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An in vitro system for developmental and genetic studies of *Leishmania donovani* phosphoglycans

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Abstract

Glycoconjugates have been shown to play important roles in *Leishmania* development. However, the ability to study these molecules and other processes would benefit greatly from improved methods for genetic manipulation and analysis of the amastigote stage. This is especially challenging for *L. donovani*, the agent of the most severe form of leishmaniasis, which can rapidly lose virulence during in vitro culture. Here we report on a clonal subline of an *L. donovani* 1S2D (LdBob or LdB), which differentiates readily from promastigotes to amastigotes in axenic culture, and maintains this ability during extended parasite cultivation in vitro. This derivative can be plated and transfected efficiently while grown as promastigotes or amastigotes. Importantly, LdB maintains the ability to differentiate while undergoing genetic alterations required for creation of gene knockouts and complemented lines. Like virulent *L. donovani*, LdB exhibits down-regulation of lipophosphoglycan (LPG) synthesis and up-regulation of A2 protein synthesis in amastigotes. We showed that knockouts of *LPG2*, encoding a Golgi GDP-mannose transporter, eliminated phosphoglycan synthesis in LdB axenic amastigotes. These and other data suggest that LdB axenic amastigotes will be generally useful as a differentiation model in studies of gene expression, virulence, glycoconjugate function and drug susceptibility in *L. donovani*.

Keywords: Protozoan parasites; In vitro differentiation; Axenic amastigotes; Transfection; Phosphoglycan molecules (PG); Lipophosphoglycan (LPG); Virulence

1. Introduction

Protozoan parasites of the genus *Leishmania* are the agents of major tropical and subtropical parasitic diseases. Depending on the parasite species as well as host factors, infections are associated with different clinical presentations, ranging from mild cutaneous lesions to severe fatal visceral disease. The parasites alternate during their life cycle between the extracellular promastigote form in the midgut of the sand fly, and the amastigote form which resides within the phagolysosomes of macrophages of their mammalian hosts. Within the sand fly, the promastigotes replicate as the procyclic form. As the time for the next sand fly bite approaches, the parasite ceases replication and differentiates into the metacyclic form, which is adapted for entry and establishment of infections in the host macrophage. Following differentiation, the amastigotes are able to replicate and cause disease. Understanding how amastigotes accomplish this is central to our understanding of leishmaniasis and efforts to develop effective chemotherapy and vaccination strategies.

Methods for the laboratory study of the insect stages of *Leishmania* are well developed. While sand fly colonies can be maintained in the laboratory, it is difficult and laborious, and these are not widely available. Fortunately, the promastigote form can be readily grown in simple media in vitro, and differentiation to metacyclic form occurs upon parasite entry into stationary phase [1]. LPG, the dominant glycoconjugate on the surface of promastigotes, undergoes

Abbreviations: LPG, lipophosphoglycan; PG, phosphoglycan; sAcP, secreted acid phosphatase; PI, propidium iodide

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structural modifications during differentiation and has long served as a convenient marker for metacyclogenesis. Metacyclics can be readily purified by both LPG dependent and independent methods, allowing investigators convenient access to these two major promastigote stages [2,3]. Correspondingly, methods for genetic manipulation of promastigotes are well developed, including plating on semisolid media, gene knockouts and expression vectors, functional rescue and transposon mutagenesis (reviewed in [4]).

In contrast to the promastigote form, methods for the study of *Leishmania* amastigotes are less developed. There are well-characterized animal models for the different disease pathologies caused by different species of *Leishmania* [5,6]. Typically amastigotes must be studied in situ in animal infections, or following infection of macrophages in vitro. While use of macrophage-like cell lines facilitates this process, often parasites fail to replicate and/or survive in a manner seen in native infections or in primary macrophage infections. Furthermore, the numbers of amastigotes that can be recovered are relatively low. For genetic studies, investigators typically must manipulate promastigotes in vitro, followed by macrophage or mouse infections to generate amastigotes for study. This is accompanied by the difficulty that promastigotes can lose virulence during the culture periods required. In our experience this factor is particularly severe for species of the *L. donovani* complex, which are responsible for the most deadly form of the disease, visceral leishmaniasis [6,7].

For many species of *Leishmania* conditions have been reported which permit the parasites to grow in vitro in forms that resemble lesion-derived amastigotes to varying degrees. These 'in vitro' amastigotes also have been termed axenic amastigotes or amastigote-like forms [8–10]. While 'in vitro' amastigotes are not perfect replicas of authentic lesion-derived amastigotes, they can be sufficiently close so as to constitute superb models, and have been used widely in studies of metabolism and gene expression (reference cited previously). New World species seem particularly amenable to adaptation for growth as amastigotes in vitro, but success has been achieved with most *Leishmania* species, a prominent exception being *L. major*. Methods have been developed for species of the *L. donovani* complex (*L. donovani*, *L. infantum* and *L. chagasi*) and these amastigotes resemble lesion amastigotes by many criteria [11–15]. However, these methods have not been widely incorporated into genetic studies of amastigotes, or the study of abundant glycolipids implicated in parasite virulence such as the PGs.

Here we report on a clonal derivative (termed Ld Bob or LdB) of the *L. donovani* 1S2D line described by Dwyer and coworkers [11,16,17], which is ideally suited for the genetic analysis of amastigotes in vitro. The LdB line is readily plated and can be transfected efficiently for genetic manipulations, while retaining its ability to differentiate repeatedly between the promastigote and axenic amastigote stages. We characterized the expression of key *Leishmania* glycoconjugates, and generated LPG-deficient parasites that retain the ability to differentiate. These data establish for the first time that knockouts in the *LPG2* gene, which encodes a Golgi GDP-mannose transporter [18], eliminate PGs in the amastigote stage as well. Our data further suggest that similar to other in vitro amastigote systems, LdB has added potential for the study of gene expression and drug susceptibility. In studies done by others, the parent of the LdB line has been shown to maintain its ability to infect human macrophage cell lines in vitro and to cause disease in the hamster model of *L. donovani* infection, suggesting that these axenic amastigotes may provide a useful platform for future studies of parasite virulence [16].

2. Methods and materials

2.1. Materials

Adenosine, RPMI 1640 vitamins solutions (cat. no. R7256), RPMI 1640 amino acid solutions (50×; cat. no. R7131), and MES, were obtained from Sigma. Glutamine, penicillin/streptomycin, phenol red solution, and fetal bovine sera, were obtained from Gibco BRL. M199 with Hanks's Salts was obtained from US Biologicals (cat. no. M2852).

2.2. Leishmania culture and differentiation in vitro

The *L. donovani* strain 1S2D (WHO designation: MHOM/SD/62/1S-CL2D; [19]) studied here was derived by D. Dwyer and had been adapted for growth as amastigotes in vitro previously [11,16,17]. A clonal line was obtained by plating and named LdBob (abbreviated LdB), and maintained in culture by cycling between promastigotes and axenic amastigotes using protocols modified from prior work. Briefly, the promastigote form was grown at 26 °C in M199 media supplemented with 10% FBS, 25 mM Hepes pH 6.9, 12 mM $NaHCO_3$, 1 mM glutamine, 1× RPMI 1640 vitamin mix, 10 μM folic acid, 100 μM adenosine, 7.6 mM hemin, 50 U/ml of penicillin, and 50 μg/ml of streptomycin. For the amastigote form, the following base media was used: 15 mM KCl; 136 mM KH_2PO_4; 10 mM $K_2HPO_4{\cdot}3H_2O$; 0.5 mM $MgSO_4{\cdot}7H_2O$; 24 mM $NaHCO_3$; 22 mM glucose; 1 mM glutamine, 1× RPMI 1640 vitamin mix, 10 μM folic acid, 100 μM adenosine, 1× RPMI amino acid mix, 5 μg/ml hemin, 50 U/ml of penicillin, and 50 μg/ml of streptomycin, 0.0005% phenol red (from a 0.5% stock; Gibco cat. no. 15100-043), and 25 mM MES. To 1 l of base media, 256 ml of fetal bovine sera (heat inactivated) was added. Then the pH was adjusted with HCl to pH 5.66 at 22 °C, yielding a final pH of 5.5 at 37° (the pH of MES is temperature dependent). Amastigote forms were grown at 37 °C with 5% CO_2.

The volume of media in an amastigote culture flask must be maintained at low levels to ensure proper aeration (maximally 5 ml in a T25 flask or 15 ml in a T75). Parasites were

converted between forms by diluting cultures 1:100 into appropriate media, every 3–4 days. In some experiments, parasites were maintained as promastigotes or amastigotes by serially passaging at a 1:100 dilution in the same media. We refer to "passage 2 promastigotes" as culture passaged twice in promastigote media from the initial amastigote form. Under the conditions used here, one passage corresponds to 6–7 cell doublings.

For plating studies, parasites were spread on appropriate media containing 1% noble agar (Difco Laboratories, cat. no. 0142-17), supplemented with 2 μg/ml biopterin. A 2× media concentrate was made, and mixed with an equal volume of 2% agar in water; repeated heating of the 2% noble agar stock was strictly avoided.

Cell density was determined using a Coulter counter. Amastigotes (and to a lesser extent promastigotes) form large aggregates that can interfere in plating and Coulter counting; these were disrupted by passing cell suspensions five times through a 27 gauge needle, and dispersal was monitored microscopically. Cell viability was measured by propidium iodide exclusion and flow cytometry [20].

For drug sensitivity determinations, parasites were maintained for at least two passages in appropriate media, and then inoculated at a concentration of 10^5 cells/ml in the presence of serial dilutions of drug. Parasite numbers were determined using a Coulter counter as previously described after an incubation of 2 days for the amastigotes and 3 days for the promastigotes. The effective concentration for 50% inhibition (EC_{50}) was defined as that drug concentration that resulted in a 50% decrease in cell number, measured at the time when control cultures lacking drug had reached 10^7/ml [21].

2.3. Western blotting and detection of secretory acid phosphatase

Cells were first washed twice in 10 ml of phosphate-buffered saline (PBS). For blotting with the anti-serum CA7AE which recognizes the phosphoglycan [Gal(β1-4)Man(α 1-PO_4) → 6] repeating disaccharide units present on LPG, PPG and secretory acid phosphatase [22], 2–3 × 10^8 cells in 1 ml of 0.15N NaCl were suspended in 50 mM Tris–HCl, pH 8, 1 mM PMSF, and sonicated on ice with a probe sonicator for 20 s. 2× SDS sample buffer with 400 mM dithiothreitol was added and debris was pelleted at 15,000 × *g* for 5 min at 25 °C [23]. 5 × 10^6 cell equivalents were loaded per lane for 12% SDS–PAGE with a 5% stacker and run at 60 V. The gel was blotted to nitrocellulose membranes and blocked overnight with 5% milk in PBS. The next day, blotting was performed using 1:1000 CA7AE followed by 1:10,000 anti-mouse conjugated to HRP. Visualization was achieved by chemiluminescence (Pierce, Supersignal). For detection of the A2 protein using monoclonal antibody C9 [24], cell suspensions in PBS were directly mixed with SDS sample buffer and boiled 10 min at 100 °C. Samples were resolved by SDS–PAGE and electroblotted onto nitrocellulose membranes. Secondary antibodies were anti-mouse IgM (1:1000) or IgG (1:1000) conjugated to horse-radish peroxidase, and binding was monitored using the ECL detection system (Amersham-Pharmacia). Log-phase (5 × 10^6/ml) culture supernatants (25 μl) were separated by non-denaturing electrophoresis in 7% acrylamide gels [23] and stained for acid phosphatase activity as described [25].

2.4. Transfection

For efficiency tests, two different electroporation conditions were used: "low voltage" (500 μF, 450 V, 2 mm cuvette; 2.25 kV/cm) or "high voltage" (25 μF, 1500 V, 4 mm cuvette; 3.75 kV/cm); [26]. The test DNA was cosmid B1660, which contains the *L. major* H region inserted into the shuttle vector cLHYG [27,28]. Following electroporation, cells were incubated 24 h in media without drug, and plated on semisolid media containing either 100 or 300 μg/ml of hygromycin B, for promastigotes and amastigotes, respectively. Uncorrected transfection efficiencies are reported and consist of colonies obtained/parasites electroporated. For *LPG2* manipulations, only the high voltage protocol was used and parasites were grown as promastigotes. For transfections of constructs with the *BSD* marker, plates contained 20 μg/ml blasticidin D, and for transfections with the *NEO* marker, plates contained 15–30 μg/ml G418.

2.5. LPG flow cytometry

For FITC-ricin staining, log-phase parasites were suspended in DMEM medium (Gibco cat. no. 11885-076) at 1.1 × 10^7 cells/ml, and incubated 5 min on ice in the presence of 5 μg/ml FITC-ricin (Sigma). For CA7AE staining, log-phase parasites were diluted in M199 media to a final concentration of 5 × 10^5 cells/ml and incubated with antibody CA7AE (dilution 1:5000) for 10 min at 25 °C. Then a fluorescein-conjugated goat anti-mouse (Jackson) was added (dilution 1:5000) for additional 10 min. Flow cytometry was performed with a Becton Dickinson FACSCalibur system. Cells incubated only with the second antibody were negative controls.

2.6. Generation of an *lpg2*$^-$ null mutant by transfection and loss of heterozygosity (LOH)

LdB promastigotes were electroporated with 10 μg of purified *LPG2::HYG* targeting fragment (2.6 kb *Eco*RV–*Pvu*II fragment from pLPG2-KO [29]), and plated on media containing 50 μg/ml hygromycin B. A homozygous mutant was recovered from the heterozygotes by a loss of heterozygosity approach (LOH; [30]). The heterozygote was grown for several passages of increasing drug pressure, ultimately to 300 μg hygromycin B/ml. From this population LPG-deficient parasites were obtained by four rounds of selection against binding to the anti-PG antibody CA7AE

[28], alternating two passages as promastigotes and one passage as amastigote. From the resulting predominantly CA7AE-unreactive population, clonal lines were obtained by plating, and LPG-deficient ones identified by flow cytometry analysis. One representative clone (*lpg2*$^{-}$ clone 6.1) was selected for further study here. Southern blot analysis confirmed the loss of the *LPG2* gene.

To restore *LPG2* expression in the LdB *lpg2*$^{-}$ mutant, transfections were performed with several different *LPG2* expression plasmids, including pLeish-LPG2 [29], pX63NEO-LPG2 [30], and pSNBR-LmLPG2 (G. Späth, unpublished data). Analysis of LPG expression showed that in general the primary transfectants did not make normal levels of LPG, for unknown reasons. Since episomal expression can be elevated by increasing the copy number [21], we used a combined FACS/drug selection protocol to recover parasites showing wild-type LPG expression. One clonal line (clone 2) of the primary *lpg2*$^{-}$/pSNBR-LmLPG2 transfectants was labeled with FITC-ricin, and the 5% of cells showing the most intense labeling were recovered by flow cytometry, and inoculated into 10–80 μg/ml G418 in the presence of 2% galactose (first passage only). The LdB *lpg2*$^{-}$/+*LPG2* parasites grown in 20 μg/ml G418 showed LPG levels similar to that of the LdB parent.

2.7. Immunofluorescence microscopy

Parasites (harvested at 5×10^6 cells/ml) were fixed in PBS containing 2% formaldehyde and 0.05% glutaraldehyde for 30 min at 25 °C, and permeabilized using ice-cold ethanol. The fixed cells were blocked with 3% BSA in 50 mM NH_4Cl in PBS for 30 min at 25 °C. Fixed cells were probed with anti-CA7AE (1:400) for 1 h at 25 °C, washed three times with PBS and incubated with anti-mouse IgG Alexa 488 (1:400) for 1 h at 25 °C as the secondary antibody. Immunofluorescence and Nomarski microscopy were performed with a Nikon microscope.

3. Results

3.1. Viability in liquid media and plating on semisolid media

L. donovani LdB promastigotes and amastigotes were grown in vitro in appropriate media as described in Section 2, where they exhibited the expected morphologies (data not shown; [11]). A representative growth curve for these cells is shown in Fig. 1A; both the promastigote and amastigote stages grew well, with doubling times of ∼9 and ∼6 h, respectively.

Cell viability is a critical factor in genetic studies, and we had encountered difficulties in this parameter with some *L. donovani* lines and protocols [12]. Using a propidium iodide dye exclusion method, the viability of both stages of LdB was high (>97%; Fig. 1B). Upon entry into stationary phase the viability decreased slightly for promastigotes (95% after 2 days; Fig. 1B), consistent with similar studies with promastigotes of *L. major* (data not shown). Maintenance of stationary phase viability is critical, since promastigotes differentiate in stationary phase to the infective metacyclic form of the parasite [3,31]. In contrast, the viability of LdB amastigotes decreased more substantially in stationary phase, dropping to less than 90% after 2 days (Fig. 1). These data suggest that for maximum viability, log-phase amastigotes should be used.

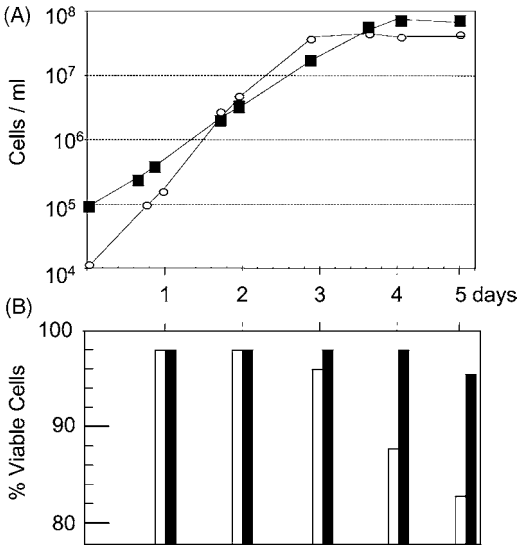

Fig. 1. Growth and viability of LdB promastigotes and amastigotes in vitro. Open and closed symbols represent LdB parasites grown under amastigote or promastigote conditions, respectively. Each point represents the average of three determinations. (A) Cell number; (B) cell viability (exclusion of PI).

Like other microbes, genetic studies of *Leishmania* benefit greatly from the incorporation of plating methods for the generation and enumeration of clonal parasite lineages. We examined the ability of LdB to generate colonies following plating of dispersed, single cell suspensions on appropriate semisolid media. Both promastigotes and amastigotes showed typical plating efficiencies for *Leishmania*, ranging from 5 to 10% for amastigotes to 12.5–14% for promastigotes (Table 1).

Table 1
Plating efficiency of *L. donovani* LdB on semisolid agar

Form plated	Semisolid media	Plating efficiency (%)
Promastigote	Promastigote	12.5–14
	Amastigote	5–8
Amastigote	Amastigote	5–10
	Promastigote	7

1000 parasites from log-phase LdB grown as promastigotes or amastigotes in appropriate media were plated on agar plates containing promastigote or amastigote media. Macroscopically-visible colonies were scored after 5 days for the amastigotes and after 7 days for the promastigotes media. The results of two independent experiments are shown.

We also examined the ability of the parasites to differentiate and form colonies simultaneously, by "cross-plating' promastigotes onto semisolid amastigote media and vice versa. In these experiments, the plating efficiencies were reduced slightly from those obtained when the media was not changed (5–8% for promastigotes plated on amastigote media, and 7% for amastigotes plated on promastigote media; Table 1). Parasites recovered from colonies on the plates were examined immediately by light microscopy and shown to have the morphology expected for the plating media (e.g. amastigotes on amastigote media plates).

3.2. Stability of amastigote differentiation during long-term culture as promastigotes

It was important to evaluate the ability of LdB to maintain the ability to differentiate in vitro, for periods sufficient for genetic manipulation. First, a freshly differentiated LdB culture was propagated as promastigotes in vitro for 1, 10 or 40 passages (these will be referred to as Pp1, Pp10 and Pp40). Then, promastigotes were plated on semisolid promastigote media, and colonies were recovered after 9 days. Cells were recovered, inoculated into amastigote growth media conditions, and scored visually for their ability to differentiate over the ensuing 48 h. A culture was scored as positive for differentiation if it grew well predominantly as amastigotes, with the percentage of promastigote forms less than 10%.

For both the Pp1 and Pp10 parasites, all 24 of the colonies tested (100%) grew and maintained the ability to differentiate into amastigotes. For the Pp40 parasites, 20 of the 24 colonies grew under amastigote growth conditions (84%), and all were able to differentiate. Therefore, LdB maintains the ability to differentiate through extended periods of culture as promastigotes, and during plating on semisolid media.

3.3. Drug susceptibility of differentiating L. donovani

Leishmania species and strains can differ greatly in their sensitivities to agents commonly used with selective markers in DNA transfections, and growth media also has a significant effect [32]. We determined the susceptibility of LdB, grown either as promastigotes or amastigotes, to several of the drugs in common usage (Table 2). For most drugs promastigotes and amastigotes showed similar susceptibilities, the exception being hygromycin B where amastigotes were nearly 10 times less susceptible (9 μg/ml versus 80 μg/ml for promastigotes versus amastigotes, respectively).

We tested the sensitivity of LdB amastigotes and promastigotes to several anti-leishmanial agents. The primary anti-leishmanial agents used clinically are pentavalent antimonial compounds [33], and the LdB amastigotes were 30-fold more susceptible to sodium stibogluconate than promastigotes (166 μg/ml versus >5000 μg/ml; Table 2). This differential stems from the ability of amastigotes to reduce SbV to toxic SbIII derivatives [34], and accordingly the susceptibility of amastigotes and promastigotes were less pronounced with SbIII (3.5 μg/ml versus 14 μg/ml; Table 2). This was also seen when promastigotes were grown in amastigote media at 26 °C, suggesting that the effect arises from parasite stage, and not the acid media pH or other factors. Similar results have been found by others with a variety of axenic *Leishmania* amastigotes [13,14,35].

Table 2
Sensitivity of *L. donovani* LdB promastigotes and axenic amastigotes to drugs used in genetic selections and for treatment

Drug	EC_{50} (μg/ml)[a]	
	Promastigote	Amastigote
Drugs used commonly in genetic manipulations		
G418	3	3
Hygromycin B	9	80
Nourseothricin	5	10
Blasticidin	5	2
Phleomycin	0.8	2
Drugs used or relevant to the clinical treatment of leishmaniasis		
SbV[b]	>5000	166
Sb3[c]	14	3.5
Allopurinol	7	62
Paromomycin	6	6

[a] Assays are described in the experimental procedures; the results are the mean of two duplicate experiments, differing by less than 10%.
[b] Sodium stibogluconate.
[c] $SbCl_3$.

Paromomycin is an aminoglycoside related to the selective agent G418, and as for this compound the susceptibilities of amastigotes and promastigotes were similar (Table 2). LdB amastigote were 10-fold less susceptible to the purine analog allopurinol (Table 2). Again, this was shown to arise from developmental stage and not media pH (data not shown).

3.4. Stable transfection of L. donovani LdB promastigotes and amastigotes

To test the ability of LdB to be transfected as either promastigotes or amastigotes, we used a 45 kb cosmid bearing ~35 kb of *L. major* DNA inserted into the *Leishmania* shuttle vector cLHYG (cosmid B1660). Cosmid vectors are used commonly in functional genetic rescue studies in *Leishmania*. We tested two electroporation conditions, a 'low voltage' protocol used commonly for *Leishmania* [21] and a recent modification termed the 'high voltage' protocol [26]. Parasites were electroporated as either promastigotes or amastigotes in the presence of cosmid B1660, and plated on homologous growth medium containing 100 or 300 μg hygromycin B/ml (promastigotes or amastigotes, respectively).

Several findings emerged (Fig. 2). The high-voltage electroporation conditions were superior for amastigotes as well as for promastigotes, giving on average 5–8-fold more colonies under equivalent conditions, as seen previously with smaller reporter constructs [26]. Increasing amounts

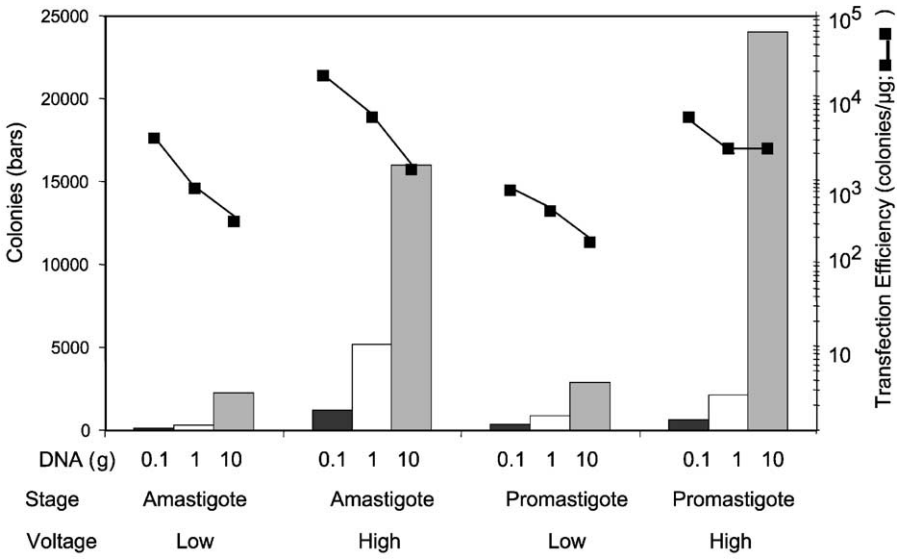

Fig. 2. Stable transfection of LdB promastigotes and amastigotes. Parasites were electroporated with the indicated amount of cosmid B1660 DNA using "low" or "high" voltage conditions (Section 2); parasites were plated on media containing 100 or 300 μg/ml hygromycin B for promastigotes and amastigotes, respectively. The number of colonies obtained (left axis) and the uncorrected transfection efficiency (colonies/μg DNA; right axis) are shown. The results shown are the average of two duplicate experiments that differ by less than 10%. Controls showed that the plating efficiency for untransfected cells was 5 and 15% for amastigotes and promastigotes on their respective media.

of exogenous DNA led to increasing numbers of colonies recovered, although efficiency/μg DNA declined 3–10-fold at the higher DNA levels, as seen previously (Fig. 2; [21]). Both promastigotes and amastigotes showed similar transfection efficiencies, with up to 15,000–25,000 colonies recovered using 10 μg DNA and the high-voltage procedure (Fig. 2). This represented an efficiency of nearly 0.1% per cell electroporated, about 10-fold less than obtained by transfection of promastigotes with smaller constructs [26].

3.5. Generation of *lpg2*$^-$ null mutant amastigotes in the LdB background

An LdB line lacking the *LPG2* gene (termed *lpg2*$^-$) was generated by standard gene replacement methods, and a loss-of-heterozygosity protocol was used to generate a homozygous knockout from a single heterozygous replacement [29,30,36]. Flow cytometry showed that these parasites lacked LPG, as judged by reactivity with β-Gal binding lectin ricin agglutinin, or the anti-PG repeat antibody CA7AE (Fig. 3 or data not shown). Loss of *LPG2* was confirmed by Southern blot analysis of the genomic DNA (data not shown). LPG expression was restored in the *lpg2*$^-$ knockout by transfection of an episomal *LPG2* expression pSNBR-LmLPG2, and FACS analysis showed that they now abundantly expressed LPG on their surface (Fig. 3, *lpg2*$^-$/+*LPG2*). The morphology of single cells by light microscopy was identical for the three lines (Fig. 4), although the *lpg2*$^-$ line had a tendency to form clumps in both stages. This has been also observed for other LPG deficient lines generated in virulent parasite backgrounds in both *L. donovani* and *L. major* (K. Zhang, R. Zufferey and G Späth, personal communications). Immunofluorescence microscopy showed that the *lpg2*$^-$ parasites lacked PGs detectable by reactivity with the CA7AE antibody (Fig. 4).

Notably, the LdB *lpg2*$^-$ mutant and its 'add-back' line *lpg2*$^-$/+*LPG2* retained the ability to differentiate fully to amastigotes when placed under appropriate growth conditions in vitro. This was established by morphological and growth criteria (Fig. 4 or data not shown), and expression of the amastigote-specific A2 protein family, which comprises multiple proteins ranging from 35 to 110 kDa [24]. Western blot analysis with anti-A2 antisera showed that as expected, LdB did not express A2 proteins when grown as promastigotes, but expressed them abundantly when grown as amastigotes (Fig. 5). The LdB *lpg2*$^-$ and *lpg2*$^-$/+*LPG2* add-back showed a similar pattern of amastigote-specific A2 protein family expression (Fig. 5). These amastigote preparations were made just 48 h after inoculation of promastigotes into amastigote growth conditions, suggesting that the rate of differentiation was comparable as well.

3.6. PG synthesis in axenic L. donovani promastigotes, amastigotes and lpg2$^-$ knockouts

Western blot analysis with the anti-PG antibody CA7AE against varying amounts of LdB amastigotes and promastigotes showed that amastigotes expressed 5–10-fold less LPG than promastigotes, using α-tubulin as a loading control (Fig. 6, lower panel). The magnitude of down-regulation was considerably less than the >1000-fold shown previously in amastigotes derived from animal infections by *L. major* and *L. donovani* [37,38]. One potential explanation was that differentiation of the parasites was incomplete,

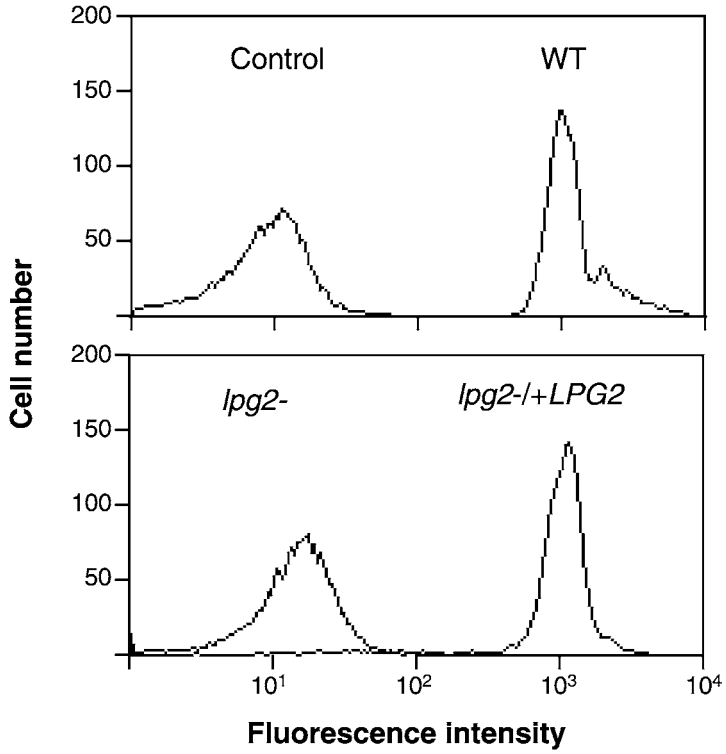

Fig. 3. Flow cytometric analysis of LPG expression of LdB and *lpg2*$^-$ derivatives. LdB (WT), *lpg2*$^-$ and *lpg2*$^-$/+*LPG2* parasites were reacted with the anti-PG antibody CA7AE as described in Section 2, and subjected to flow cytometry. In this experiment, the *lpg2*$^-$/+*LPG2* 'add-back' was LdB *lpg2*$^-$ transfected with pLeish-LPG2 [29] and grown in 50 μg/ml blasticidin. Control refers to WT parasites where CA7AE was omitted.

with promastigotes remaining in the ostensibly 'amastigote' preparation. However, immunofluorescence microscopy or flow cytometry with the anti-PG antibody CA7AE suggested that the amastigote population was homogeneous (Fig. 4 and data not shown).

We examined two other protein families that bear the same PG repeat modification as LPG. PPGs are typically large proteins, some of which localize to the parasite surface by GPI anchors [39,40]. In standard SDS–PAGE, PPGs typically run in the stacking gel/interface region (Figs. 6 and 7).

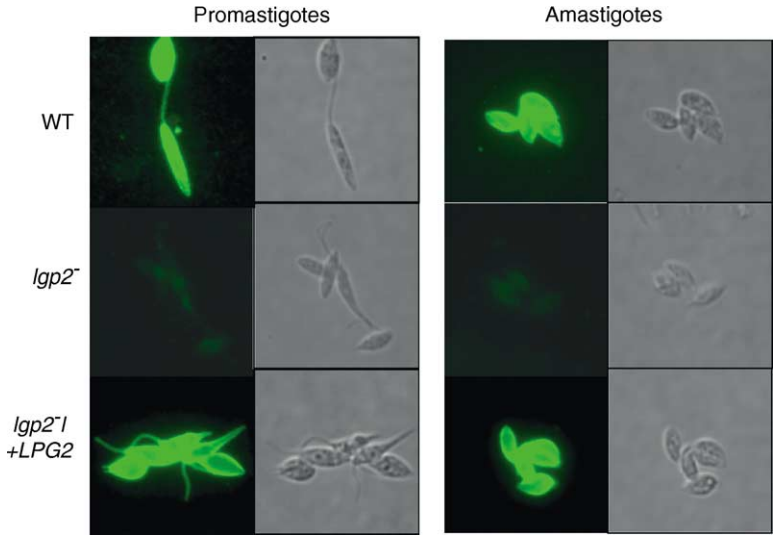

Fig. 4. PG expression in WT and *lpg2*$^-$ LdB promastigotes and amastigotes. Promastigotes or amastigotes of LdB (WT), *lpg2*$^-$ and *lpg2*$^-$/+*LPG2* lines were visualized by Nomarski or immunofluorescence analysis with anti-PG antibody CA7AE. The exposure times for *lpg2*$^-$ promastigotes and amastigote immunofluorescence image was 4 s, for all other lines it was 1 s.

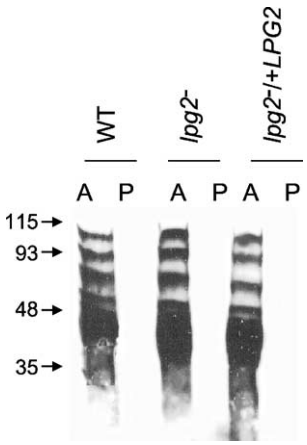

Fig. 5. Expression of the A2 protein family in promastigotes and amastigotes of LdB and *lpg2*$^{-}$ derivatives. 5×10^6 LdB cells grown as amastigotes (A) or promastigotes (P) were subjected to Western blotting with anti-*L. donovani* A2 monoclonal antibody. The migration of molecular weight markers (in kDa) are shown. Ponceau S staining of the blotted membrane showed equivalent protein loading (not shown).

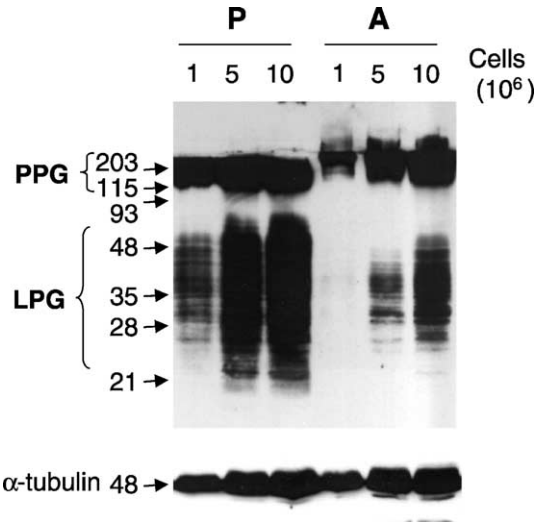

Fig. 6. Western blot analysis of phosphoglycan expression in LdB promastigotes and amastigotes. LdB promastigotes (P) or amastigotes (A) were grown in appropriate media to log-phase, and extracts corresponding to 1×10^6, 5×10^6 or 10×10^6 cells were subjected to Western blot analysis for PG expression with the CA7AE monoclonal antibody (upper panel) or with anti-α-tubulin antibody (lower panel). The regions corresponding to LPG and PPG are shown in brackets, and the migration of molecular weight markers (in kDa) is indicated.

PPG was expressed by amastigotes, at levels somewhat lower than in promastigotes (Fig. 6). Secretory acid phosphatase (sAP) is encoded by a small gene family and this protein is secreted into the growth media by both promastigotes and amastigotes [41]. sAP was detected following non-denaturing electrophoresis and staining for enzymatic activity. In vitro, sAP was secreted by both stages (Fig. 7C), and the electrophoretic mobility of amastigote sAP was slower than promastigote sAP (Fig. 7C), in agreement with previous findings with *L. donovani* amastigotes grown in macrophages [42].

As expected from previous studies of *lpg2*$^{-}$ mutants derived in avirulent backgrounds, the LdB promastigotes

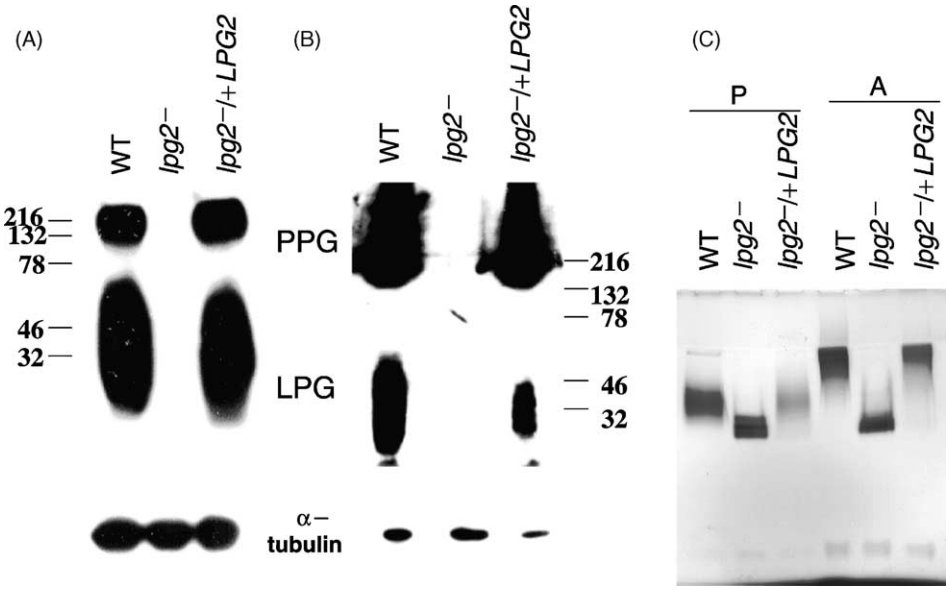

Fig. 7. Phosphoglycan expression in *lpg2*$^{-}$ and 'add-back' LdB derivatives. LdB, *lpg2*$^{-}$ and *lpg2*$^{-}$/+*LPG2* were grown in appropriate media to log-phase. (Panel A) Promastigotes. Parasites were grown in promastigote media and extracts corresponding to 1×10^6 cells were subjected to Western blot analysis for PG expression with the CA7AE monoclonal antibody (upper panel) or with anti-α-tubulin (lower panel). The regions corresponding to LPG and PPG are marked, and molecular weight markers (kDa) are shown. (Panel B) In vitro amastigotes. Parasites were grown in amastigote media and analyzed as described in panel A. (Panel C) sAP. Culture media supernatants from promastigotes (P) and amastigotes (A) were separated by non-denaturing electrophoresis and stained for acid phosphatase activity. In this system mobility is not related in a simple way to molecular weight.

completely lacked PGs, as judged by a lack of reactivity with anti-PG antisera (Figs. 7A and 4). Significantly, we were able to assess PG synthesis in the amastigote stage for the first time, and as for promastigotes, Western blot analysis showed that the *lpg2*$^{-}$ amastigotes lacked PG-containing glycoconjugates (Fig. 7B). For sAP, both promastigote and amastigote forms of *lpg2*$^{-}$ LdB expressed normal levels of sAP, whose electrophoretic mobilities were the same, and both faster than that of wild-type LdB (Fig. 7C). In *L. donovani* promastigotes we showed previously that this electrophoretic shift accompanies loss of PG repeats [30]. The similar mobility of promastigote and amastigote sAP in the Ld *lpg2*$^{-}$ mutant argues that it likewise completely lacks all PG repeats.

In combination with the LPG results above, these data show for the first time that the LdB *lpg2*$^{-}$ amastigotes lack all PGs. This suggests that they do not express a stage-specific GDP-mannose transport activity necessary for the assembly of PGs, redundant with LPG2. For both parasite stages, re-expression of *LPG2* restored LPG and PPG synthesis to wild-type levels (Fig. 7A and B; lanes *lpg2*$^{-}$/+*LPG2*), and restored the mobility of the sAP to that of WT (Fig. 7C).

4. Discussion

4.1. Properties of LdB advantageous to genetic studies of *Leishmania* differentiation and virulence

In this work we have described a clonal line of *L. donovani* (LdBob or LdB), whose properties render it suitable for studies of *Leishmania* differentiation in vitro. While other strains or sublines of this species have been described previously, including derivatives of the 1S2D parent of LdB, in our experience LdB performs better and more reproducibly in the properties examined. Moreover, the protocols and LdB line described here are robust enough to work well in at least four different laboratories thus far. Relevant properties of LdB established in this work are summarized below, and other properties of the parental *L. donovani* 1S2D line are described by Dwyer and coworkers [16].

4.1.1. In vitro differentiation to amastigote-like forms

LdB differentiates readily between both developmental stages in vitro, into forms that resemble authentic *L. donovani* amastigotes reasonably well. Besides morphological criteria, this was shown by studies of several stage-specific markers including LPG, sAP and the A2 protein family (Figs. 4–7), and drug susceptibility (Table 2). In vitro amastigotes abundantly expressed the A2 protein family, whereas this protein was absent in promastigotes. The overall expression of the *Leishmania* glycoconjugates studied resembled that of authentic amastigotes, as discussed more extensively in Section 4.2.

4.1.2. Viability and growth

Both stages of the parasite showed rapid growth in appropriate media during in vitro culture (Fig. 1A). Importantly, the viability of the cells was high, although upon entry into stationary phase amastigote viability was decreased 20% (Fig. 1B). This may arise from the tendency of the parasites to form clumps and aggregate at the bottom of the flask. Both of these criteria are key parameters in distinguishing healthy in vitro amastigotes from unhealthy cells, which typically round up and appear superficially similar to amastigotes.

4.1.3. Plating efficiency

Both forms of the parasite could be plated on semisolid media with good efficiencies, and ‘cross-platings’ work with little reduction in efficiencies.

4.1.4. High transfection efficiency

The LdB line showed high stable transfection efficiencies as both promastigote and amastigotes, yielding upwards of 20,000 colonies/transfection under appropriate conditions (Fig. 4). The efficiencies obtained were comparable to those obtained previously with promastigotes of an avirulent derivative of *L. donovani* 1S [28], and with constructs smaller than the cosmid tested here the efficiency can be as high as 3%/electroporated cell [26]. In previous studies we showed that the transient transfection efficiency of LdB promastigotes was also high (∼20%; [26]). These high efficiencies are of great value in a variety of genetic approaches, including functional genetic rescue with genomic DNA libraries in cosmids or other vectors [43]. The ability to modulate the transfection efficiency allows the investigator to choose the conditions appropriate for generating knockout mutants (where the input DNA levels should be kept relatively low in order to minimize the homologous integration of multiple copies; [44]).

4.1.5. Stability of differentiation phenotype during in vitro culture and transfection

A significant challenge in genetic studies of parasite virulence is the tendency of *Leishmania* to lose virulence during in vitro culture. In controlled tests in which LdB was passed exclusively in the promastigote form, we found that the ability to differentiate to amastigotes in vitro was stable through 20 passages of routine promastigote culture, and only showed a slight reduction after 40 passages. Importantly, the ability to differentiate was maintained through one round of stable transfection, followed by a loss of heterozygosity protocol to generate *lpg2*$^{-}$ mutations, and then after reintroduction of LPG2 constructs and restoration of LPG biosynthesis (Figs. 4, 5, and 7). In this regards LdB differs from other lines of *L. donovani* used previously in genetic studies of LPG biosynthesis [45]. Transfection of the parental line of LdB with episomal expression constructs has also been used to probe the effects of calreticulin domains in macrophage survival [46].

4.1.6. Maintenance of virulence in macrophage and animal infections

Of particular significance is the fact that this line of *L. donovani* been shown to maintain its ability to infect human macrophage cell lines in vitro, and to cause disease in the hamster model of *L. donovani* infection [16], despite having been grown in extensively in culture (with regular cycling between stages in vitro). In preliminary studies involving mouse infections, LdB shows virulence comparable to standard fully virulent *L. donovani* lines (M. Wilson, SG and SMB, unpublished data). Collectively, these data suggest that these axenic amastigotes may provide a useful platform in the future for the study of parasite virulence.

Overall, the properties of LdB suggest that it will provide an excellent model for the study of many processes relevant to *L. donovani* biology, allowing investigators to overcome previous limitations for working with *L. donovani*, both in vitro and in vivo. This may include the examination of gene expression during developmental transitions through DNA microarray and proteomic approaches, especially since it seems likely that good synchronicity may be possible following initiation of differentiation of LdB from promastigote to amastigote differentiation (or vice versa) in vitro. Another example of this concerns our studies of LPG biosynthetic genes, discussed below. Other investigators have emphasized the importance of studying drug susceptibility and metabolism in the amastigote form of the parasite, and have shown that in many cases in vitro amastigotes offer good models for this [13–15,34]. In this respect LdB seems to offer similar advantages; for example, LdB amastigotes are much more sensitive to the pentavalent antimonials than promastigotes (Table 2), as seen in amastigotes grown in macrophages.

4.2. LdBob and the study of amastigote glycoconjugates

Our data show that the LdB line is a suitable platform for biochemical and genetic studies probing the expression of *Leishmania* glycoconjugates in the amastigote stage. LdB amastigotes showed down-regulation of LPG expression, although it was ‘leaky’ relative to lesion amastigotes (10-fold rather than >1000-fold down-regulated; [37,38]). LdB amastigotes maintained expression of sAP levels comparable to that of promastigotes, and showed a reduction in sAP electrophoretic mobility relative to promastigotes comparable to that seen in lesion amastigotes [41,42]. While the structural basis underlying decreased sAP mobility was not determined, previous studies suggest that this may arise from increasing numbers of PG repeats [30,47]. Our studies also suggest that LdB amastigotes maintain high levels of PPG as well, as seen previously in *L. mexicana* and *L. major* [48].

We then applied these data to the question of whether *L. donovani* has an amastigote-specific pathway for GDP-mannose transport, distinct from that mediated by *LPG2* which has been studied extensively in promastigotes [18,30]. Previous work has focused on genes implicated in promastigote-specific pathways (such as those leading to LPG synthesis) or ones involved in both stages (such as PG biosynthesis). However, amastigotes are known to differ in the abundance and types of glycoconjugates expressed, suggesting that amastigote-specific steps and regulation occur as well [49]. Importantly, the effects of *lpg2*$^{-}$ null mutants on PG expression had not been previously examined in amastigotes from any species [50]. Our studies rule out the possibility of an amastigote-specific LPG2-independent GDP-mannose transporter activity, here in *L. donovani* and also in *L. major* [51]. It is significant that the LdB parasites showed that even in a relatively ‘leaky’ LPG background, PG expression was abolished in amastigotes (Fig. 6).

This observation may be relevant in futures studies seeking to probe the difference in survival of *lpg2*$^{-}$ parasites seen in different parasite species [52]. While *lpg2*$^{-}$ null mutants in *L. mexicana* remain virulent, this mutation completely abrogates the ability of *L. major* to replicate as amastigotes within macrophages and cause disease [50–52]. This suggested that different species vary in their emphasis on various parasite molecules for survival and virulence, and their interactions with host defenses and the immune response [52]. Since previous *lpg2*$^{-}$ knockouts of *L. donovani* had lost the ability to differentiate in culture, and their add-back mutants remain avirulent, it has not been possible to test the effect of *lpg2*$^{-}$ mutations on virulence (unpublished data; [29,30]). With the availability of *lpg2*$^{-}$ LdB mutants we are now in a position to probe the role of PG expression in *L. donovani* virulence for the first time. These lengthy studies are now in progress (M. Wilson, SG and SMB).

In summary, because of their ease of cultivation, resemblance to authentic amastigotes, ability to be propagated and genetically manipulated extensively in vitro while maintaining virulence and differentiation, LdB axenic amastigotes constitute a valuable tool for the investigation of many processes in *L. donovani*. This is especially important given that this species is responsible for the most severe, fatal form of leishmaniasis.

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References

[1] Sacks DL. Metacyclogenesis in *Leishmania* promastigotes. Exp Parasitol 1989;69:100–3.

[2] Späth GF, Beverley SM. A lipophosphoglycan-independent method for isolation of infective *Leishmania* metacyclic promastigotes by density gradient centrifugation. Exp Parasitol 2001;99:97–103.
[3] Sacks DL, Perkins PV. Identification of an infective stage of *Leishmania* promastigotes. Science 1984;223:1417–9.
[4] Beverley SM. Genetic and genomic approaches to the analysis of *Leishmania* virulence. In: Marr JM, Nilsen T, Komuniecki R, editors. Molecular and medical parasitology. New York: Academic Press; 2003. p. 111–22.
[5] Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to *Leishmania major* in mice. Nat Immunol 2002;2:845–58.
[6] Molyneux DH, Ashford RW. The biology of *Trypanosoma* and *Leishmania*, parasites of man and domestic animals. London: Taylor and Francis; 1983.
[7] De T, Roy S. Infectivity and attenuation of *Leishmania donovani* promastigotes: association of galactosyl transferase with loss of parasite virulence. J Parasitol 1999;85:54–9.
[8] Pan AA, Duboise M, Eperon S, Rivas L, Hodgkinson V, Traub-Cseko Y, et al. Developmental life cycle of *Leishmania*: cultivation and characterization of cultured extracellular amastigotes. J Euk Microbiol 1993;40:213–23.
[9] Gupta N, Goyal N, Rastogi AK. In vitro cultivation and characterization of axenic amastigotes of *Leishmania*. Trends Parasitol 2001;17:150–3.
[10] Bates PA. Axenic culture of *Leishmania* amastigotes. Parasitol Today 1993;9:143–6.
[11] Joshi M, Dwyer DM, Nakhasi HL. Cloning and characterization of differentially expressed genes from in vitro-grown 'amastigotes' of *Leishmania donovani*. Mol Biochem Parasitol 1993;58:345–54.
[12] Saar Y, Ransford A, Waldman E, Mazareb S, Amin-Spector S, Plumblee J, et al. Characterization of developmentally-regulated activities in axenic amastigotes of *Leishmania donovani*. Mol Biochem Parasitol 1998;95:9–20.
[13] Sereno D, Lemesre JL. Axenically cultured amastigote forms as an in vitro model for investigation of antileishmanial agents. Antimicrob Agents Chemother 1997;41:972–6.
[14] Sereno D, Cavaleyra M, Zemzoumi K, Maquaire S, Ouaissi A, Lemesre JL. Axenically grown amastigotes of *Leishmania infantum* used as an in vitro model to investigate the pentavalent antimony mode of action. Antimicrob Agents Chemother 1998;42:3097–102.
[15] Sereno D, Roy G, Lemesre JL, Papadopoulou B, Ouellette M. DNA transformation of *Leishmania* infantum axenic amastigotes and their use in drug screening. Antimicrob Agents Chemother 2001;45:1168–73.
[16] Debrabrant A, Joshi M, Pimenta PFP, Dwyer DM, in preparation.
[17] Duncan R, Alvarez R, Jaffe CL, Wiese M, Klutch M, Shakarian A, et al. Early response gene expression during differentiation of cultured *Leishmania donovani*. Parasitol Res 2001;87:897–906.
[18] Ma D, Russell DG, Beverley SM, Turco SJ. Golgi GDP-mannose uptake requires *Leishmania* LPG2. A member of a eukaryotic family of putative nucleotide-sugar transporters. J Biol Chem 1997;272:3799–805.
[19] Dwyer DM. Antibody-induced modulation of *Leishmania donovani* surface membrane antigens. J Immunol 1976;117:2081–91.
[20] Jacobs DB, Pipho C. Use of propidium iodide staining and flow cytometry to measure anti-mediated cytotoxicity: resolution of complement-sensitive and resistant target cells. J Immunol Methods 1983;62:101–8.
[21] Kapler GM, Coburn CM, Beverley SM. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. Mol Cell Biol 1990;10:1084–94.
[22] Tolson DL, Turco SJ, Beecroft RP, Pearson TW. The immunochemical structure and surface arrangement of *Leishmania donovani* lipophosphoglycan determined using monoclonal antibodies. Mol Biochem Parasitol 1989;35:109–18.
[23] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press; 1989. p. 1–1000.
[24] Zhang WW, Charest H, Ghedin E, Matlashewski G. Identification and overexpression of the A2 amastigote-specific protein in *Leishmania donovani*. Mol Biochem Parasitol 1996;78:79–90.
[25] Katakura K, Kobayashi A. Acid phosphatase activity of virulent and avirulent clones of *Leishmania donovani* promastigotes. Infect Immun 1988;56:2856–60.
[26] Robinson K, Beverley SM. Improved methods for transfection and tests of RNA interference (RNAi) activity in *Leishmania*. Mol Biochem Parasitol 2003;128:217–28.
[27] Cotrim PC, Garrity LK, Beverley SM. Isolation of genes mediating resistance to inhibitors of nucleoside and ergosterol metabolism in *Leishmania* by overexpression/selection. J Biol Chem 1999;274:37723–30.
[28] Ryan KA, Dasgupta S, Beverley SM. Shuttle cosmid vectors for the trypanosomatid parasite *Leishmania*. Gene 1993;131:145–50.
[29] Scianimanico S, Desrosiers M, Dermine JF, Meresse S, Descoteaux A, Desjardins M. Impaired recruitment of the small GTPase rab7 correlates with the inhibition of phagosome maturation by *Leishmania donovani* promastigotes. Cell Microbiol 1999;1:19–32.
[30] Descoteaux A, Luo Y, Turco SJ, Beverley SM. A specialized pathway affecting virulence glycoconjugates of *Leishmania*. Science 1995;269:1869–72.
[31] Sacks D, Kamhawi S. Molecular aspects of parasite-vector and vector-host interactions in leishmaniasis. Annu Rev Microbiol 2001;55:453–83.
[32] Descoteaux A, Garraway LA, Ryan KA, Garrity LK, Turco SJ, Beverley SM. Identification of genes by functional complementation in the protozoan parasite *Leishmania*. In: Adolph KW, editor. Methods in molecular genetics, vol. 3. San Diego: Academic Press; 1994. p. 22–48.
[33] Berman JD. Human leishmaniasis: clinical, diagnostic, and chemotherapeutic developments in the last 10 years. Clin Infect Dis 1997;24:684–703.
[34] Shaked-Mishan P, Ulrich N, Ephros M, Zilberstein D. Novel intracellular SbV reducing activity correlates with antimony susceptibility in *Leishmania donovani*. J Biol Chem 2001;276:3971–6.
[35] Ephros M, Bitnun A, Shaked P, Waldman E, Zilberstein D. Stage-specific activity of pentavalent antimony against *Leishmania donovani* axenic amastigotes. Antimicrob Agents Chemother 1999;43:278–82.
[36] Gueiros-Filho FJ, Beverley SM. Selection against the dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) locus as a probe of genetic alterations in *Leishmania major*. Mol Cell Biol 1996;16:5655–63.
[37] McConville MJ, Blackwell JM. Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani*. Characterization of the promastigote and amastigote glycolipids. J Biol Chem 1991;266:15170–9.
[38] Turco SJ, Sacks DL. Expression of a stage-specific lipophosphoglycan in *Leishmania major* amastigotes. Mol Biochem Parasitol 1991;45:91–9.
[39] Foth B, Piani A, Curtis JM, T., I, McConville M, Handman E. *Leishmania major* proteophosphoglycans exist as membrane-bound and soluble forms and localise to the cell membrane, the flagellar pocket and the lysosome. Int J Parasitol 2002;32:1701–8.
[40] Ilg T, Montgomery J, Stierhof YD, Handman E. Molecular cloning and characterization of a novel repeat-containing *Leishmania major* gene, *PPG1*, that encodes a membrane-associated form of proteophosphoglycan with a putative glycosylphosphatidylinositol anchor. J Biol Chem 1999;274:31410–20.
[41] Ellis SL, Shakarian AM, Dwyer DM. *Leishmania*: amastigotes synthesize conserved secretory acid phosphatases during human infection. Exp Parasitol 1998;89:161–8.

[42] Doyle PS, Dwyer DM. *Leishmania*: immunochemical comparison of the secretory (extracellular) acid phosphatases from various species. Exp Parasitol 1993;77:435–44.
[43] Ryan KA, Garraway LA, Descoteaux A, Turco SJ, Beverley SM. Isolation of virulence genes directing surface glycosyl-phosphatidylinositol synthesis by functional complementation of *Leishmania*. Proc Natl Acad Sci USA 1993;90:8609–13.
[44] Cruz A, Beverley SM. Gene replacement in parasitic protozoa. Nature 1990;348:171–3.
[45] Beverley SM, Turco SJ. Lipophosphoglycan (LPG) and the identification of virulence genes in the protozoan parasite *Leishmania*. Trends Microbiol 1998;6:35–40.
[46] Debrabant A, Lee N, Pogue GP, Dwyer DM, Nakhasi HL. Expression of calreticulin P-domain results in impairment of secretory pathway in *Leishmania donovani* and reduced parasite survival in macrophages. Int J Parasitol 2002;32:1423–34.
[47] Lippert DN, Dwyer DW, Li F, Olafson RW. Phosphoglycosylation of a secreted acid phosphatase from *Leishmania donovani*. Glycobiology 1999;9:627–36.
[48] Ilg T. Proteophosphoglycans of *Leishmania*. Parasitol Today 2000;16:489–97.
[49] Ilgoutz SC, McConville MJ. Function and assembly of the *Leishmania* surface coat. Int J Parasitol 2001;31:899–908.
[50] Ilg T, Demar M, Harbecke D. Phosphoglycan repeat-deficient *Leishmania* mexicana parasites remain infectious to macrophages and mice. J Biol Chem 2001;276:4988–97.
[51] Späth G, Lye LF, Segawa H, Sacks DL, Turco SJ, Beverley SM. Genetic separation of the acute and persistent phases in mouse infections by *Leishmania major* [submitted for publication].
[52] Turco SJ, Späth GF, Beverley SM. Is lipophosphoglycan a virulence factor? A surprising diversity between *Leishmania* species. Trends Parasitol 2001;17:223–6.