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Functional Characterization of Nucleoside Transporter Gene Replacements in *Leishmania donovani*

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Abstract

Leishmania donovani express two nucleoside transporters of non-overlapping ligand selectivity. To evaluate the physiological role of nucleoside transporters in *L. donovani*, homozygous null mutants of the genes encoding the LdNT1 adenosine-pyrimidine nucleoside transporter and the LdNT2 inosine-guanosine transporter were created singly and in combination by single targeted gene replacement followed by selection for loss-of-heterozygosity. The mutant alleles were verified by Southern blotting, and the effects of gene replacement on transport phenotype were evaluated by rapid sampling transport measurements and by drug resistance profiles. The $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ mutants were all capable of proliferation in defined culture medium supplemented with any of a spectrum of purine nucleobases or nucleosides, except that a $\Delta ldnt2$ lesion conferred an inability to efficiently salvage exogenous xanthosine, a newly discovered ligand of LdNT2. Each of the three knockout strains was viable as promastigotes and axenic amastigotes and capable of maintaining an infection in J774 and bone marrow-derived murine macrophages. These genetic studies demonstrate: 1) that *L. donovani* promastigotes, axenic amastigotes, and tissue amastigotes are viable in the absence of nucleoside transport; 2) that nucleoside transporters are not essential for sustaining an infection in mammalian host cells; and 3) that the phagolysosome of macrophages is likely to contain purines that are not LdNT1 or LdNT2 ligands, i.e., nucleobases. Furthermore, the $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ knockouts offer a unique genetically defined null background for the biochemical and genetic characterization of nucleoside transporter genes and cDNAs from phylogenetically diverse species and of genetically manipulated *LdNT1* and *LdNT2* constructs.

Keywords

Transport; transporters; purines; pyrimidines; infectivity

Abbreviations

ENT, equilibrative nucleoside transporter; LOH, loss-of-heterozygosity; PCR, polymerase chain reaction; FBS, fetal bovine serum; UTR, untranslated region; ORF, open reading frame; PBS, phosphate buffered saline; PRT, phosphoribosyltransferase

1. Introduction

Leishmania donovani is the etiologic agent of visceral leishmaniasis, a devastating and often fatal disease. *Leishmania* species are digenetic protozoan parasites in which the extracellular,

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flagellated promastigote exists in the phlebotomine sandfly vector, and the intracellular amastigote resides within the phagolysosome of macrophages and reticuloendothelial cells of the mammalian host. The current arsenal of drugs used in the treatment of leishmaniasis – or for that matter any parasitic disease – is far from ideal, and the need for more efficacious and selective anti-leishmanial drugs that exploit fundamental biochemical differences between parasite and host is acute.

Perhaps the most striking metabolic discrepancy between parasites and their mammalian hosts is the disparate mechanisms by which they generate purine nucleotides. Whereas mammalian cells synthesize purine nucleotides from amino acids and 1-carbon compounds, all of the protozoan parasites studied to date are incapable of *de novo* synthesis of the purine ring [1]. Thus, each genus of parasite expresses a unique complement of purine salvage enzymes that enable the parasite to scavenge preformed purines from the host milieu. The initial step in purine acquisition by the parasite involves the translocation of host purines across the parasite surface membrane, a process that is mediated by nucleoside and nucleobase transporters.

Nucleoside permeation into *L. donovani* is carried out by two high affinity transporters with non-overlapping ligand specificity, LdNT1 and LdNT2. LdNT1 transports adenosine and pyrimidine nucleosides, whereas LdNT2 is selective for inosine and guanosine [2,3]. Mutants genetically deficient in either LdNT1 (TUBA5) or LdNT2 (FBD5) activity have been created by negative selection, and the genes for these permeases were subsequently cloned from a cosmid library by functional rescue of these nucleoside-transport-deficient *Leishmania* [2–4]. Based on their primary structures and predicted membrane topologies, LdNT1 and LdNT2 belong to the equilibrative nucleoside transporter (ENT) family, although subsequent electrophysiological investigations revealed each to be an electrogenic proton symporter [5].

The TUBA5 and FBD5 parasites were originally derived after mutagenesis and selection in tubercidin, an adenosine analog, or formycin B, an inosine isomer, respectively [4]. Southern blot analyses revealed no gross gene rearrangements at the *LdNT1* and *LdNT2* loci in the nucleoside transport-deficient *L. donovani*, and Northern blot analyses indicated that the *LdNT1* and *LdNT2* transcripts were expressed at normal levels in TUBA5 and FBD5 cells, respectively [2,3]. Subsequent sequence analyses of the mutant alleles revealed the basis of the genetic deficiencies to be either point mutations within *LdNT1* and *LdNT2* that cripple the permeation mechanisms of the encoded permeases or a nonsense mutation within one *ldnt2* allele [6,7]. Because of the potential for residual nucleoside transporter activity in TUBA5 and FBD5 parasites and the possibility that functionally incompetent transporters are generated or accumulate in some subcellular milieu within the nucleoside transport-deficient lines, the TUBA5 and FBD5 cells do not provide an optimal null background for transfection studies with nucleoside transporter genes or cDNAs.

Thus, to assess the consequences of a complete deficiency in LdNT1 and LdNT2 activity on the ability of *L. donovani* to transport, salvage, and proliferate in various purines, $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ knockouts were isolated by a combination of targeted gene replacement and selection for loss-of-heterozygosity (LOH). Mutants lacking *LdNT1* could not transport adenosine but were capable of growing on all purine nucleosides and nucleobases tested. Mutants possessing a $\Delta ldnt2$ lesion failed to transport inosine and xanthosine but were capable of robust growth on inosine and guanosine but not xanthosine. All three knockout strains were capable of transforming into axenic amastigotes and establishing an infection in murine macrophages. These studies establish that nucleoside transport is not essential for the viability of either parasite life cycle stage, intimates that purine nutrition within the intracellular milieu of the macrophage does not require nucleosides, and provides an ideal vehicle for future structure-function studies on LdNT1 and LdNT2 and for the expression of heterologous

transporters genes and cDNAs within a genetic background completely devoid of nucleoside transport mechanisms.

2. Materials and Methods

2.1. Chemicals, materials, and reagents

[³H]inosine (38.4 Ci/mmol) and [³H]xanthosine (8.6 Ci/mmol) were purchased from Moravak Biochemicals, Inc. (Brea, CA), [³H]adenosine (40 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [α -³²P]dCTP (3000 Ci/mmol) was supplied by MP Biomedicals (Irvine, CA). All restriction and DNA modifying enzymes were bought from either Invitrogen (Carlsbad, CA), Gibco-BRL Life Technologies Inc. (Gaithersburg, MD), or New England Biolabs, Inc. (Beverly, MA), and the polymerase chain reaction (PCR) protocols were performed using the Advantage™ HF-2 PCR kit from BD Biosciences (Franklin Lakes, NJ). Synthetic oligonucleotides were acquired from Invitrogen. The pX63-HYG [8] and pX63-PHLEO [9] leishmanial vectors that harbor genes conferring hygromycin and phleomycin resistance, respectively, were generous gifts from Dr. Stephen M. Beverley (Washington University School of Medicine). Hygromycin, tubercidin, and formycin B were obtained from Sigma-Aldrich (St. Louis, MO). All other materials, chemicals, and reagents were of the highest purity commercially available.

2.2 Parasite cell culture

The *L. donovani* LdBob strain [10] was provided by Dr. Stephen M. Beverley (Washington University, St. Louis, MO). LdBob promastigotes were routinely cultured at 26 °C in purine-replete M199-based medium [10] or in Dulbecco's modified Eagle-*Leishmania* (DME-L) medium [11] in which the bovine serum albumin was replaced with 10% dialyzed fetal bovine serum (FBS), 1 mM glutamine, 1 X RPMI 1640 vitamin mix, 10 μ M folate, and 100 μ M xanthine. Once isolated, the Δ *ldnt1* and Δ *ldnt2* strains were continuously cultured in 50 μ g/ml hygromycin and 50 μ g/ml phleomycin, respectively. The Δ *ldnt1*/ Δ *ldnt2* double knockout was maintained in both 50 μ g/ml hygromycin and 50 μ g/ml phleomycin. Single cell cloning of promastigotes was accomplished in semi-solid medium on plates as described [11], and axenic amastigotes were grown at 37 °C as reported [10].

2.3. Oligonucleotide primers

Primers used in the PCR-based amplification of 5' and 3' flanking segments of the *L. donovani* *LdNT1* and *LdNT2* loci are designated by the flank (either 5' or 3'), the targeted locus, and orientation. The restriction sites in the PCR primers that were employed in the subcloning of the amplified products into vectors are underlined:

5'-*LdNT1*-Sense, 5'-AAGCTTGAGGTGCCCCGCGATTGTTG-3';

5'-*LdNT1*-Antisense, 5'-GTCGACGGAATGGGAGAAGAGAG-3';

3'-*LdNT1*-Sense, 5'-TCTAGAGGATCATCGTAGCGGCGTC;

3'-*LdNT1*-Antisense, 5'-AGATCTAGCAGCGGGCACAAGGTG;

5'-*LdNT2*-Sense, 5'-CATTAAAGCTTCCCTACTTGCCTTGCTG-3';

5'-*LdNT2*-Antisense, 5'-
TTCAAAGCTTAGTTTAAATCAGTCAGAGTAACTCAGTAAG-3';

3'-*LdNT2*-Sense; 5'-TTCCCCGGGTCTGGCCTAACTCTCTTTGTTTG-3';

3'-*LdNT2*-Antisense, 5'-CTCAGATCTCAAAAAAAAAACAGGGAGAAG-3'.

2.4. DNA manipulations

Isolation of genomic DNA and Southern blotting were achieved using standard procedures [12]. Hybridization probes were the full length coding sequences for *LdNT1* and *LdNT2* and a probe to a portion of the 5' untranslated region (UTR) of each open reading frame (ORF), all of which were generated by PCR and gel-purified using a QIAEX II kit (Qiagen, Valencia, CA). The plasmids from which the hybridization probes were amplified harbored the appropriate sequences that had been cloned into the TOPO-TA vector (Invitrogen™).

Overlapping and complementary mutagenic primers used to eliminate a 5' BglII site within the 5' flank of the *LdNT1* gene were mutation primer-A, 5'-CGTGCCGTTTCGTAGATATTGTCCTCACGTCAGAC-3', and mutation primer-B, 5'-GTCTGACGTGAGGACAATATCTACGAAACGGCACG-3'. PCR mutagenesis was accomplished as described [13].

2.5. Molecular constructs for replacement of *LdNT1* and *LdNT2*

LdNT1 [2] and *LdNT2* [3] were originally isolated from a cosmid library of *L. donovani* genomic DNA. 5' and 3' flanking regions were identified by restriction mapping, sequencing, and Southern blotting of both genomic and cosmid DNA and then amplified from genomic DNA using PCR for subcloning into targeting vectors. Standard PCR conditions (25 cycles; 94 °C for 30 s, 62 °C for 30 s, 72 °C for 60–90 s) for amplification of *L. donovani* genomic DNA sequences were implemented. Sense and antisense primers used in the PCR were 5'-*LdNT1*-Sense and 5'-*LdNT1*-Antisense for the 479 bp *LdNT1* 5' flanking region, 3'-*LdNT1*-Sense and 3'-*LdNT1*-Antisense for the 539 bp *LdNT1* 3' flanking region, 5'-*LdNT2*-Sense and 5'-*LdNT2*-Antisense for the 750 bp *LdNT2* 5' flanking region, and 3'-*LdNT2*-Sense and 3'-*LdNT2*-Antisense for the 589 bp *LdNT2* 3' flanking region. PCR products for the four flanking regions were separated on agarose gels, purified using a QIAEX II kit (Qiagen), digested with the appropriate restriction enzymes, and either inserted directly (*LdNT2* flanks) into the germane sites of the pX63-HYG plasmid or ligated first into the TOPO-TA vector and then transferred into the relevant restriction sites within pX63-HYG. Each of the two flanks of both loci were ligated sequentially into pX63-HYG, and the gene targeting constructs were designated pX63-HYG- Δ *ldnt1* and pX63-HYG- Δ *ldnt2*, respectively.

To create the pX63-PHLEO- Δ *ldnt2* targeting vector, it was first necessary to mutate a Sac I restriction site within the 5' flank of *LdNT2*. Overlapping mutagenic primers to eliminate the Sac I site within the *LdNT2* 5' flank were 5'-CACTCGAAGTGCTTTTGTAGCTCCCATCGTGAGG-3' and 5'-CCTCACGATGGGAGCTCCAAAAGCTCTTCGAGTG-3', and PCR mutagenesis was accomplished as described [13]. Then, the HYG and PHLEO genes were excised from the mutagenized pX63-HYG- Δ *ldnt2* and pX63-PHLEO vectors, respectively, by digestion with Xho I and Sac I, and the PHLEO gene was ligated into the Xho I/Sac I sites of pX63-HYG- Δ *ldnt2* from which the HYG gene had been removed.

2.6. Transfections

Parasites were transfected by electroporation using conditions analogous to those previously reported [14]. The targeting constructs were cleaved with either Hind III and Bgl II (*LdNT1*) or Nhe I and Bgl II (*LdNT2*) to release the linear targeting fragments from pX63-HYG- Δ *ldnt1*, pX63-HYG- Δ *ldnt2*, and pX63-PHLEO- Δ *ldnt2*. Linearized targeting DNAs were designated by the plasmid from which they were cleaved without the initial letter: X63-HYG- Δ *ldnt1* from pX63-HYG- Δ *ldnt1*, X63-HYG- Δ *ldnt2* from pX63-HYG- Δ *ldnt2*, and X63-PHLEO- Δ *ldnt2* from pX63-PHLEO- Δ *ldnt2*. Cells were maintained in nonselective liquid

medium for 24 h after transfection and then plated on semi-solid agar under appropriate selective conditions.

2.7. Gene replacements

To isolate *LdNT1/ldnt1* and *LdNT2/ldnt2* heterozygotes, wild type parasites were transfected with X63-HYG- Δ *ldnt1* or X63-HYG- Δ *ldnt2* and selected on plates containing 50 μ g/ml hygromycin B. Colonies were picked after 2-3 weeks, expanded in medium containing the selective agent, and heterozygosity was established by Southern blotting [12] using appropriate 5' flanking probes. To obtain the Δ *ldnt1* and Δ *ldnt2* null mutants, the *LdNT1/ldnt1* and *LdNT2/ldnt2* heterozygotes were plated in the presence of 5 μ M tubercidin or 1 μ M formycin B, respectively. The Δ *ldnt1*/ Δ *ldnt2* double mutant was created within the Δ *ldnt2* background by first generating the *LdNT1/ldnt1*/ Δ *ldnt2* clone after transfecting the Δ *ldnt2* knockout with X63-HYG- Δ *ldnt1* and selecting in 50 μ g/ml hygromycin. The Δ *ldnt1*/ Δ *ldnt2* double knockout was then obtained from the *LdNT1/ldnt1*/ Δ *ldnt2* strain by selection in 5 μ M tubercidin. All mutant strains created by targeted gene replacement were maintained in selective medium containing the drugs from which it and its progenitors were selected.

2.8. Transport assays

Nucleoside transport measurements were accomplished in phosphate buffered saline (PBS) using a previously described oil-stop method [15]. Rates of transport were determined for 1.0 μ M [3 H]adenosine (0.40 Ci/mmol), 1.0 μ M [3 H]inosine (0.38 Ci/mmol), and 10 μ M [3 H]xanthosine (0.086 Ci/mmol). The apparent K_m for xanthosine uptake was calculated by determining the rate of xanthosine uptake at six different concentrations of the nucleoside.

2.9 Sensitivities to tubercidin and formycin B

The sensitivities of the Δ *ldnt1*, Δ *ldnt2*, and Δ *ldnt1*/ Δ *ldnt2* knockouts to tubercidin (7-deazaadenosine), a cytotoxic ligand of LdNT1 [4,16], and formycin B (7-hydroxy-3- β -D-ribofuranosylpyrazolo-[4,3-d]pyrimidine), an anti-leishmanial pyrazolopyrimidine nucleoside isomer of inosine [17], were evaluated as described [7,18]. The EC_{50} values are the effective concentrations of drug that inhibit growth by 50%.

2.10. Purines as nutrients

To evaluate the abilities of the Δ *ldnt1*, Δ *ldnt2*, and Δ *ldnt1*/ Δ *ldnt2* cells to grow in various purines, wild type and mutant parasites were washed several times with PBS and resuspended in purine-deplete DME-L-based medium that lacked the bovine serum albumin and purine components but that was supplemented with 10% dialyzed FBS. 1.0 ml aliquots of cells were seeded at a concentration of 5×10^4 parasites/ml and incubated for 7–10 days in this DME-L-based medium to which various purine supplements were added to a final concentration of 100 μ M. Cells were enumerated visually by hemacytometer.

2.11 . Macrophage infectivity assays

Prior to infection, 2×10^5 J774 murine macrophages (ATCC, Manassas, VA) were incubated overnight in 4-well Lab-TekII[®] Chamber Slides (Nalge Nunc International Corp., Naperville, IL) containing 1.0 ml RPMI medium supplemented with 10% heat inactivated FBS, 4 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in a humidified 5 % CO₂ atmosphere. After 16 hr, unattached macrophages were removed by washing the chamber slides twice with RPMI medium, and 2.0×10^6 stationary-phase *L. donovani* promastigotes were added to each chamber. After 4 hr, adherent macrophages were washed three times with RPMI to remove any remaining extracellular promastigotes, after which fresh growth medium was added at 24 and 48 hrs. The level of infection at 72 hr was assessed by staining macrophage and parasite nuclei with propidium iodide as described [19]. Parasites were detected on a Zeiss

Axiovert 200M scope (Carl Zeiss Microimaging, Thornwood, NY) using a 60X oil immersion lens. The preparation and infection of murine bone marrow-derived macrophages was as reported [20]. Pictures were taken with an AxioCam MRm camera (Carl Zeiss Microimaging) and parasites enumerated visually.

3. Results

3.1. Southern blot analysis of the gene replacements

$\Delta ldn1$, $\Delta ldn2$, and $\Delta ldn1/\Delta ldn2$ parasites were created within LdBob, a strain of *L. donovani* that is capable of transformation into axenic amastigotes and that retains its capacity to infect mammalian macrophages [10]. The $\Delta ldn1$ and $\Delta ldn2$ single knockouts were each generated after a single round of targeted gene replacement followed by selection for LOH. Functional heterozygotes were first generated by homologous recombination after transfection with vectors containing 5'- and 3'-flanking regions of either *LdNT1* or *LdNT2* encompassing a drug resistance marker, and homozygous null mutants ($\Delta ldn1$ or $\Delta ldn2$) were then isolated from the heterozygotes by negative selection in media containing a cytotoxic transporter ligand. Each of the gene replacements within the $\Delta ldn1/\Delta ldn2$ double knockout was constructed by the same strategy. First, the $\Delta ldn2$ mutant was created from wild type parasites after a single round of gene replacement to create the *LdNT2/ldn2* heterozygote followed by selection in formycin B. The *LdNT1/ldn1/ $\Delta ldn2$* line was generated after an additional round of gene replacement with the *LdNT1* targeting vector followed by selection for LOH in tubercidin to isolate the $\Delta ldn1/\Delta ldn2$ double knockout.

To confirm the gene replacement events in the genetically manipulated strains, Southern blotting of genomic DNA from wild type, heterozygous, and homozygous knockout parasites was performed (Fig. 1). Southern blot analysis using the *LdNT1* and *LdNT2* ORFs as hybridization probes revealed the loss of both wild type alleles in each of the null mutant lines, whereas the *LdNT2/ldn2* and *LdNT1/ldn1/ $\Delta ldn2$* heterozygotes retained at least one wild type copy of the gene that hybridized to the radiolabelled probe. The nature of the replacements in the heterozygotes was also confirmed by probing with either the 3' UTR from *LdNT1* or the 5' UTR from *LdNT2*, an analysis that was also performed after clonal isolates of the heterozygotes were initially obtained (data not shown). The hybridization signals all corresponded to the size of the restriction fragments predicted from the sequence of the *LdNT1* and *LdNT2* ORFs and adjacent 5' and 3' UTRs [2,3].

3.2. Transport phenotypes of the null mutants

In order to assess the phenotypic consequences of the genetic lesions, nucleoside transport assays were performed on wild type and mutant parasites using naturally occurring nucleoside ligands of the two permeases [2–4]. Wild type parasites efficiently transported exogenous [³H]adenosine and [³H]inosine, whereas the genetic lesions resulted in null transport phenotypes for ligands of the affected gene product (Fig. 2). Thus, the $\Delta ldn1$ and $\Delta ldn1/\Delta ldn2$ lines were unable to transport adenosine (Fig. 2, panel A), while the $\Delta ldn2$ and $\Delta ldn1/\Delta ldn2$ strains could not transport inosine (Fig. 2, panel B). Interestingly, adenosine transport into $\Delta ldn2$ cells was markedly up-regulated compared to wild type parasites. The mechanism for this apparent compensation is unknown.

3.3. Drug resistance profiles of the knockout lines

Previous analyses of nucleoside transport have indicated that tubercidin and formycin B are cytotoxic ligands for *LdNT1* and *LdNT2*, respectively [2–4]. To confirm the null transport phenotypes of the knockout lines, their sensitivities to tubercidin and formycin B were compared to that of wild type *L. donovani*. The effective concentrations of tubercidin that inhibited growth by 50% (EC₅₀ value) were 957- and 3,428-fold greater for the $\Delta ldn1$ and

$\Delta ldnt1/\Delta ldnt2$ strains than for wild type parasites (Table 1). Similarly, the calculated EC₅₀ values of the $\Delta ldnt2$ and $\Delta ldnt1/\Delta ldnt2$ null mutants to formycin B were 2,442- and 5,428-fold greater than the EC₅₀ value of the wild type parasites for the drug (Table 1). The reason that the double knockout exhibits a slightly and consistently greater resistance to tubercidin and formycin B than the pertinent single knockout is not known, but the data imply that both toxic nucleoside analogs could be very low affinity ligands for the other transporter.

3.4. Growth phenotype of the $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ promastigotes

The ability of $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ promastigotes to grow in various purines was compared. Whereas wild type and $\Delta ldnt1$ promastigotes could grow in medium supplemented with any of the added purine nucleobases or nucleosides tested, including hypoxanthine, adenine, guanine, xanthine, and their corresponding ribonucleosides, the $\Delta ldnt2$ and $\Delta ldnt1/\Delta ldnt2$ promastigotes could not proliferate in xanthosine as the sole purine nutrient (Fig. 3).

3.5. Xanthosine transport by *L. donovani*

The inability of $\Delta ldnt2$ and $\Delta ldnt1/\Delta ldnt2$ promastigotes to undergo a normal rate of growth in xanthosine implied that xanthosine is an heretofore undiscovered LdNT2 ligand. A comparison of xanthosine transport into wild type, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ parasites demonstrated that the uptake of xanthosine into wild type parasites was essentially absent in the two null mutants harboring the $\Delta ldnt2$ lesion (Fig. 4A). Substrate saturation curves with wild type *L. donovani* revealed that [³H]xanthosine transport displayed Michaelis-Menten kinetics with an apparent K_m value of ~43 μM (Fig. 4B).

3.6. Transformation of wild type, $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ promastigotes to axenic and intracellular amastigotes

The ability of $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ promastigotes to transform into axenic amastigotes was then assessed by shifting the parasites from promastigote to axenic amastigote growth medium. All three knockout lines experienced the same morphological transformation as the wild type LdBob line and were capable of continuous growth at 37 °C and pH 5.5. The axenic amastigotes could be transformed back to the flagellated promastigotes by the appropriate shifts in medium, temperature, and pH. The nucleoside transport phenotypes of all amastigote cell lines were equivalent to their promastigote counterparts (data not shown). Therefore, purine nucleoside transport in amastigotes also proceeds via LdNT1 and LdNT2. The ability of the null transport mutants to infect murine macrophages was also assessed. Wild type, $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ parasites were all capable of infecting and sustaining infections of J774 murine macrophages to roughly equivalent extents (Fig. 5). Similar data were obtained with bone marrow-derived macrophages as well (data not shown).

4. Discussion

To evaluate the contributions that LdNT1 and LdNT2 play in purine salvage in both life cycle stages of the parasite, $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ knockouts were constructed within a virulent *L. donovani* background using well-established gene replacement approaches [8,21–23]. Although mutational approaches have previously enabled the isolation and functional characterization of LdNT1- and LdNT2-deficient *L. donovani*, (TUBA5 and FBD5 strains, respectively) the defective genes in the TUBA5 and FBD5 strains harbored point mutations that rendered the encoded polypeptides transport-incompetent. TUBA5 and FBD5 cells synthesize *ldnt1* and *ldnt2* transcripts, respectively, and transfectants encompassing the mutant alleles tagged with green fluorescent protein at the NH₂-terminus produce transporter capable of targeting to the parasite cell surface [6,7]. Comparatively, therefore, the knockout lines, unlike the mutationally derived TUBA5 and FBD5 cells, provide an authentic null background to assess the role of nucleoside transporters in purine salvage, proliferation, and survival within

the mammalian host. Furthermore, the knockout strains provide clean backgrounds for transfection studies of heterologous or mutant transporters that are untainted by endogenously produced mutant protein. Finally, it should be noted that the $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ knockouts described in this investigation were generated in an *L. donovani* clone that is capable of sustaining an infection in host macrophages, thereby enabling a practical test of transporter function in amastigotes. The FBD5 and TUBA5 promastigotes were isolated from an avirulent, noninfectious strain of *L. donovani* [4] and therefore, the requirement for LdNT1 and LdNT2 involvement in promastigote transformation to the amastigote, infectivity of mammalian host cells, and their ability to proliferate as amastigotes could not be assessed.

The nucleoside transporter gene knockouts retained the ability to transform into axenic amastigotes and to infect and proliferate within murine macrophages. Thus, $\Delta ldnt1$, $\Delta ldnt2$, nor $\Delta ldnt1/\Delta ldnt2$ genotypes did not confer purine auxotrophy or loss of infectivity. Therefore, neither LdNT1 nor LdNT2, alone or in combination, is essential for promastigote or amastigote viability. The ability of $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ parasites to establish an infection in macrophages, where they reside in the phagolysosome, strongly implies that this mammalian host cell compartment is capable of supplying purines to meet the nutritional needs of the pathogen that are not ligands of either LdNT1 or LdNT2. These purines, presumably nucleobases, are transported into the parasite by other transporters, such as LdNT3 (J. Galazka and B. Ullman, unpublished), a homolog of the LmaNT3 nucleobase transporter that has been characterized from *L. major* [24]. Once inside the parasite, purine nucleobases can be salvaged to the nucleotide level by three phosphoribosyltransferase (PRT) enzymes, hypoxanthine-guanine PRT, adenine PRT, and xanthine PRT, and then distributed among the internal nucleotide pools by purine interconversion enzymes.

Unsurprisingly, the transport and growth phenotypes of the $\Delta ldnt1$ and $\Delta ldnt2$ were similar to the previously characterized TUBA5 and FBD5 cells, respectively, [4] and were therefore not analyzed in great detail. These characterizations did however reveal one unusual and previously unobserved feature of great utility: that parasites harboring a $\Delta ldnt2$ null genotype were incapable of efficient growth in xanthosine, a 6-oxypurine nucleoside, although they grew perfectly well on inosine and guanosine, two other LdNT2 ligands. Transport studies on wild type LdBob revealed xanthosine to be a relatively low affinity ligand for LdNT2 with a K_m value for the nucleoside ~40-fold and 15-fold greater than those previously obtained for LdNT2 for inosine and guanosine, respectively [3]. The molecular underpinnings for the discrepant growth of $\Delta ldnt2$ parasites in the three nucleoside ligands of LdNT2 are unclear, although it can be surmised that parasites and/or their culture medium is/are capable of cleaving inosine and guanosine but not xanthosine to their corresponding nucleobases. These nucleobases, hypoxanthine and guanine, can enter *L. donovani* through LdNT1- and LdNT2-independent mechanisms [4], e.g., LdNT3. Whether this nucleoside cleavage is catalyzed by residual mammalian purine nucleoside phosphorylase in the medium, by one of the nucleoside hydrolase activities that have been described in *L. donovani* [25], or some other activity will require additional dissection. The growth dependence on xanthosine, however, offers for the first time a positive selection for transport proficiency within the $\Delta ldnt2$ background, further enabling a genetic analysis of transport function in these parasites.

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Fig. 1.

Southern blot analysis of $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ knockouts. Genomic DNA from wild type (lane 1), *LdNT2/ldnt2* (lane 2), $\Delta ldnt2$ (lane 3), $\Delta ldnt1$ (lane 4), *LdNT1/ldnt1/Δldnt2* (lane 5), and $\Delta ldnt1/\Delta ldnt2$ (lane 6) parasites was digested with Eco R1, fractionated on 0.8% agarose gels, and blotted onto nylon membranes. Blots were hybridized under high stringency conditions with probes to the *LdNT1* ORF (panel A), the *LdNT1* 3' UTR (panel B), the *LdNT2* ORF (panel C), or the *LdNT2* 5'-UTR (panel D).

Fig. 2.

Transport of adenosine and inosine by $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ knockouts. The abilities of wild type (•), $\Delta ldnt1$ (○), $\Delta ldnt2$ (•), and $\Delta ldnt1/\Delta ldnt2$ (□) parasites to transport 1 μ M [3 H]adenosine (panel A) or 1 μ M [3 H]inosine (panel B) as a function of time was measured. Results are expressed as mean $\pm$ standard deviation ($n = 2$).

Fig. 3.

Growth phenotypes of wild type and knockout promastigotes. The abilities of wild type and knockout parasites to proliferate in purine-defined DME-L-based medium as a function of purine source is depicted.

Fig. 4.

Xanthosine transport in wild type and mutant *L. donovani*. The abilities of wild type (▪), Δ ldnt2 (▲), and Δ ldnt1/ Δ ldnt2 (▼) to transport 10 μM [³H]xanthosine as a function of time were assessed (panel A). Xanthosine transport as a function of ligand concentration was also assessed (panel B). The data reported in the figure are the results of two independent experiments.

Fig. 5.

Parasitemia of wild type and mutant lines. Infectivity assays using J774 murine macrophages were performed as described in Materials and Methods. The numbers of wild type, $\Delta ldnt1$, $\Delta ldnt2$, and $\Delta ldnt1/\Delta ldnt2$ parasites in J774 macrophages were enumerated visually after staining with propidium iodide. One hundred macrophages from each infection were counted. The averages and standard errors of two independent experiments are depicted.

Table 1

EC₅₀ values of wild type, $\Delta ldn1$, $\Delta ldn2$, and $\Delta ldn1/\Delta ldn2$ promastigotes for tubercidin and formycin B. The results are averages and standard errors for two independent measurements. The relative resistance of the $\Delta ldn1$, $\Delta ldn2$, and $\Delta ldn1/\Delta ldn2$ cell lines to both drugs compared to wild type parasites is indicated in parentheses.

	<u>Tubercidin (nM)</u>	<u>Formycin B (nM)</u>
Wild type	9.2 ± 2.7	1.62 ± 0.55
$\Delta ldn1$	8,800 ± 900 (957)	2.33 ± 0.76 (1.44)
$\Delta ldn2$	5.33 ± 0.96 (0.58)	3,956 ± 1389 (2442)
$\Delta ldn1/\Delta ldn2$	34,300 ± 4400 (3728)	8,793 ± 2598 (5428)