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Short communication

Differential modulation of macrophage response elicited by glycoinositolphospholipids and lipophosphoglycan from *Leishmania* (*Viannia*) *shawi*

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ABSTRACT

In this work, some aspects of the glycobiology of *Leishmania shawi* were examined, as it is a causative agent of cutaneous leishmaniasis in the New World. Additionally, the interaction of *L. shawi*'s main glycoconjugates [lipophosphoglycan (LPG) and glycoinositolphospholipids (GIPLs)] with macrophages was evaluated *in vitro*. *L. shawi* LPG was devoid of side-chains in its repeat units, whereas monosaccharide analysis showed that GIPLs were suggestive of mannose-rich (type I or hybrid). In order to evaluate the biological roles of those molecules, BALB/c resident peritoneal macrophages were incubated with these glycoconjugates for 24 h, and the levels of nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-12p70 and IL-10, were determined. In general, the GIPLs exhibited a greater proinflammatory role than the LPGs did. However, for the first time, the GIPLs from this species were able to trigger the production of IL-10, an anti-inflammatory cytokine. In conclusion, *L. shawi* glycoconjugates were able to interact with the innate immune compartment. These data reinforce the role of parasite glycoconjugates during parasite and host cell interactions.

1. Introduction

Leishmania shawi was first identified in 1989, parasitizing the skin of monkeys and the livers of sloths in the Amazon region [1]. In the majority of human cases, this species induces one single cutaneous lesion, but generalized lesions have also been recorded [2]. Most of the studies conducted on the interaction of this species with the vertebrate host were performed in mice. In BALB/c and C57BL/6 mice, this parasite induced large lesions, accompanied by an intense inflammatory infiltrate, with necrosis areas, and a high degree of parasitism [3]. In BALB/c mice, the pathology induced by *L. shawi* was associated with large amounts of IL-10 and TNF-α expressed by both $CD4^+$ and $CD8^+$ T lymphocytes, suggesting a detrimental role of antiinflammatory and inflammatory cytokines in determining the disease phenotype [4]. By the other side, *Leishmania major* induces pathology in BALB/c mice with high amounts of Th2 cytokines and reinforces the immunological differences among New and Old World strains of *Leishmania* sp. [5].

The glycoconjugate antigens from *Leishmania* have the potential to modulate APC, enabling parasite attachment to macrophages, modulating cytokine production as well as the production of nitrogen intermediates [6]. The most studied glycoconjugates are lipophosphoglycan (LPG) and the glycoinositolphospholipids (GIPLs). Biochemically, LPG has four distinct domains: (i) a well conserved GPI anchor composed of 1-O-alkyl-2-lyso-phosphatidylinositol (PI); (ii) a core composed of Gal(α1–6)Gal(α1–3)Galf(β1–3)[Glc(α1)PO_4]Man(α1–3)Man(α1–4)-GlcN(α-1) heptasaccharide; (iii) a portion of disaccharide repeats of the Gal(β1–4)Man(α1)PO_4 units; and (iv) a terminal neutral oligosaccharide (cap) [6]. The main polymorphisms in LPG are located in the repeat units and caps. For example, in the New World species *Leishmania braziliensis* and *Leishmania infantum*, the LPGs are devoid of side chains, or they may exhibit one–three β-glucoses as side chains, respectively [7–9]. These variations have implications in their interactions with murine macrophages, where *L.* (*V.*) *braziliensis* LPG exhibited a more proinflammatory role than *L.* (*L.*) *infantum* did [10]. On the other hand, GIPLs are low molecular weight molecules that cover the parasite's surface and are similar to LPG in sharing a common lipid backbone and a glycan motif containing up to 7 sugars. The basic GIPL structure is a Manα1–4GlcN linked to an alkyl-acylglycerol through a phosphatidylinositol (PI) residue. Depending on a variety of fatty acid substitutions in the lipid anchor and monosaccharide substitutions in the glycan core moiety, GIPLs can be classified into one of three groups: Type I GIPLs (mannose-rich) are characterized by having an α1,6-mannose residue linked to the Manα1–4GlcN motif. Type II GIPLs (galactose-rich) have

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a much more heterogeneous lipid composition with C18:0, C22:0, C24:0, and C26:0 fatty acids. They are characterized by having an α1,3-mannose residue linked to the Manα1–4GlcN motif, which is similar to the glycan core of LPG. The third group is the hybrid-type GIPLs, and these share common features with both type I and type II GIPLs, as they have mannose residues located on both the C-3 and C-6 positions of the Manα1–4GlcN motif [6,11]. Recently, it was reported that GIPLs from *L. braziliensis* (type II) and *L. infantum* (type I/hybrid) were inhibitory molecules, significantly impairing the production of NO and IL-12, even in the presence of IFN-γ and lipopolysaccharides (LPS) [11]. These data, together with LPG, suggest that *Leishmania* glycoconjugates could have antagonistic effects on host cells. However, nothing is known about *L. shawi* glycoconjugates. Therefore, this report aimed to purify and preliminarily characterize the biochemical structure of LPG and GIPL of *L. shawi*, and to analyze their effects on the peritoneal macrophages of BALB/c mice.

The *L.* (*V.*) *shawi* (MHOM/BR/1996/M15789) strain was isolated from a patient with American tegumentary leishmaniasis in Buriticupu County, State of Maranhão, Brazil, and identified using monoclonal antibodies and multilocus enzyme electrophoresis at the Evandro Chagas Institute in Belém, State of Pará, Brazil. The parasites were maintained in BALB/c mice, and they were isolated and grown in M199 medium, as described [12].

LPG extraction was performed as described with solvent E (H_2O/ethanol/diethylether/pyridine/NH_4OH; 15:15:5:1:0.017) and purified using a phenyl-sepharose column [13]. For GIPLs, cells were harvested and subjected to a methanol:chloroform:water extraction (10:10:3). This material was loaded onto an octyl-sepharose column and purified following a gradient of 1-propanol in 0.1 M ammonium acetate buffer (5–60%). The LPG and GIPL purities were tested for LPS traces as previously described [10], and the results showed a total absence of LPS (data not shown). Purified LPG was subjected to mild acid hydrolysis (0.02 N HCl, 5 min, 100 °C) to depolymerize the repeat units. Water-soluble fractions were partitioned using 1-butanol and repeat units were treated with alkaline phosphatase (15 mM Tris buffer, pH 9.0, 1 U, 16 h, 37 °C). The neutral repeat units were desalted by passage through a two-layered column of AG50W-X12 (H^+) over AG1-X8 (acetate) [14]. Samples were subjected to fluorophore-assisted carbohydrate electrophoresis (FACE), as described [15], and the gel was visualized under ultraviolet (UV) light. Oligoglucose ladders (G_1–G_7) were used as standards (Fig. 1a). As expected, a band co-migrating with the standard oligo-glucose ladder, Glc_2, indicates the presence of the disaccharide repeats of the Gal(β1–4)Man(α1)PO_4, common to all LPGs [13]. This indicates that the LPG of *L. shawi* is devoid of side chains, and it is very similar to *L. braziliensis* LPG [8] or *L. infantum* LPG (type I) [9]. In order to characterize the monosaccharide composition of GIPLs, the sugar motif was subjected to strong acid hydrolysis (2 N trifluoroacetic acid, 3 h, 100 °C). Samples were desalted, as described above, and subjected to FACE [9] using monosaccharides (D-galactose, D-glucose, and D-mannose) (Sigma-Aldrich) as standards (Fig. 1b). In this experiment, the GIPLs of *L. braziliensis* (type II) and *L. infantum* (type I) were used as controls. The band profiles were analyzed by densitometry, and as expected, the GIPL of *L. infantum* (type I) exhibited a prominent band of mannose (>90%), whereas this band was fainted (<30%) in *L. braziliensis* GIPL (type II). Similar to *L. infantum* GIPL, *L. shawi* GIPL was also mannose-rich (~50%), suggesting that this species may possess type I or hybrid GIPL (Figure 1b).

The next step was to evaluate whether those features of the LPG and GIPLs from *L.* (*V.*) *shawi* could modulate the interaction with murine peritoneal macrophages. BALB/c mice peritoneal macrophages (10^5) were cultured in a sterile 96-well plate in RPMI medium and exposed to LPS (0.1 μg/well — positive control); LPG and GIPLs (5 or 10 μg/well); live *L.* (*V.*) *shawi* promastigotes (MOI 5:1); and only with RPMI 1640 medium (negative control). After 24 h, culture supernatants were collected for IL-12p70, TNF-α and IL-10 measurements using sandwich enzyme-linked immunosorbent assay, which was performed in accordance with the manufacturer's recommendations (e-Biosciences); NO determination was performed using the Griess reaction (Sigma). Statistical analysis was performed using the non-parametric Kruskal–Wallis test followed by Dunn's post hoc test for multiple comparisons. GraphPad Prism version 5 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze the results. The differences were considered statistically significant at a 5% significance level ($P < 0.05$). These results were expressed as the mean ± standard deviation of three independent experiments.

Similar to our previous observations [10], peritoneal macrophages from BALB/c mice incubated with LPG did not produce considerable amounts of IL-12 (Fig. 2a). On the other hand, GIPL-treated macrophages produced higher amounts of IL-12 compared to LPG and untreated controls ($P < 0.05$), moreover macrophages incubated with live parasites did not produce IL-12. Unlike what was observe with *L. infantum* and *L. braziliensis* GIPLs [11], this is the first time that a glycoconjugate from *Leishmania* was able to trigger the production of this cytokine. GIPL-treated macrophages also exhibited higher levels of TNF-α compared to LPG, especially in concentrations of 10 μg/well (Fig. 2b); live parasites did not trigger TNF-α production in macrophages ($P < 0.05$). Interestingly, LPG, GIPLs, and live parasites were able to trigger the production of IL-10 (Fig. 2c), a feature that was not observed for the *L. braziliensis* and *L. infantum* glycoconjugates [10,11]. Consistent with our previous observations [13,14], live parasites were not able to trigger the production of IL-12 and TNF-α, since *Leishmania* parasites inhibit the activation of macrophages. This inhibition could

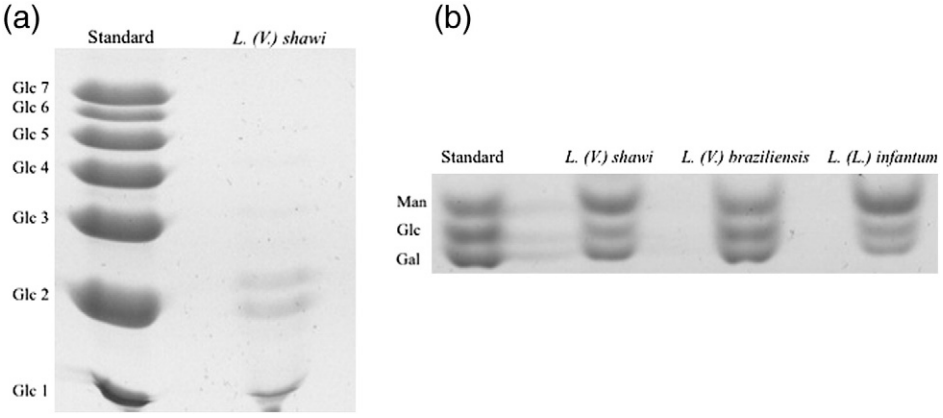

Fig. 1. Preliminary analysis of the lipophosphoglycan (LPG) and glycoinositolphospholipids (GIPLs) of *Leishmania shawi*. (a) FACE of the LPG repeat units: lane 1, oligoglucose ladder represented by G_2–G_7; lane 2, *L. shawi* repeat units (M15789 strain). (b) FACE analysis of GIPL monosaccharides: lane 1, monosaccharide standards; lane 2, *L. shawi* (M15789 strain); lane 3, control strain of *L. braziliensis* (M2903 strain); and lane 4, control strain of *L. infantum* (BH46 strain). Man, mannose; Gal, galactose; Glc, glucose.