Published in final edited form as:

Mol Biochem Parasitol. 2016 August ; 208(2): 74–83. doi:10.1016/j.molbiopara.2016.06.008.

GMP reductase and genetic uncoupling of adenylate and guanylate metabolism in *Leishmania donovani* parasites

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Abstract

Purine acquisition is an essential nutritional process for *Leishmania*. Although purine salvage into adenylate nucleotides has been investigated in detail, little attention has been focused on the guanylate branch of the purine pathway. To characterize guanylate nucleotide metabolism in *Leishmania* and create a cell culture model in which the pathways for adenylate and guanylate nucleotide synthesis can be genetically uncoupled for functional studies in intact cells, we created and characterized null mutants of *L. donovani* that were deficient in either GMP reductase alone (*gmpr*) or in both GMP reductase and its paralog IMP dehydrogenase (*gmpr/ impdh*). Whereas wild type parasites were capable of utilizing virtually any purine nucleobase/nucleoside, the *gmpr* and *gmpr/ impdh* null lines exhibited highly restricted growth phenotypes. The *gmpr* single mutant could not grow in xanthine, guanine, or their corresponding nucleosides, while no purine on its own could support the growth of *gmpr/ impdh* cells. Permissive growth conditions for the *gmpr/ impdh* necessitated both xanthine, guanine, or the corresponding nucleosides, and additionally, a second purine that could serve as a source for adenylate nucleotide synthesis. Interestingly, GMPR, like its paralog IMPDH, is compartmentalized to the leishmanial glycosome, a process mediated by its COOH-terminal peroxisomal targeting signal. The restricted growth phenotypes displayed by the *L. donovani gmpr* and *gmpr/ impdh* null mutants confirms the importance of GMPR in the purine interconversion processes of this parasite.

Graphical abstract

The restricted growth phenotypes displayed by the *Leishmania donovani gmpr* and *gmpr/ impdh* null mutants confirms the importance of GMPR in the purine interconversion processes of this parasite.

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Keywords

Leishmania; purine; salvage; guanylate; adenylate; uncoupling

1. Introduction

Leishmania donovani, the causative agent of visceral leishmaniasis, is a digenetic protozoan parasite that exists as a flagellated extracellular promastigote within sandflies of the *Phlebotomus* and *Lutzomyia* genera and as immotile intracellular amastigotes within macrophages and other reticuloendothelial cells of infected mammalian hosts. Visceral leishmaniasis, if untreated, is a terminal disease. Unfortunately, the current assortment of anti-leishmanial drugs is far from ideal due to toxicity, invasive routes of administration, and emergent resistance [1–3]. Thus, the need for new drugs and for the identification of new chemotherapeutic targets is acute. One pathway that presents an attractive drug target is that for purine salvage because, unlike mammals, all protozoan parasites lack the enzymatic machinery for purine biosynthesis *de novo* and, therefore, the salvage of host purines is an indispensable nutritional requirement [4,5]. Accordingly, each genus of protozoan parasite has evolved a unique complement of transporters and enzymes that enables it to acquire purines from their hosts.

The purine salvage pathway of *Leishmania* is perhaps the most complex and convoluted among all the protozoan parasites [6–8]. As a consequence, virtually any purine nucleobase or nucleoside can serve as the sole source of purine to support growth of wild type parasites [4–7]. In addition to a multiplicity of purine interconversion enzymes [4–7,9], *Leishmania* expresses four enzymes capable of salvaging preformed purines to the nucleotide level: hypoxanthine-guanine phosphoribosyltransferase (HGPRT), xanthine phosphoribosyltransferase (XPRT), adenine phosphoribosyltransferase, and adenosine kinase [3,5,10,11]. A systematic genetic analysis of the functional roles of the four salvage enzymes in *L. donovani* has confirmed that none of these enzymes, by itself, is necessary for adenylate and guanylate nucleotide production [12,13]. However, a conditionally lethal *hgprt* *xprt* double knockout in *L. donovani* exhibited a highly restrictive growth phenotype in which promastigotes could not propagate on any purine in the absence of pharmacologic intervention by 2'-deoxycofomycin (dCF), which served to prevent deamination of 6-aminopurine sources. This finding demonstrated that either HGPRT or

XPRT alone is sufficient and necessary for parasite survival and growth [12–15]. The *hgprt⁻ xpirt* strain was also extremely compromised in its capacity to infect mice [14,16].

The strikingly aberrant growth and infectivity phenotype of the *hgprt⁻ xpirt* null mutant implied that the six nucleotide interconversion enzymes that function to balance adenylate and guanylate nucleotide pools could serve as bottlenecks for interconversion of salvaged purines into the intracellular nucleotide pools. These six enzymes include adenylosuccinate synthetase (ADSS) and adenylosuccinate lyase (ASL) that convert IMP to AMP, IMP dehydrogenase (IMPDH) and GMP synthetase that convert IMP to GMP, and GMP reductase (GMPR) and AMP deaminase that respectively back convert GMP and AMP to IMP (Fig. 1) [5,7,17–21]. The importance of the nucleotide interconversion pathways in growth and infectivity was validated by the isolation and characterization of *adss* and *asl* and *impdh* null mutants [22,23]. The *adss* and *asl* parasites exhibited growth phenotypes essentially indistinguishable from that of the previously characterized *hgprt⁻ xpirt* double knockout, whereas the *impdh* cells could utilize guanine, guanosine, xanthine or xanthosine, all guanylate precursors, to fulfill their purine requirements [14,22,23]. All three null mutants exhibited reduced infectivity phenotypes in macrophages; however, only *asl* parasites were severely incapacitated in their ability to establish a visceral infection in the mouse model, whereas parasite burdens in mice inoculated with *adss* or *impdh* cells were robust [22,23].

GMPR and IMPDH from *L. donovani* are paralogous proteins that possess a cystathionine- β -synthase (CBS) domain that is involved in nucleotide sensing and enzyme regulation [24], as well as a COOH-terminal tri-peptide peroxisomal targeting signal-1 (PTS1) that directs these enzymes to the glycosome [21,23,25,26], a peroxisomal-like microbody found in *Leishmania* and related trypanosomatids [27–30]. Recombinant *L. donovani* GMPR and IMPDH [21,24], as well as their recombinant counterparts from the related trypanosomatid *Trypanosoma brucei* [31,32], have been expressed, purified, characterized, and shown to exhibit unique kinetic, as well as structural, features distinct from their mammalian counterparts. Because the growth and infectivity profiles of the *impdh* parasites indicated that these knockout lines utilized GMPR to back-convert guanylate nucleotides into IMP that could be used to produce adenylate nucleotides [23], we used targeted gene replacement strategies to introduce a *gmp^r* lesion into wild type *L. donovani* in an effort to further validate the utility of the GMPR protein as a functional component of the nucleotide interconversion pathway. The *gmp^r* knockout was subsequently used as a background strain to create an *L. donovani gmp^r impdh* double null mutant in which the adenylate and guanylate branches of purine metabolism were genetically uncoupled. Unlike wild type *L. donovani*, the *gmp^r* line failed to grow on xanthine, guanine, or guanosine as a purine source, while the *gmp^r impdh* double mutant could not grow in any purine on its own. Permissive conditions for growth of the *gmp^r impdh* double knockout required at least two purines, one providing a source of guanylate nucleotides and one a source of adenylate nucleotides. These findings confirm the general model for purine interconversion and metabolism in *L. donovani* and validate the functional utility of GMPR and IMPDH, which may serve as checkpoints to balance adenylate and guanylate nucleotide production in these parasites [24].

2. Materials and methods

2.1. Chemicals and Reagents

Purine bases and nucleosides were purchased from Sigma–Aldrich® (St. Louis, MO), or Fisher Scientific (Pittsburgh, PA), and restriction enzymes were bought from New England Biolabs (Beverly, MA). The TOPO® TA Cloning® kit and pCR®2.1-TOPO® vector were procured from Thermo Fisher Scientific (Waltham, MA). IMPDH and ODC antisera were produced in guinea pigs or rabbits, respectively, as previously reported [21,33], and the mouse monoclonal anti- α -tubulin (DM1A) antibody was obtained from EMD Millipore (Billerica, MA). Goat anti-rabbit HRP-conjugated and goat anti-mouse HRP-conjugated secondary antibodies were purchased from Thermo Fisher Scientific (Waltham, MA), and the goat anti-guinea pig HRP-conjugated antibody was acquired from Santa Cruz Biotechnology (Dallas, TX). The pX63-PHLEO, pX63-PAC, pX63-NEO, pX63-HYG, and pXG-BSD leishmanial expression vectors harboring the phleomycin/bleomycin binding protein (PHLEO), puromycin *N*-acetyltransferase (PAC), neomycin resistance (NEO), hygromycin phosphotransferase (HYG), and blasticidin deaminase (BSD) markers [34–36], respectively, were constructed and supplied by Dr. Stephen M. Beverley (Washington University, St. Louis, MO), and the pQE-80 vector was acquired from Qiagen (Valencia, CA). The goat anti-rabbit Oregon Green-, goat anti-guinea pig Rhodamine Red conjugated secondary fluorescent antibodies, and SYBR® Green were obtained from Thermo Fisher Scientific. All other chemicals and reagents were of the highest quality commercially accessible.

2.2. Axenic Parasite cell culture

The LdBob strain [37] adapted from the 1S2D clone of wild type *L. donovani* [38,39] was originally obtained from Dr. Beverley. LdBob promastigotes were cultured at 26 °C in modified Dulbecco's Modified Eagle Medium-*Leishmania* (DME-L) [40] medium [12] that was supplemented with 5% FBS. Plating methods and single cell cloning protocols for *L. donovani* promastigotes have been described previously [40,41]. Wild type and *gmpr* parasites were routinely cultivated in 100 μ M hypoxanthine as the purine nutrient, and the *gmpr*[p*GMPR*] and *gmpr*[p*gmpr* SKL] cell lines were propagated in 100 μ M guanine. The *impdh* knockout parasites were maintained in 100 μ M xanthine, while the *gmpr*/*impdh* double knockout strain was grown in 100 μ M hypoxanthine and 100 μ M xanthine. Axenic amastigotes were propagated at 37 °C as detailed [12,37,38] in 100 μ M of the same growth-permissive purines in which the corresponding promastigotes were grown. All cell lines were routinely cycled between promastigotes and axenic amastigotes [37] with their respective purine additions.

2.3. GMPR and IMPDH cloning and sequencing

The *Leishmania infantum* GMPR (LinJ.17.0870) was identified in the GeneDB database (<http://www.genedb.org>) by BLAST searches using the human GMPR1 protein sequence (NP_006868.3) [42,43]. The *Leishmania donovani* GMPR ORF was amplified by PCR from *L. donovani* 1S2D genomic DNA with the primers designed against the *L. infantum* coding sequence (Table S1), cloned into TOPO® TA and sequenced. IMPDH was cloned and sequenced as described [17,18]

2.4. DNA manipulations and Southern blotting

Genomic DNA from $5 \times 10^7 - 1 \times 10^8$ parasites was isolated using the Qiagen DNeasy Blood and Tissue Kit (Waltham, MA), digested with appropriate restriction enzymes, fractionated on 0.8% agarose gels, transferred to nylon membranes, and Southern blotting analyses performed using conventional protocols [9]. Hybridization probes were the full length coding sequences for *GMPR* and *IMPDH*, both of which were generated by PCR, separated on 0.8% agarose gels, and gel-purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI). The template plasmids from which the hybridization probes were amplified harbored the full-length genes that had been previously cloned into the TOPO® TA Cloning® Kit with pCR™2.1-TOPO® vector (Thermo Fisher Scientific).

2.5. Gene targeting and complementation constructs

The 5' and 3' UTRs of *GMPR* were PCR-amplified from *L. donovani* genomic DNA using primers designed against the flanking regions of the *L. infantum* *GMPR* sequence that had been obtained from GeneDB (<http://www.genedb.org>). The PCR-generated flanking regions of *L. donovani* *GMPR*, containing either HindIII/SalI (5' UTR) or SmaI/BglII (3' UTR) restriction sites (Table S1) were ligated into the TOPO® TA vector and sequenced. The pX63-HYG- *gmpr* targeting plasmid used to create the *gmpr* cell line was generated by excising the 5' or 3' UTR from the TOPO® TA vector with HindIII/SalI or SmaI/BglII, respectively, followed by sequential insertion of the 5' or 3' UTR into the corresponding restriction sites within the pX63-HYG vector [35] that had also been digested with HindIII/SalI, followed by digestion with SmaI/BglII. Due to the presence of a SmaI restriction site in the pX63-PAC vector [34], a different strategy was used to generate the pX63-PAC- *gmpr* replacement plasmid. Briefly, the pX63-HYG- *gmpr* plasmid was digested with HindIII/BamHI, while the pX63-PAC vector was digested with HindIII/BglII, and the compatible DNA fragments containing either the 5' and 3' *GMPR* UTRs and the plasmid backbone or the PAC drug resistance cassette were ligated together to form the pX63-PAC- *gmpr* replacement plasmid. Complementation constructs that facilitated the expression of either *GMPR* or mis-localized *gmpr* SKL in parasites containing a *gmpr* null mutation were created using the pXG-BSD leishmanial expression vector [36]. The *GMPR* ORF was amplified via PCR from genomic DNA with sense and antisense primers that added a 5' SmaI or 3' BamHI restriction site, respectively (Table S1), cloned into the pCR™2.1-TOPO® vector, and sequenced to ensure fidelity. To generate a mutant *gmpr* in which the last three amino acids (SKL) that comprise the PTS1 of *GMPR* were deleted, an antisense primer that encompassed a BamHI restriction site, an artificially introduced in-frame stop codon and amino acids 486–489 of the *GMPR* ORF was designed (Table S1). This antisense primer was used in conjunction with the sense primer described above to PCR-amplify the *gmpr* SKL fragment which was then subcloned into the pCR™2.1-TOPO® vector, and sequenced to ensure fidelity. The *GMPR* and *gmpr* SKL fragments were excised from the pCR™2.1-TOPO® vector via digestion with Sma/BamHI, gel purified and ligated into the pXG-BSD blasticidin resistance expression plasmid [36] that had also been digested with SmaI/BamHI and gel purified. Both the wild type pXG-BSD-*GMPR* and truncated pXG-BSD-*gmpr* SKL constructs were verified by sequencing. The *impdh* targeting constructs were generated as described using the pTRG vector and 4-way multi-fragment ligation system that was designed by our laboratory [23,44].

2.6. Creation of null mutants and complemented strains

5×10^7 LdBob promastigotes were transfected using standardized electroporation conditions [7]. Electroporated parasites were maintained for 24 h in liquid medium prior to plating on semi-solid DME-L-based growth medium supplemented with a permissive purine at a concentration of 100 μ M and the appropriate drugs. The drug concentrations employed for the genetic manipulations were 50 μ g/ml hygromycin, 50 μ g/ml phleomycin, 20 μ g/ml puromycin, 20 μ g/ml Geneticin (G418), and 20 μ g/ml blasticidin, as applicable for the selective marker. The pX63-HYG- *gmpr* and pX63-PHLEO- *gmpr* knockout vectors were linearized by digestion with HindIII and BglIII while the pTRG-PAC- *impdh* and pTRG-NEO- *impdh* constructs were excised with PacI, and the fragments harboring the drug resistance cassette and the UTRs from the gene of interest were gel purified just prior to electroporation. The linearized targeting constructs used to introduce the genetic lesions were designated by the name of the plasmid minus the initial letter, e.g., X63-HYG- *gmpr* from pX63-HYG- *gmpr*. A *GMPR/ gmpr::HYG (GMPR/gmpr)* heterozygote was generated after transfecting wild type LdBob with X63-HYG- *gmpr*, and the *gmpr::HYG/ gmpr::PHLEO (gmpr)* null mutant was isolated after transfection of the *GMPR/gmpr* heterozygote with X63-PHLEO- *gmpr* followed by selection in hygromycin, phleomycin and 100 μ M hypoxanthine as a purine source. The IMPDH/ *impdh::PAC* heterozygote was then created into the *gmpr* background using the linearized TRG-PAC- *impdh* targeting construct, and the *gmpr/ IMPDH/ impdh* cell line was subsequently transfected with TRG-NEO- *impdh*, selected in hygromycin, phleomycin, puromycin, and G418 with 100 μ M concentrations of both hypoxanthine and xanthine to generate the *gmpr/ impdh::PAC/ impdh::NEO (gmpr/ impdh)* double knockout. The pXG-BSD-*GMPR* and pXG-BSD-*gmpr* SKL plasmids were used to generate the *gmpr[pGMPR]*, *gmpr[pgmpr SKL]*, *gmpr/ impdh[pGMPR]* and *gmpr/ impdh[pgmpr SKL]* complemented cell lines from the *gmpr* and *gmpr/ impdh* knockouts, respectively. The complemented cell lines were selected in blasticidin, and 100 μ M guanine was the added purine. Southern blot analysis was used to verify the genotypes of the heterozygous, knockout, and complemented strains as described above.

2.7. Antibody generation and western blotting

The L. major GMPR, which shows 98% amino acid sequence identity to LdGMPR was used for antibody production as previously described [24]. Open Biosystems (Huntsville, AL) was contracted to produce polyclonal LmGMPR antiserum in rabbits using purified recombinant LmGMPR as an immunogen and standard injection protocols. Antiserum to the *L. donovani* IMPDH antiserum was produced in guinea pigs as described [21], and mouse monoclonal anti- α -tubulin antibody (DM1A) was purchased from EMD Chemicals (Gibbstown, NJ). Western blotting was performed via standard protocols [45] using goat anti-rabbit HRP-conjugated, goat anti-guinea pig HRP-conjugated, and goat anti-mouse HRP conjugated secondary antibodies (Thermo Fisher Scientific).

2.8. Growth phenotypes

To evaluate the capabilities of the *gmpr*, *gmpr[pGMPR]*, *gmpr[pgmpr SKL]*, *impdh*, *gmpr/ impdh*, *gmpr/ impdh[pGMPR]*, and *gmpr/ impdh[pgmpr SKL]* cells to

multiply in different purines, wild type and transgenic parasites were washed several times with PBS, resuspended at a density of 5×10^4 parasites/ml in modified DME-L [12] containing 100 μ M of one or more purines, supplemented with 5% dialyzed FBS, seeded in 1.0 ml aliquots into 24-well tissue culture plates (Sarstedt Inc., Newton, NC), and incubated at 26 °C. Parasites were allowed to grow for 7–10 days then enumerated visually via hemacytometer. Parasite growth in adenine or adenosine was evaluated in the presence of 20 μ M dCF, an inhibitor of the *L. donovani* adenine aminohydrolase [46–49], in order to prevent deamination of the 6-aminopurines to hypoxanthine and inosine. Without added dCF, adenine and adenosine are converted to hypoxanthine and inosine, respectively, over the course of the 7–10 day experiment, so dCF is obligatory for preservation of the added 6-aminopurines in these growth experiments. Additionally, growth experiments were conducted with the *gmp^r/imp^{dh}* cell line in which the concentration of hypoxanthine or xanthine was fixed at 25 μ M and the concentrations of xanthine or hypoxanthine were varied from 1.56 to 100 μ M, respectively, and the final cell density was assessed using a SYBR® Green assay [50,51]. Briefly, SYBR® Green was diluted to 1X concentration in 1% Triton X-100 in PBS, and incubated with cells for 60 min while shaking. Fluorescence was quantified with a Molecular Devices SpectraMax M2 plate reader using wavelengths of 485_{Ex} and 538_{Em}.

2.9. Macrophage infections

J774 culture-form murine macrophages (ATCC, Manassas, VA) were seeded at a density of 2×10^5 cells per well into 4-well Nunc™ Lab-Tek™ II Chambered Coverglass slides (Thermo Fisher Scientific) placed in a 37 °C 5% CO₂ incubator and allowed to adhere for 8 h. The infection protocol with wild type, *gmp^r*, *gmp^r[pGMPR]*, *gmp^r[pgmp^r SKL]*, *imp^{dh}*, *gmp^r/imp^{dh}*, and *gmp^r/imp^{dh}[pGMPR]* parasites was performed as detailed [12,14]. 72 h post-infection, amastigotes were stained with the Diff-Quik kit (International Medical Equipment Inc., San Marcos, CA) and enumerated visually on a Zeiss Axiovert 200 inverted microscope (Carl Zeiss Microimaging, Thornwood, NY).

2.10. Subcellular localization

GMPR was localized in *L. donovani* promastigotes by indirect immunofluorescence as described [21,22,25,26] using a 1:500 dilution of anti-LmGMPR antibody and a 1:10,000 dilution of secondary goat anti-rabbit Oregon Green-conjugated antibody (Thermo Fisher Scientific). Parasites were costained with guinea pig antibodies to the *L. donovani* IMPDH protein (1:200) and goat anti-guinea pig Rhodamine Red-conjugated (1:10,000) secondary antibody (Thermo Fisher Scientific) to stain glycosomes. IMPDH has been previously validated as a glycosomal enzyme [23]. Cells were photographed on a Zeiss Axiovert 200 inverted microscope (Carl Zeiss Microimaging) using a 63× oil immersion lens and a Zeiss AxioCam MR camera coupled with Axiovision 4.2 software (Carl Zeiss Microimaging) and images were compiled using Adobe Photoshop Creative Suite 4.

3. Results

3.1. Confirmation of the *gmpr* genotype

Southern blot analysis of genomic DNA from wild type, *GMPR/gmpr* heterozygotes, *gmpr* knockouts, *gmpr*[p*GMPR*] or *gmpr*[p*gmpr* SKL] complemented parasites probed with the full length *GMPR* ORF confirmed the expected genotypes of all five cell lines (Fig. 2A). The ~ 3300 bp *Sma*I fragment excised from wild type or *GMPR/gmpr* genomic DNA encompasses the full length *GMPR* ORF as well as 123 bp and 1690 bp of the 5' and 3'-UTR's, respectively. The absence of a hybridization signal in genomic DNA from *gmpr* parasites confirmed the null genotype of this cell line, and episomal complementation of the *gmpr* cell line with either [p*GMPR*] or [p*gmpr* SKL] was verified by the presence of a 1479 or 1470 bp *Sma*I/BamHI fragments, respectively. Western blot analysis with polyclonal anti-GMPR antisera confirmed the specificity of the reagent, the absence of GMPR protein in the *gmpr* mutant, and the presence of GMPR in wild type, *gmpr*[p*GMPR*] and *gmpr*[p*gmpr* SKL] parasites. Equal loading of the blot was confirmed by probing the same samples with anti-tubulin antibody (Fig. 2B).

3.2. Growth phenotype of the *gmpr* null mutant

The impact of a *gmpr* lesion on the nutritional requirements of *L. donovani* promastigotes was evaluated by assessing the capacity of wild type, *gmpr*, and *GMPR*-complemented promastigotes to grow in medium supplemented with one of a selection of purines (Fig. 3). Growth in adenine or adenosine was evaluated in the presence of 20 µM dCF, which acts to prevent deamination of these 6-aminopurines into hypoxanthine or inosine, respectively, over the course of the growth experiments [46–49]. Whereas wild type, promastigotes could multiply in medium supplemented with any of the purine nucleobases or nucleosides tested, the *gmpr* null mutant could not propagate on guanine, guanosine, xanthine, or xanthosine as a sole external source of purine. Episomal complementation of *gmpr* parasites with *gmpr*[p*GMPR*] rescued the restrictive growth phenotypes in these 6-oxopurines. Furthermore, the *gmpr*[p*gmpr* SKL] complemented cell line was also capable of robust growth in guanine, xanthine, and their corresponding ribonucleosides, indicating that deletion of the SKL COOH-terminal PTS1 tripeptide did not negatively impact the fitness of parasites expressing mislocalized *gmpr* SKL protein (Fig. 3).

3.3. Confirmation of the *gmpr/ impdh* genotype

To investigate the consequences of genetically uncoupling the adenylate and guanylate branches of the purine salvage pathway, we generated an *L. donovani gmpr/ impdh* double knockout. The *impdh* mutation was created in the *gmpr* background after two rounds of targeted gene replacement and selection in permissive growth conditions that required the inclusion of two separate purines in the growth medium, one that could be metabolized to adenylate nucleotides and the other utilized to make guanylate nucleotides. The genotype of the resulting double knockout parasites was established by Southern blot analysis of genomic DNA from wild type and mutant parasites digested with *Sma*I or *Sma*I/BamHI and hybridized to the *GMPR* ORF as a probe (Fig. 4A). The ~ 3300 bp wild type *GMPR* *Sma*I fragment encompassed the *GMPR* ORF, as well as a portion of the 5' and 3' flanking regions, and the 1479 bp *Sma*I/BamHI fragment included either the *GMPR* or *gmpr* SKL

ORFs that had been excised from the [pXG-BSD] episomes. The absence of a signal in the lanes with *gmp^r*, *gmp^r IMPDH/impdh*, or *gmp^r impdh* DNA confirmed the *gmp^r* genotype in those strains. The same genomic DNA samples were digested with EcoRV/PstI and probed with the full length *IMPDH* sequence (Fig. 4B). The *IMPDH* ORF, as well as a portion of the *IMPDH* 5' and 3' UTRs, was excised from the genomic DNA and are within an approximately 2300 bp band in lanes containing genomic DNA from wild type, *gmp^r*, and *gmp^r IMPDH/impdh* parasites. The lack of signal in the *gmp^r impdh*, *gmp^r impdh[pGMPR]*, and *gmp^r impdh[p^{gmp^r}* SKL] cell lines confirmed the replacement of both *IMPDH* alleles in these strains (Fig. 4B). Western blot analysis the genotype of the *gmp^r impdh* knockouts and verified that the *gmp^r impdh[pGMPR]* and *gmp^r impdh[p^{gmp^r}* SKL] complemented cells produce the GMPR protein (Fig. 4C). The same samples were probed with anti-IMPDH antibody to confirm the presence of the IMPDH protein in wild type and *gmp^r* parasites and the absence of IMPDH in *impdh*, *gmp^r impdh*, *gmp^r impdh[pGMPR]*, and *gmp^r impdh[p^{gmp^r}* SKL] cell lysates. Both blots were also hybridized with anti-ODC antibody to demonstrate equal loading.

3.4. Growth phenotype of *gmp^r/ impdh* knockouts

The capacity of the *gmp^r impdh* double knockout to utilize various purines was then compared to that of the wild type, *gmp^r*, *impdh*, *gmp^r impdh[pGMPR]*, and *gmp^r impdh[p^{gmp^r}* SKL] cell lines (Fig. 5A). Whereas wild type promastigotes could grow in medium with any of the purine nucleobases, nucleosides, or combinations tested, the *gmp^r impdh* parasites could not propagate in medium supplemented with any individual purine, including hypoxanthine or xanthine (Fig. 5A). The *gmp^r impdh* double knockout could, however, grow robustly when hypoxanthine and xanthine were combined in the growth medium (Fig. 5A). The *gmp^r* control, as shown in Fig. 3, could not propagate in medium supplemented with either guanosine or xanthine but was capable of robust growth in either hypoxanthine or inosine and in adenine or adenosine in the presence of 20 μ M dCF (Fig. 5A). In contrast, the *impdh* null mutant was able to grow in guanine, guanosine or xanthine but not in adenine, adenosine hypoxanthine or inosine, although both *gmp^r* and *impdh* promastigotes grew robustly, as expected, in medium supplemented with a mixture of 100 μ M hypoxanthine and 100 μ M xanthine (Fig. 5A). The growth phenotypes of the complemented *gmp^r impdh[pGMPR]* and *gmp^r impdh[p^{gmp^r}* SKL] cell lines mirrored those of *impdh* parasites, indicating that the GMPR and *gmp^r* SKL proteins produced in these complemented cell lines provided the parasites with adequate levels of GMPR or *gmp^r* SKL to sustain their growth in guanine, guanosine, or xanthine, regardless of the subcellular location of the protein.

The *gmp^r* and *impdh* lesions in the *gmp^r impdh* double knockout effectively disconnect the adenylate and guanylate nucleotide biosynthetic branches in that strain affording the opportunity to dissect the relative contributions of both branches to nucleotide-driven cellular functions. In this study, we exploited this genetic disengagement of the purine branches in the *gmp^r impdh* cell line to establish its nutritional requirements for hypoxanthine, which can only be incorporated into adenylate nucleotides, and xanthine, exclusively a source of guanylate nucleotides. Thus, growth of *gmp^r impdh* promastigotes was determined by varying the concentrations of hypoxanthine and xanthine

added to the culture medium in the presence of either 25 μ M xanthine or 25 μ M hypoxanthine, respectively (Fig. 5B). Noticeably, the requirements for xanthine to sustain *gmpr¹ impdh* growth in the presence of 25 μ M hypoxanthine were substantially lower than those for xanthine in the presence of 25 μ M hypoxanthine (Fig. 5B).

3.5. Macrophage infections

To ascertain whether the *gmpr* lesion compromised the capacity of *L. donovani* to establish an infection in mammalian macrophages, the abilities of wild type, knockout cell lines, and complemented promastigotes to infect murine J774 culture form macrophages were appraised (Fig. 6). Whereas wild type, *gmpr*[p*GMPR*] and *gmpr*[p*gmpr* SKL] parasites were capable of sustaining comparable and robust infections in J774 cells after 72 h, amastigote loads in macrophages infected with either the *impdh* or *gmpr* lines were reduced by ~25%, while amastigote burdens of macrophages infected with *gmpr¹ impdh* promastigotes were ~50% of levels achieved with wild type parasites (Fig. 6). The *gmpr¹ impdh*[p*GMPR*] and *gmpr¹ impdh*[p*gmpr* SKL] parasites had amastigote loads in J774 cells that were equivalent to that obtained with the *impdh* single knockout.

3.6. Immunolocalization of GMPR

The *L. donovani* GMPR accommodates an archetypical PTS1 signal sequence (SKL). To confirm that this topogenic signal is targeting GMPR to the glycosome, immunofluorescence assays were performed on wild type, *gmpr*, *gmpr*[p*GMPR*] and *gmpr*[p*gmpr* SKL] promastigotes (Fig. 7). Immunofluorescence analysis of wild type (Fig. 7, panels a–d) and *gmpr*[p*GMPR*] cells (Fig. 7, panels e–h) revealed a punctate staining pattern for GMPR protein in both of these cell lines. The glycosomal milieu of GMPR in wild type and *gmpr*[p*GMPR*] cells was corroborated by co-staining with anti-IMPDH antibodies (Fig. 7, panels i–j). Conversely, the staining pattern for the *gmpr* SKL protein in the *gmpr*[p*gmpr* SKL] parasites was diffuse (Fig. 7, panels m–p). The absence of both GMPR and IMPDH in the *gmpr¹ impdh* cells was also confirmed in these immunofluorescence assays (Fig. 7, panels q–t).

4. Discussion

By virtue of serving as the key enzyme that initiates the back-conversion of guanylate nucleotides to adenylate nucleotides, GMPR plays a prominent role in ensuring a proper equilibrium between guanylate and adenylate nucleotide pools in cells. The role of GMPR in maintaining the nucleotide balance in protozoan parasites is particularly significant because these organisms do not synthesize purine nucleotides *de novo* and must rely on host purines to meet their nutritional needs and to maintain and proportion their intracellular nucleotide levels. In contrast to the mammalian GMPR, the leishmanial GMPR accommodates CBS motifs. These regulatory elements are found within a variety of channels and enzymes from bacteria to humans and have been speculated to sense changes in their nucleotide environments via the binding of adenosyl motifs, which in turn affects their activity [52]. In contrast to previous studies, the single CBS domain of the *Leishmania* GMPR, which is composed of two tandemly arranged ~60 amino acid repeat CBS motifs, has been characterized biochemically and shown to bind GTP, GMP, and ATP. Binding of GTP and

GMP resulted in GMPR activation, whereas ATP binding caused GMPR inhibition [24]. These nucleotide-induced kinetic alterations were also accompanied by changes in the quaternary structure of GMPR [24]. A similar analysis of the CBS domain of the paralogous *L. donovani* IMPDH also demonstrated nucleotide interaction, although IMPDH activity was inhibited by GTP and unaffected by ATP [24]. The biochemical analysis of the CBS domain of GMPR highlights the central role of this enzyme in nucleotide metabolism as a regulatory checkpoint between guanylate and adenylate metabolism in *Leishmania*.

Despite the detailed *in vitro* characterization of the *Leishmania* GMPR enzyme [24], its role in intact parasites has not been evaluated. To test its metabolic significance, an *L. donovani* *gmpr* knockout was created by targeted gene replacement and characterized, establishing that GMPR is not essential for parasite viability under permissive conditions. As anticipated, *gmpr* promastigotes failed to grow when xanthine, guanine, or guanosine was provided as the sole purine source (Fig. 3) because a GMPR deficiency prevents conversion of these purines to adenylate nucleotides (see Fig. 1). Thus, GMPR must be essential for guanylate nucleotide incorporation into the amastigote adenylate nucleotide pool in infected hosts. All other purines were permissive for promastigote growth since adenylate nucleotide transformation to guanylate nucleotides remained intact in *gmpr* cells. The genetic lesion only slightly impacted the ability of *L. donovani* to infect macrophages (Fig. 6). Complementation of the *gmpr* lesion with an ectopic *GMPR* circumvented both the pronounced growth and slight infectivity deficits, and proper GMPR localization did not seem necessary for these phenotypic reversals, as the *gmpr*[*pgmpr* SKL] expressing mislocalized *gmpr* in which the PTS1 triad had been deleted did not express the growth or infectivity shortfalls of the *gmpr* null line (Figs. 3, 6 and 7). It should be noted that the episomally encoded GMPR and *gmpr* in the *gmpr*[*pGMPR*] and *gmpr*[*pgmpr* SKL] cells, respectively, were overexpressed compared to wild type GMPR, and this overexpression could have masked any fitness or growth defect conferred by cytosolic mislocalization of the *gmpr* in the *gmpr*[*pgmpr* SKL] parasites in our experiments (Fig. 2B). However, the glycosomal milieu of other glycosomal enzymes such as HGPRT, XPRT, and IMPDH also does not seem to be essential for their function in either the promastigote or amastigote forms since PTS1 deletion mutants of ectopic *hgprt*, *xprt*, and *impdh* genes all resulted in mistargeting of their encoded proteins to the cytosol but complemented phenotypic deficiencies in the corresponding null mutant lines at expression levels comparable to wild type *L. donovani* [21,23,25,26]. Consequently, it remains unclear what the biological advantage is for kinetoplastids to compartmentalize some of the enzymes involved in purine salvage and metabolism in the glycosome.

The ability to generate independent *gmpr* and *impdh* *L. donovani* clones offered the opportunity to create a conditionally lethal *gmpr* *impdh* double knockout line in which the adenylate and guanylate branches could be genetically uncoupled. Because the *gmpr* *impdh* null mutant, unlike wild type *L. donovani*, could not interconvert adenylate and guanylate nucleotides, the double knockout line could not be propagated in any single purine nucleobase or nucleoside provided to the culture medium although the cell line grew robustly in a combination of hypoxanthine and xanthine (Fig. 5A), which could serve as sources of adenylate and guanylate nucleotides, respectively (Fig. 1). The genetic uncoupling of the adenylate and guanylate nucleotide branches enabled us to determine that

the amount of purine required to fulfill adenylate nucleotide requirements in *L. donovani* promastigotes was considerably greater than that required to fulfill guanylate nucleotide requirements, presumably due to the relatively higher concentrations of intracellular adenylate nucleotides in the parasite (Fig. 5B). This genetic disjunction of adenylate and guanylate nucleotide synthesis in *gmpri* *impdh* cells has also been instrumental in the evaluation of the consequences of selective nutritional depletion of parasite adenylate or guanylate nucleotides on fundamental biological processes such as the stress response of *Leishmania* to purine depletion [53]. Exploiting this strategy, Martin *et al.* proved that the purine stress response in which the *L. donovani* proteome is remodeled could be attributed to selective depletion of the adenylate nucleotide pathway [53].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to acknowledge Dr. Nicola Carter, Ph.D., Dr. Phillip Yates, Ph.D. and Dr. Jessica L. Martin, Ph.D. for their insightful discussions and experimental design ideas regarding the *gmpri* *impdh* double mutant.

Funding Sources

This work was supported by National Institutes of Health Grant AI023682 and Canadian Institutes of Health Research (CHIR) (MOP 238294-11) (AJ)

Abbreviations

HGPRT	hypoxanthine-xanthine phosphoribosyltransferase
XPRT	xanthine phosphoribosyltransferase
dCF	2'-deoxycoformycin
ADSS	adenylosuccinate synthetase
ASL	adenylosuccinate lyase
IMPDH	IMP dehydrogenase
GMPT	GMP reductase
CBS	cystathionine-β-synthase
PTS1	peroxisomal targeting signal-1
PHLEO	phleomycin/bleomycin binding protein
PAC	puromycin <i>N</i> -acetyltransferase
NEO	neomycin resistance
HYG	hygromycin phosphotransferase
BSD	blasticidin deaminase

DME-L	Dulbecco's modified eagle medium-Leishmania
G418	Geneticin

References

1. Croft SL, Coombs GH. Leishmaniasis—current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol.* 2003; 19:502–8. [PubMed: 14580961]
2. Croft SL, Sundar S, Fairlamb AH. Drug resistance in leishmaniasis. *Clin Microbiol Rev.* 2006; 19:111–26. [PubMed: 16418526]
3. Krenitsky TA, Koszalka GW, Tuttle JV, Adamczyk DL, Elion GB, Marr JJ. Purine salvage enzymes in man and *Leishmania donovani*. *Adv Exp Med Biol.* 1979; 122B:51–6. [PubMed: 121040]
4. Looker DL, Berens RL, Marr JJ. Purine metabolism in *Leishmania donovani* amastigotes and promastigotes. *Mol Biochem Parasitol.* 1983; 9:15–28. [PubMed: 6199667]
5. Marr JJ, Berens RL, Nelson DJ. Purine metabolism in *Leishmania donovani* and *Leishmania braziliensis*. *Biochim Biophys Acta.* 1978; 544:360–71. [PubMed: 719006]
6. Berens, RL.; Krug, Edward C.; Marr, Joseph J. Purine and Pyrimidine Metabolism. In: Marr, JJ.; Muller, M., editors. *Biochemistry and Molecular Biology of Parasites*. Academic Press Ltd; London, England; San Diego, CA: 1995. p. 89-117.
7. LaFon SW, Nelson DJ, Berens RL, Marr JJ. Purine and pyrimidine salvage pathways in *Leishmania donovani*. *Biochem Pharmacol.* 1982; 31:231–8. [PubMed: 7059364]
8. Marr, JJ.; Muller, M. *Biochemistry and molecular biology of parasites*. Academic Press; London; San Diego: 1995.
9. Looker DL, Marr JJ, Berens RL. Mechanisms of action of pyrazolopyrimidines in *Leishmania donovani*. *J Biol Chem.* 1986; 261:9412–5. [PubMed: 2424909]
10. Datta AK, Bhaumik D, Chatterjee R. Isolation and characterization of adenosine kinase from *Leishmania donovani*. *J Biol Chem.* 1987; 262:5515–21. [PubMed: 3032935]
11. Tuttle JV, Krenitsky TA. Purine phosphoribosyltransferases from *Leishmania donovani*. *J Biol Chem.* 1980; 255:909–16. [PubMed: 7356667]
12. Boitz JM, Ullman B. *Leishmania donovani* singly deficient in HGPRT, APRT or XPRT are viable in vitro and within mammalian macrophages. *Mol Biochem Parasitol.* 2006; 148:24–30. [PubMed: 16597468]
13. Hwang HY, Ullman B. Genetic analysis of purine metabolism in *Leishmania donovani*. *J Biol Chem.* 1997; 272:19488–96. [PubMed: 9235951]
14. Boitz JM, Ullman B. A conditional mutant deficient in hypoxanthine-guanine phosphoribosyltransferase and xanthine phosphoribosyltransferase validates the purine salvage pathway of *Leishmania donovani*. *J Biol Chem.* 2006; 281:16084–9. [PubMed: 16603734]
15. Hwang HY, Gilberts T, Jardim A, Shih S, Ullman B. Creation of homozygous mutants of *Leishmania donovani* with single targeting constructs. *J Biol Chem.* 1996; 271:30840–6. [PubMed: 8940067]
16. Boitz JM, Ullman B. Amplification of adenine phosphoribosyltransferase suppresses the conditionally lethal growth and virulence phenotype of *Leishmania donovani* mutants lacking both hypoxanthine-guanine and xanthine phosphoribosyltransferases. *J Biol Chem.* 2010; 285:18555–64. [PubMed: 20363738]
17. Wilson K, Beverley SM, Ullman B. Stable amplification of a linear extrachromosomal DNA in mycophenolic acid-resistant *Leishmania donovani*. *Mol Biochem Parasitol.* 1992; 55:197–206. [PubMed: 1359409]
18. Wilson K, Collart FR, Huberman E, Stringer JR, Ullman B. Amplification and molecular cloning of the IMP dehydrogenase gene of *Leishmania donovani*. *J Biol Chem.* 1991; 266:1665–71. [PubMed: 1671039]
19. Spector T, Jones TE, Elion GB. Specificity of adenylosuccinate synthetase and adenylosuccinate lyase from *Leishmania donovani*. Selective amination of an antiprotozoal agent. *J Biol Chem.* 1979; 254:8422–6. [PubMed: 468834]

20. Spector T, Jones TE. Guanosine 5'-monophosphate reductase from *Leishmania donovani*. A possible chemotherapeutic target. *Biochem Pharmacol*. 1982; 31:3891–7. [PubMed: 7159467]
21. Dobie F, Berg A, Boitz JM, Jardim A. Kinetic characterization of inosine monophosphate dehydrogenase of *Leishmania donovani*. *Mol Biochem Parasitol*. 2007; 152:11–21. [PubMed: 17173987]
22. Boitz JM, Strasser R, Yates PA, Jardim A, Ullman B. Adenylosuccinate Synthetase and Adenylosuccinate Lyase Deficiencies Trigger Growth and Infectivity Deficits in *Leishmania donovani*. *J Biol Chem*. 2013; 288:8977–90. [PubMed: 23404497]
23. Fulwiler AL, Boitz JM, Gilroy C, Yates PA, Jardim A, Ullman B. IMP dehydrogenase deficiency in *Leishmania donovani* causes a restrictive growth phenotype in promastigotes but is not essential for infection in mice. *Mol Biochem Parasitol*. 2011; 180:123–6. [PubMed: 21907738]
24. Smith S, Boitz J, Chidambaram ES, Chatterjee A, Ait-Tihyaty M, Ullman B, Jardim A. The Cystathionine-beta-synthase domains on the guanosine 5'-monophosphate reductase and inosine 5'-monophosphate dehydrogenase enzymes from *Leishmania* regulate enzymatic activity in response to guanylate and adenylate nucleotide levels. *Mol Microbiol*. 2016
25. Shih S, Hwang HY, Carter D, Stenberg P, Ullman B. Localization and targeting of the *Leishmania donovani* hypoxanthine-guanine phosphoribosyltransferase to the glycosome. *J Biol Chem*. 1998; 273:1534–41. [PubMed: 9430693]
26. Zarella-Boitz JM, Rager N, Jardim A, Ullman B. Subcellular localization of adenine and xanthine phosphoribosyltransferases in *Leishmania donovani*. *Mol Biochem Parasitol*. 2004; 134:43–51. [PubMed: 14747142]
27. Michels PA, Hannaert V, Bringaud F. Metabolic aspects of glycosomes in trypanosomatidae – new data and views. *Parasitol Today*. 2000; 16:482–9. [PubMed: 11063859]
28. Opperdoes FR, Szikora JP. *In silico* prediction of the glycosomal enzymes of *Leishmania major* and trypanosomes. *Mol Biochem Parasitol*. 2006; 147:193–206. [PubMed: 16546274]
29. Parsons M. Glycosomes: parasites and the divergence of peroxisomal purpose. *Mol Microbiol*. 2004; 53:717–24. [PubMed: 15255886]
30. Parsons M, Furuya T, Pal S, Kessler P. Biogenesis and function of peroxisomes and glycosomes. *Mol Biochem Parasitol*. 2001; 115:19–28. [PubMed: 11377736]
31. Bessho T, Okada T, Kimura C, Shinohara T, Tomiyama A, Imamura A, Kuwamura M, Nishimura K, Fujimori K, Shuto S, Ishibashi O, Kubata BK, Inui T. Novel Characteristics of Trypanosoma brucei Guanosine 5'-monophosphate Reductase Distinct from Host Animals. *PLoS Negl Trop Dis*. 2016; 10:e0004339. [PubMed: 26731263]
32. Bessho T, Morii S, Kusumoto T, Shinohara T, Noda M, Uchiyama S, Shuto S, Nishimura S, Djikeng A, Duszenko M, Martin SK, Inui T, Kubata KB. Characterization of the novel Trypanosoma brucei inosine 5'-monophosphate dehydrogenase. *Parasitology*. 2013; 140:735–45. [PubMed: 23369253]
33. Jiang Y, Roberts SC, Jardim A, Carter NS, Shih S, Ariyanayagam M, Fairlamb AH, Ullman B. Ornithine decarboxylase gene deletion mutants of *Leishmania donovani*. *J Biol Chem*. 1999; 274:3781–8. [PubMed: 9920931]
34. Freedman DJ, Beverley SM. Two more independent selectable markers for stable transfection of *Leishmania*. *Mol Biochem Parasitol*. 1993; 62:37–44. [PubMed: 8114824]
35. Cruz A, Coburn CM, Beverley SM. Double targeted gene replacement for creating null mutants. *Proc Natl Acad Sci U S A*. 1991; 88:7170–4. [PubMed: 1651496]
36. Goyard S, Beverley SM. Blasticidin resistance: a new independent marker for stable transfection of *Leishmania*. *Mol Biochem Parasitol*. 2000; 108:249–52. [PubMed: 10838227]
37. Goyard S, Segawa H, Gordon J, Showalter M, Duncan R, Turco SJ, Beverley SM. An in vitro system for developmental and genetic studies of *Leishmania donovani* phosphoglycans. *Mol Biochem Parasitol*. 2003; 130:31–42. [PubMed: 14550894]
38. Debrabant A, Joshi MB, Pimenta PF, Dwyer DM. Generation of *Leishmania donovani* axenic amastigotes: their growth and biological characteristics. *Int J Parasitol*. 2004; 34:205–17. [PubMed: 15037106]
39. Dwyer DM. A monophasic medium for cultivating *Leishmania donovani* in large numbers. *J Parasitol*. 1972; 58:847–8. [PubMed: 5057248]

40. Iovannisci DM, Ullman B. High efficiency plating method for *Leishmania* promastigotes in semidefined or completely-defined medium. *J Parasitol.* 1983; 69:633–6. [PubMed: 6631633]
41. Iovannisci DM, Ullman B. Single cell cloning of *Leishmania* parasites in purine-defined medium: isolation of drug-resistant variants. *Adv Exp Med Biol.* 1984; 165(Pt A):239–43. [PubMed: 6720386]
42. Aslett M, Aurrecochea C, Berriman M, Brestelli J, Brunk BP, Carrington M, Depledge DP, Fischer S, Gajria B, Gao X, Gardner MJ, Gingle A, Grant G, Harb OS, Heiges M, Hertz-Fowler C, Houston R, Innamorato F, Iodice J, Kissinger JC, Kraemer E, Li W, Logan FJ, Miller JA, Mitra S, Myler PJ, Nayak V, Pennington C, Phan I, Pinney DF, Ramasamy G, Rogers MB, Roos DS, Ross C, Sivam D, Smith DF, Srinivasamoorthy G, Stoeckert CJ Jr, Subramanian S, Thibodeau R, Tivey A, Treatman C, Velarde G, Wang H. TriTrypDB: a functional genomic resource for the Trypanosomatidae. *Nucleic Acids Res.* 2010; 38:D457–62. [PubMed: 19843604]
43. Deng Y, Wang Z, Ying K, Gu S, Ji C, Huang Y, Gu X, Wang Y, Xu Y, Li Y, Xie Y, Mao Y. NADPH-dependent GMP reductase isoenzyme of human (GMPR2). Expression, purification, and kinetic properties. *Int J Biochem Cell Biol.* 2002; 34:1035–50. [PubMed: 12009299]
44. Fulwiler AL, Soysa DR, Ullman B, Yates PA. A rapid, efficient and economical method for generating leishmanial gene targeting constructs. *Mol Biochem Parasitol.* 2011; 175:209–12. [PubMed: 21055426]
45. Sambrook, J.; Russell, DW. *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.: 2001.
46. Kidder GW, Nolan LL. Adenine aminohydrolase: occurrence and possible significance in trypanosomid flagellates. *Proc Natl Acad Sci U S A.* 1979; 76:3670–2. [PubMed: 291031]
47. Iovannisci DM, Goebel D, Allen K, Kaur K, Ullman B. Genetic analysis of adenine metabolism in *Leishmania donovani* promastigotes. Evidence for diploidy at the adenine phosphoribosyltransferase locus. *J Biol Chem.* 1984; 259:14617–23. [PubMed: 6501311]
48. Boitz JM, Strasser R, Hartman CU, Jardim A, Ullman B. Adenine aminohydrolase from *Leishmania donovani*: unique enzyme in parasite purine metabolism. *J Biol Chem.* 2012; 287:7626–39. [PubMed: 22238346]
49. Boitz JM, Ullman B. Adenine and adenosine salvage in *Leishmania donovani*. *Mol Biochem Parasitol.* 2013; 190:51–5. [PubMed: 23845934]
50. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrob Agents Chemother.* 2004; 48:1803–6. [PubMed: 15105138]
51. Ortiz D, Guiguemde WA, Johnson A, Elya C, Anderson J, Clark J, Connelly M, Yang L, Min J, Sato Y, Guy RK, Landfear SM. Identification of Selective Inhibitors of the *Plasmodium falciparum* Hexose Transporter PfHT by Screening Focused Libraries of Anti-Malarial Compounds. *PLoS One.* 2015; 10:e0123598. [PubMed: 25894322]
52. Ignoul S, Eggermont J. CBS domains: structure, function, and pathology in human proteins. *Am J Physiol Cell Physiol.* 2005; 289:C1369–78. [PubMed: 16275737]
53. Martin JL, Yates PA, Boitz JM, Koop DR, Fulwiler AL, Cassera MB, Ullman B, Carter NS. A Role for Adenine Nucleotides in the Sensing Mechanism to Purine Starvation in *Leishmania donovani*. *Mol Microbiol.* 2016 Apr 7. [Epub ahead of print]. doi: 10.1111/mmi.13390

GMP reductase (GMPR) is essential for guanylate nucleotide salvage from the host

gmpr promastigotes cannot grow on xanthine, guanine, or guanosine

A *gmpr*⁻ *impdh* mutant cannot interconvert adenylate and guanylate nucleotides

A *gmpr*⁻ *impdh* double knockout cannot grow on a single purine source

Fig. 1. Purine metabolism in *Leishmania*

The schematic depicts the enzymatic machinery in *Leishmania* parasites involved in the salvage and interconversion of metabolites that produce the adenylyate and guanylate monophosphate nucleotides. 1, GMP reductase; 2, inosine monophosphate dehydrogenase; 3, GMP synthase; 4, xanthine phosphoribosyltransferase; 5, guanine deaminase; 6, hypoxanthine-guanine phosphoribosyltransferase; 7, adenylosuccinate synthetase; 8, adenylosuccinate lyase; 9, AMP deaminase; 10, adenine phosphoribosyltransferase; 11, adenine aminohydrolase; AMP, adenosine monophosphate; GMP, guanosine monophosphate; IMP, inosine monophosphate; XMP, xanthine monophosphate; Ade, adenine; Hyp, hypoxanthine; Xan, xanthine; Gua, guanine.

Fig. 2. Southern and Western analysis of *gmp* knockouts

(A) Total genomic DNA from wild type (lane 1), *GMPR/gmp* heterozygotes (lanes 2 and 3), *gmp* (lane 4), *gmp*[pGMPR] (lane 5), or *gmp*[pgmp SKL] (lane 6) parasites was digested with *Sma*I (lanes 1–4) or *Sma*I/*Bam*HI (lanes 5 and 6), fractionated on a 0.8% agarose gel, transferred to nylon membranes, and hybridized under stringent conditions with a probe containing the full length *GMPR* ORF. (B) Lysates from exponentially growing wild type (lane 1), *gmp* (lane 2), *gmp*[pGMPR] (lane 3) or *gmp*[pgmp SKL] (lane 4) parasites were loaded onto a 4–20% polyacrylamide gel and analyzed by immunoblotting using monospecific, polyclonal anti-GMPR antibodies that had been produced in rabbits. Equal loading of samples was verified by stripping and reprobing the same membrane with commercially available mouse anti- α -tubulin monoclonal antibody.

Fig. 3. Growth phenotype of *gmpr* knockouts

The ability of wild type, *gmpr*, *gmpr*[pGMPR], or *gmpr*[pgmpr SKL] to grow in defined medium containing different purine sources was evaluated. Parasites were seeded at a density of 5×10^4 /ml into medium containing 100 μ M purine, incubated for 7–10 days and enumerated via hemacytometer. Parasite proliferation in adenine (*Ade*), or adenosine (*Ado*), was evaluated in the presence of 20 μ M dCF.

Fig. 4. Southern and Western analysis of *gmpr/ impdh* knockouts

Total genomic DNA from wild type (lane 1), *impdh* (lane 2), *gmpr* (lane 3), *gmpr/ IMPDH/impdh* (lane 4), *gmpr/ impdh* (lane 5), *gmpr/ impdh*[pGMPR] (lane 6), or *gmpr/ impdh*[pgmpr SKL] (lane 7), parasites was digested with SmaI (Panel A, lanes 1–5), SmaI/BamHI (Panel A, lanes 6 and 7, Panel B, lanes 6 and 7), or EcoRV/PstI (Panel B, lanes 1–5) fractionated on a 0.8% agarose gel, and blotted onto nylon membrane. The blots were hybridized under stringent conditions with a probe containing either the full length *GMPR* (A) or *IMP DH* ORF (B). (C) Western blot analysis was performed on total cell lysates from wild type (lane 1), *impdh* (lane 2), *gmpr* (lane 3), *gmpr/ impdh* (lane 4), *gmpr/ impdh*[pGMPR] (lane 5), or *gmpr/ impdh*[pgmpr SKL] (lane 6), using monospecific, anti-GMPR or anti-IMP DH antibodies, and equal loading of the samples was verified by probing the same membranes with anti-ODC antibody.

Fig. 5. Growth phenotype of *gmpr*/ *impdh* knockouts

(A) Wild type, *impdh*, *gmpr*, *gmpr*/ *impdh*, *gmpr*/ *impdh*[p*GMPR*] or *gmpr*/ *impdh*[p*gmpr* SKL] parasites were inoculated at a density of 5×10^4 /ml into medium containing 100µM purine, incubated for 7–10 days and enumerated via hemacytometer. Parasite proliferation in adenine (*Ade*), or adenosine (*Ado*), was evaluated in the presence of 20 µM dCF. (B) Final cell density was assessed in a fixed concentration of 25 µM hypoxanthine (*Hyp*) (■) or xanthine (*Xan*) (▲), while the concentration of the other purine was varied from 1.56–100 µM, and fluorescence units were measured using a SYBR® Green assay.

Fig. 6. Macrophage infections

Culture form J774 murine macrophages were seeded at a density of 2×10^5 cells/ml into 4-well chamber slides, and infected with stationary phase *L. donovani* parasites at a 10:1 ratio. The ability of wild type, *impdh*, *gmpr*, *gmpr*[pGMPR], *gmpr*[pgmpr SKL], *gmpr*/*impdh*, *gmpr*/*impdh*[pGMPR], or *gmpr*/*impdh*[pgmpr SKL], parasites to infect macrophages was assessed via hemacytometer.

Fig. 7. Localization of GMPR

Wild type (a–d), *gmpr* (e–h), *gmpr*[pGMPR] (i–l), *gmpr*[p*gmpr* SKL] (m–p), or *gmpr* *impdh* (q–t) *L. donovani* promastigotes were subjected to immunofluorescence analysis using rabbit anti-GMPR (a, e, i, m, and q) or guinea pig anti-IMPDPH (b, f, j, n, and r) monospecific, polyclonal antibodies. Goat anti-rabbit Oregon Green-conjugated and goat anti-guinea pig Rhodamine Red-conjugated secondary antibodies were used to detect the GMPR and IMPDPH primary antibodies, respectively. Overlays of the Oregon Green and

Rhodamine Red staining are shown in panels *c, g, k, o* and *s*. Parasites were also stained with DAPI (*d, h, l, p, and t*).

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