity. Δ*impdh* parasites were created from previously reported *IMPDH/impdh* strains [18] by targeted gene replacement in the *Ld*Bob *L. donovani* strain that retains its ability to infect macrophages and mice after prolonged culture [7,19]. Targeting constructs were those described previously [18]. To confirm the genetic lesions, Southern and western analyses were performed. (A) Total genomic DNA (~5 μg) from wild type *L. donovani* (lane 1), *IMPDH/impdh*-1 (lane 2), Δ*impdh*-1 (lane 3), *IMPDH/impdh*-2 (lane 4) and Δ*impdh*-2 (lane 5) was digested with XholI, fractionated on a 1% agarose gel, and blotted onto a nylon membrane. The blot was hybridized under high stringency conditions using the *IMPDH* coding sequence as the probe. (B) Lysates of exponentially growing wild type, Δ*impdh*-1, Δ*impdh*-2, Δ*impdh*-2[pRP-*IMPDH*] and Δ*impdh*-2[pRP-*impdh*ΔAKM] promastigotes were analyzed by immunoblotting using anti-IMPDH monospecific polyclonal antisera [13]. The amount of protein loaded onto each lane was normalized by blotting with antisera to the *L. donovani* arginase enzyme. (C) The ability of wild type, Δ*impdh*-1, Δ*impdh*-2, Δ*impdh*-2[pRP-*IMPDH*] and Δ*impdh*-2[pRP-*impdh*ΔAKM] promastigotes to proliferate in eight different purine sources was evaluated. Exponentially growing parasites were seeded at 5×10^4 cells/mL in DME-L medium supplemented with 5% dialyzed fetal bovine serum and one of the indicated purines at a concentration of 100 μM. Cell viability was then assessed using Alamar Blue, a redox indicator that yields a colorimetric change upon its reduction triggered by metabolic activity [20]. Growth for each cell line in each purine was calculated as the mean of three replicates. Data for Δ*impdh*-1[pRP-*IMPDH*] and *Δimpdh*-1[pRP-*impdh*ΔAKM] promastigotes were essentially identical to those obtained with their Δimpdh-2 counterparts and are not depicted. (D) Five groups of five Balb/c mice were infected with either 5×10^6 wild type, Δ*impdh*-1, Δ*impdh*-2, Δ*impdh*-2[pRP-*IMPDH*] or Δ*impdh*-2[pRP-*impdh*ΔAKM] stationary phase promastigotes. The mice were sacrificed 4 weeks post-infection and the parasite loads in liver and spleens were quantified using limiting dilution. The limiting dilution medium for all cell lines was modified DME-L [6] containing 5% FBS and 100 μM xanthine as the purine source. Data presented are the average results and standard error from five animals.