The Role of Lipophosphoglycan in *Leishmania mexicana* parasite Infectivity of using Balb/c and C57 Macrophages model

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Submission date: 20.01.2025 Electronic publishing date: 23.2.2025

Abstract: Leishmania species are recognized to be pathogenic parasites that infect almost 15 million people globally, and up to date there is no vaccine available to stop the spread of Leishmania infection. For *Leishmania mexicana* to lose its virulence, the parasite culture must undergo in vitro passaging, little is known about the process underlying the reduction of parasite virulence. Thus, the purpose of this work was to look into the expression of lipophosphoglycan (LPG), as a virulence factor, during *L. mexicana* in vitro culturing and assessing the parasite's capacity to infect target cells. Results illustrated the extreme downregulation in LPG expression on the surface of promastigotes. Since anti-LPG monoclonal antibody staining showed LPG expression was significantly less expressed in passage 20 (P20) compared to its expression in passage 1 (P1). In addition, this was confirmed by flow cytometry analysis, which also showed that LPG was highly expressed in P1 compared to P20. Moreover, an in vitro infectivity assay was performed using Balb/c and C57 macrophages. Both cells were infected with *L. mexicana* promastigotes from P1 and P20 for 24 hours. The results illustrated the ability of P1, but not P20, to transform into the non-flagellated amastigotes sage inside infected cells. Our results concluded the significant role of LPG protein in *L. mexicana* virulence and its ability to induce infection.

Keywords: Leishmania, Lipophosphoglycan, Virulence, Down-regulation, Passage number

Introduction

In many countries, Leishmania species infection is a major health concern, according to reports, more than 20 species of this parasite cause Leishmaniasis, a clinical condition that affects both humans and animals. [1,2]. Leishmania species employ several virulent factors, including lipophosphoglycan (LPG), metalloprotease glycoprotein 63 (GP63), LACK (Leishmania homologue of receptor for activated C kinase), and chitinase (CHT1) genes, to influence the host immunological responses. GP63, which is mostly found on the surface of promastigotes and amastigotes, has been discovered it play a role in attracting the APC to the location of the sandfly's bite which leads to promastigots' attachment to the host cells [3,4]. LPG has a crucial role in the parasite's survival inside the midgut of sand flies. According to [5,6], the promastigotes *Leishmania major* adhesion to the *Phlebotomus papatasi* midgut was dramatically reduced by the use of anti-LPG antibodies. In the mammalian host, LPG blocks the complement system from creating pores, allowing the promastigotes to enter silently host cells, and delays phagosome development, [7,8]. It's interesting to note that LPG from *Leishmania mexicana* stimulated PKC in C57 macrophages but reduced PKC's enzymatic activity in Balb/c macrophages. This may explain the susceptibility of Balb/c mice to *L. mexicana*, [9,10]. Moreover, it was discovered that *L. major* containing a mutant LPG1 gene was unable to infect macrophages in vivo or in vitro. However, the parasite successfully recovered its virulence after the LPG1 gene was reintroduced [11,12]. Collectively, these investigations highlight the significance of LPG as a virulence factor in Leishmania due to its function in the parasite's attachment to the sand fly's midgut and in promoting its survival and persistence within host cells. Fresh parasites (P1) obtained from Balb/c mice and passaged 20 times in vitro (P20) lost their virulence and infectivity, as was previously documented [13]. The study's goal is to confirm if changes in LPG expression in P20 promastigotes decrease virulence. The expression of LPG on promastigotes will be investigated using immunofluorescence and flow cytometry, as well as promastigotes from P1 and P20 interaction with Balb/c and C57 macrophages.

Materials and methods

The strain of *L. mexicana* (MNYC/BZ/62/M379) was obtained as described by [13]. Immunofluorescence staining was used to compare the expression of LPG protein on the surface of P1 and P20 L. mexicana promastigotes cultured at 25°C. One million parasites were washed twice using 500µl PBS and 500µl of blocking buffer. Cells were incubated with the monoclonal primary mouse anti-LPG antibody CA7AE at 4°C overnight, control cells were incubated without primary antibody. The next day, the cells were washed twice with PBS, and 0.5µl of secondary antibody a goat-anti-mouse IgG (1:1000) was added to the target cells at room temperature for

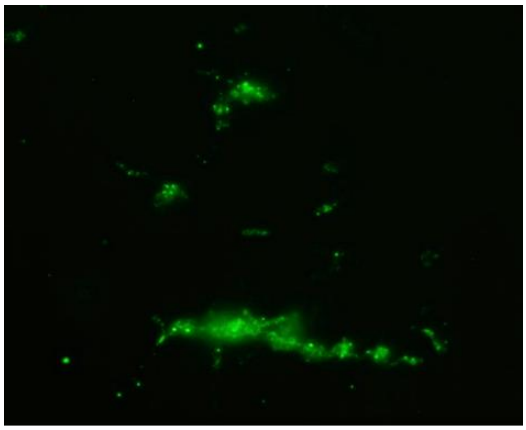
one hour. After three washes with 500µl PBS, cells were fixed with 50µl of cold 4% w/v paraformaldehyde (Sigma, UK). Slides were examined by EVOS FL microscope (40X magnification). The expression of LPG on the surface of P1 and P20 promastigotes was further examined by the flow cytometry technique. The above antibodies were used to stain promastigotes from P1 and P20 for one hour at 4°C with monoclonal primary mouse anti-LPG antibody CA7AE, after washing with PBS, the goat-anti-mouse IgG FITC secondary antibody was added for thirty minutes at room temperature. Cells were then washed twice. A 400µl of sheath fluid was added to the pellet, the cells were examined using a Gallios flow cytometer (Beckman Coulter, UK).

Parasites infectivity: Balb/c and C57 macrophages were used to test the infectivity of *L. mexicana* promastigotes P1 and p20. Briefly, A sterile knife and tweezers were used to remove the tibias and femurs from the hind limbs of 6–12-week-old Balb/c and C57 mice in order to obtain their bone marrow. The bone-marrow cells were flushed out using RPMI 1640 medium. After being washed, the cells were incubated in T25 using BM-DC media. After that, the cells were cultured for six days at 37°C with 5% CO2 and 95% humidity. After the bone-marrow cells of Balb/c and C57 mice had differentiated into macrophages, *L. mexicana* promastigotes P1 and P20 were added with a ratio of 01:20 macrophages to promastigotes, respectively, for 24 hours at 37°C with 5% CO2, 95% humidity. The means ± SEM are used to represent the results. GraphPad Prism software (version 7) was used for statistical analysis of the data, and the one-way ANOVA test was used to determine statistical significance.

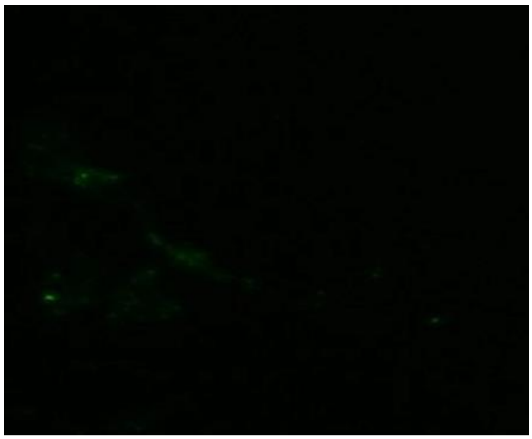
Results

LPG expression on the surface of P1 and P20 *L. mexicana*. The impact of in vitro 20 passages on the expression of LPG on the surface of promastigotes was investigated using *L. mexicana* Anti-LPG monoclonal antibody. As explained in the methods section, one million P1 and P20 promastigotes were stained with monoclonal primary antibodies. The results demonstrate that the P20 promastigotes (Figure 1.b) had a significant decrease in LPG expression compared to P1 promastigotes (Figure 1.a).

The down-regulation of LPG expression was further confirmed using flow cytometry. As stated in the methods section, 500,000 P1 and P20 cells were cultivated for six days at 25°C and stained with primary anti-LPG antibodies CA7AE. Following analysis using Kaluza software, the findings showed that P1 promastigotes had higher levels of LPG expression compared to P20 promastigotes (Figure 2).



A - *Leishmania mexicana P1*



B - *Leishmania mexicana P20*

Figure1: Impact of in vitro cultivation on LPG protein expression on *Leishmania mexicana* promastigotes' surface: EVOS FL microscopy was used to examine the labelled cells with anti-LPG monoclonal antibodies. (A) revealed that P1 promastigotes expressed more LPG than P20 promastigotes (B)

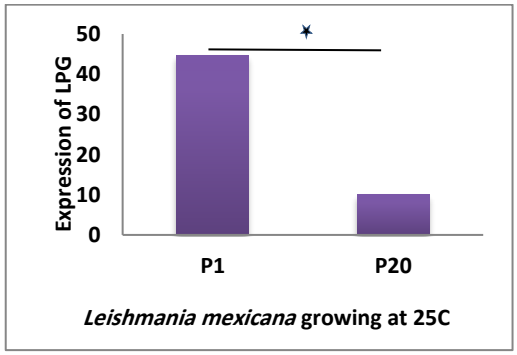


Figure 2: Flow cytometry analysis of LPG expression in P1 and P20 *Leishmania mexicana*. Promastigotes were immunofluorescence stained with anti-LPG primary antibody and scanned for LPG expression. *P<0.05 according to the Mann-Whitney U test. The data is displayed as mean + SEM.

Parasite infectivity. To evaluate parasite infectivity, the interaction method between the parasite from P1 and P20 promastigotes with bone marrow-derived Balb/c and C57 macrophages was used, as described in the method section. Cells were harvested, subjected to differentiation media for six days, and then infected with $20x10^6$ P1 and P20 *L. mexicana* promastigotes for twenty-four hours before being examined using an EVOS FL microscope. Results presented in Figure(3), clearly showed that P1 promastigotes were able to successfully change into amastigotes inside parasitophous and heavily infect both types of macrophages, however, the parasite from P20 has completely failed to perform any parasitophorosis

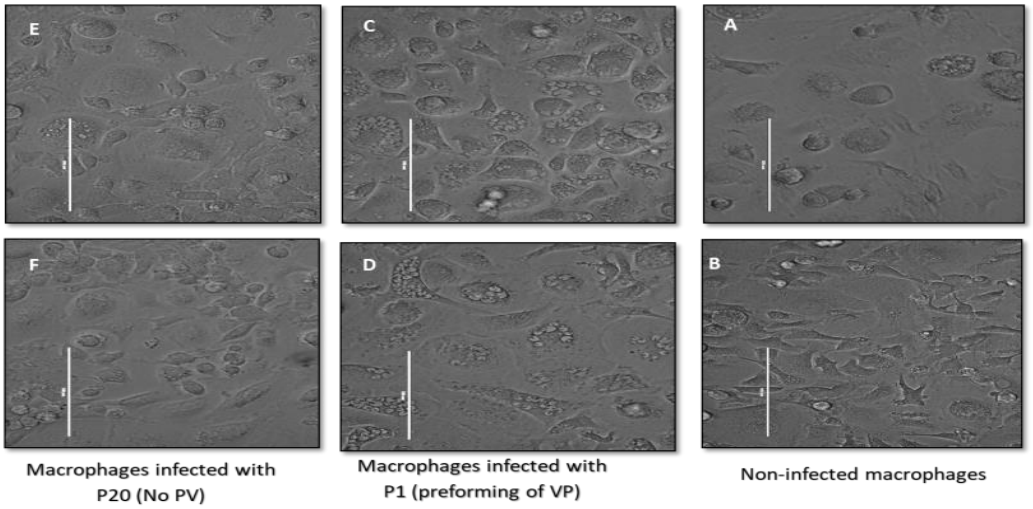


Figure 3: *Leishmania mexicana* infectivity in C57 and Balb/c macrophages. P1 and P20 *L. mexicana* promastigotes were introduced into C57 and Balb/c macrophages at a 1:20 (cell: parasite) ratio. Pictures (a) and (b) depict non-infected C57 and Balb/c macrophages, respectively. (c) and (d) After a 24-hour infection, P1 promastigotes completely change into amastigotes inside C57 and Balb/c macrophages. The failure of P20 promastigotes to develop into amastigotes within C57 and Balb/c macrophages is shown in (e) and (f).

Discussion

The effect of culturing *L. mexicana* in vitro on the morphology and some related virulence genes was well established by [13]. The modulation of LPG expression on the surface of P1 and P20 promastigotes was investigated by immunofluorescence assay and flow cytometry. Results of immunofluorescence revealed that most of the P1 promastigotes represented LPG protein on their surface as detected by the green fluorescent stain. Moreover, almost the entire surface of P1 promastigotes appeared to be enriched with LPG, reflecting its high expression. These findings were further confirmed by flow cytometry results that quantified a more than twofold decline in the percentage of LPG on the surface of P1 promastigotes compared to P20 promastigotes (Figures 1-2).

Our findings that LPG proteins are membrane-anchored proteins that are extremely unique to the promastigote stage are agreed with [14,15]. Conversely, after P1 promastigotes passed in vitro 20 times, LPG expression appeared to extremely decrease. This suggests that a decrease in LPG expression was brought about by extended promastigotes sub-culturing. These findings concurred with those of [13], who found that P20 *L. mexicana* promastigotes had downregulated LPG2. Additionally, after serial passaging, *L. major* showed a decrease in LPG expression [16].

The findings also showed that, unlike P20 promastigotes, which stayed in the promastigote stage and were unable to infect Balb/c or C57 macrophages, P1 promastigotes were able to infiltrate both host cells and change into amastigotes within PVs, figure 3. This was in line with [17] who used *L. donovani* promastigotes from varying passage numbers (5, 10, and 25) to infect hamsters. The parasite's virulence was associated with the hamsters' decreased weight. According to their findings, parasites from passage 5 cause infection, whereas those from passages 10 and 25 did not cause any weight loss.

Additionally, a recent study by [18] revealed that serial passaging in vitro resulted in the loss of *L. donovani* infectivity in vivo, along with the inability of promastigotes to develop into amastigotes in Balb/c mice. Furthermore, because promastigotes from passages 11 and 21 were substantially less numerous after 30 days of infection, scientists hypothesized that the parasite's capacity to survive inside the spleen and liver of Balb/c mice is heavily dependent on the passage number.

Furthermore, because P20 promastigotes were unable to infect human U937 monocytes, [13] verified that *L. mexicana* promastigotes had lost their virulence after 20 in vitro passages. According to reports, LPG inhibits protein kinase C, a crucial signaling molecule that initiates numerous defense mechanisms, including the production of nitric oxide and oxidative burst, and

stimulates the complement system to create C3b and C3bi opsonins. The promastigotes enter the macrophages silently thanks to both processes [19,20]. Nevertheless, [21,22] claimed that LPG-deficient *L. mexicana* was able to live, multiply, and infect Balb/c macrophages both in vitro and in vivo.

The reduction of phagolysosome formation is thought to be essential for the promastigotes' survival inside the macrophages. LPG helps delay the phagolysosomal fusion, as previously mentioned [23], [8]. Therefore, the significant decrease of LPG in P20 promastigotes, which results in phagolysosomal fusion, may be the cause of the promastigotes' incapacity to live and infect the macrophages. According to [13], P20 promastigotes showed higher expression of Rab7 and Rab9, which are directly correlated with phagolysosomal maturation, than P1 promastigotes, which showed a delay in phagolysosome maturation.

Conclusion

This study concluded that *L. mexicana* promastigotes at P20 lose their virulence when their expression of LPG protein is suppressed, this could suggest a relationship between the level of LPG expression and the parasite pathogenicity since the promastigotes from P20 have failed to produce amastigote stage inside mammalian cells.

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