Vaccination with *Leishmania mexicana* LPG induces PD-1 in CD8⁺ and PD-L2 in macrophages thereby suppressing the immune response: A model to assess vaccine efficacy

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**A B S T R A C T**

*Leishmania* lipophosphoglycan (LPG) is a molecule that has been used as a vaccine candidate, with contradictory results. Since unsuccessful protection could be related to suppressed T cell responses, we analyzed the expression of inhibitory receptor PD-1 in CD8⁺ and CD4⁺ lymphocytes and it is ligand PD-L2 in macrophages of BALB/c mice immunized with various doses of *Leishmania mexicana* LPG and re-stimulated *in vitro* with different concentrations of LPG. Vaccination with LPG enhanced the expression of PD-1 in CD8⁺ cells. Activation molecules CD137 were reduced in CD8⁺ cells from vaccinated mice. *In vitro* re-stimulation enhanced PD-L2 expression in macrophages of healthy mice in a dose-dependent fashion. The expression of PD-1, PD-L2 and CD137 is modulated according to the amount of LPG used during immunization and *in vitro* re-stimulation. We analyzed the expression of these molecules in mice infected with 1 × 10⁵ or 1 × 10⁶ *L. mexicana* promastigotes and re-stimulated *in vitro* with LPG. Infection with 1 × 10⁵ parasites increased the PD-1 expression in CD8⁺ and diminished PD-L2 in macrophages. When these CD8⁺ cells were re-stimulated *in vitro* with LPG, simulating a second exposure to parasite antigens, PD-1 expression increased significantly more, in a dose dependent fashion. We conclude that CD8⁺ T lymphocytes and macrophages express inhibition molecules according to the concentrations of *Leishmania* LPG and to the parasite load. Vaccination with increased amounts of LPG or infections with higher parasite numbers induces enhanced expression of PD-1 and functional inactivation of CD8⁺ cells, which can have critical consequences in leishmaniasis, since these cells are crucial for disease control. These results call for pre-vaccination evaluations of potential immunogens, specifically where CD8 cells are required, since inhibiting molecules can be induced after certain thresholds of antigen concentrations. We propose that the analysis of PD-1 and PD-L2 are useful tools to monitor the optimal dose for vaccination candidates.

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1. Introduction

*Leishmania* lipophosphoglycan (LPG), one of the principal molecules of the parasite, modulates the immune response. LPG is a ligand for TLR2 in NK cells regulating their IFN-γ and TNF-α production [1]. In mast cells and macrophages LPG modulates TLR2 and protein kinase-alpha (PKC-α), respectively [2,3]. CD4⁺ lymphocytes define *Leishmania* infections, where a Th-1 aids parasite control and Th-2 response favors disease progression in mouse models [4]. A major role in the defense against *Leishmania* is played by CD8⁺ cells, both by IFN-γ production and cytotoxicity [5–7]. Activation of CD8⁺ and CD4⁺ lymphocytes is regulated by PD-1, an inhibition receptor whose two ligands are PD-L1 (B7-H1) and PD-L2 (B7-DC) [8,9]. The recognition of PD-1 by either ligand leads to a functional exhaustion of CD8⁺ lymphocytes, characterized by reduced proliferation, the absence of cytokine production and a failure to exert cytotoxicity [10,11]. Yet some evidence also suggests that these molecules modulate CD8⁺ cells during *Leishmania mexicana* infections. A reduction of CD8⁺ lymphocytes has been observed in patients with diffuse cutaneous leishmaniasis (DCL),

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infected with \textit{L. mexicana}. These cells showed enhanced expression of PD-1 and were hampered in their effector mechanisms, being non-responsive in their cytokine production and showing limited cytotoxicity when confronted with autologous \textit{Leishmania}-infected macrophages\cite{12,13}. In a model of experimental chronic visceral leishmaniasis caused by \textit{Leishmania donovani}, CD8\(^+\) cells were found to show phenotypic markers of functional exhaustion\cite{14}. PD-L2 is a ligand for PD-1 displayed on dendritic cells and macrophages, both of which are host cells for \textit{Leishmania}\cite{9}. For protection against \textit{Leishmania} infections, a fine-tuned regulation leading to CD8\(^+\) cell activation is crucial, which includes the induction of co-stimulatory signals and activation molecules such as CD137, favoring cell survival, and the inhibition of PD-1 to avoid cellular anergy.

LPG has been widely used as a vaccine candidate against leishmaniasis, with contradicting results. Thus, subcutaneous immunization with LPG has failed to protect BALB/c mice against \textit{Leishmania amazonensis} infections, exacerbating the disease by enhanced TGF-β and IL-10 production\cite{15}. The administration of anti-LPG antibodies or the intranasal administration of LPG was shown to revert this effect\cite{16}.

One of the main pitfalls during vaccination schemes that end unsuccessfully is the use of given antigen concentrations, without previous analysis as to whether this immunogen induces inhibitory or activation molecules. Furthermore, the diverse protection models vary widely in parasite numbers used during the infection challenge, which also accounts for possible contradicting results. To gain insight into the unpredictable outcomes of the different LPG vaccination models, we analyzed if different \textit{L. mexicana} LPG concentrations showed diverse modulation of the inhibitory PD-1 molecule expression in T lymphocytes and PD-L2 expression in macrophages. Additionally, we analyzed the influence of the parasite load on the expression of these molecules.

2. Material and methods

2.1. Animals

Male BALB/c mice aged to 6–8 weeks were bred and housed at the animal facilities of the Departamento de Medicina Experimental of the Medical Faculty, UNAM, following the National Ethical Guidelines for Animal Health NOM-062-ZOO-1999 and the guidelines recommended for animal care by the Ethical Committee of the Medical School of the UNAM.

2.2. \textit{Leishmania mexicana} culture

\textit{L. mexicana} parasites were grown in RPMI-1640 medium (Life Technologies, Gaithersburg, MA, USA), supplemented with 10\% heat-inactivated FBS at 28\(^\circ\)C. Metacyclic promastigotes were harvested at late log phase (5 day culture).

2.3. Lipophosphoglycan purification

Lipophosphoglycan was purified from \textit{L. mexicana} as previously described\cite{1}.

2.4. Vaccination and infection

For vaccination assays, LPG was suspended in sterile PBS at a final concentration of 1 \(\mu\)g/\(\mu\)L. Mice received three subcutaneous injections (insulin syringe, needle 31 G BD) in the dorsum containing 10 or 100 \(\mu\)g of LPG or 100 \(\mu\)L PBS as control, at a 15 day interval. The protection assay was carried out 20 days after the last vaccination. Mice were infected subcutaneously (insulin syringe, needle 31 G BD) with \(1 \times 10^5\) \textit{L. mexicana} promastigotes in the ear dermis. The lesion was measured weekly with a Vernier. For infection analysis, non-vaccinated mice were infected with \(1 \times 10^4\) or \(1 \times 10^5\) promastigotes and sacrificed prior to ulceration of the lesions.

2.5. Peritoneal cells

Mice were sacrificed by cervical dislocation. The peritoneal cavity was infused with 10\% of cold sterile PBS pH 7.4 and lightly massaged. The peritoneal fluid was collected and centrifuged at 800 \(\times\) g for 10 min at 4\(^\circ\)C. The cells were cultured 2 h in RPMI 1640 (supplemented with 100U/mL penicillin and 100U/mL streptomycin) containing 10\% (v/v) heat-inactivated FBS (RPMI–FBS) at 37\(^\circ\)C with 5\% CO\(_2\). Macrophages (1 \(\times\) \(10^6\)/mL) were maintained in 24-well cell culture plates (Corning). Different LPG concentrations (1, 5 or 10 \(\mu\)g) were added, and a negative control contained only culture medium. After 24 h the cells were harvested and analyzed by flow cytometry.

2.6. Spleenocyte purification

The spleen was aseptically removed and placed in a Petri dish containing cold PBS. The tissue was disrupted in a 100 \(\mu\)m nylon cell strainer (BD Falcon) and the isolated cells were centrifuged at 800 \(\times\) g for 10 min at 4\(^\circ\)C. Cells were separated by Ficoll–Hypaque gradient (Sigma) and mononuclear cells were washed twice with PBS and placed in 6-well plates (Corning) at 5 \(\times\) \(10^6\) cells per well and stimulated with 1, 5 or 10 \(\mu\)g \textit{L. mexicana} LPG during 24 h.

2.7. Flow cytometry

The extracellular expression of PD-1, CD137, PD-L2 and PD-L1 was analyzed in stimulated or non-stimulated peritoneal macrophages and mononuclear cells (1 \(\times\) \(10^6\) cells/mL) were suspended in 100 \(\mu\)L FACS buffer (BD Biosciences cat. 342003) containing CD16/32 antibodies for 10 min on ice. After washing, cells were stained in 50 \(\mu\)L FACS buffer containing fluorochrome-labeled antibodies specific for CD3e (BD Pharmingen cat. 553066), CD8a (BD Pharmingen, cat. 551162), CD4 (BD Pharmingen, cat. 552775), CD137 (BD Pharmingen cat. 558976), F4/80 (Biolegend, cat. 122615), PD-1 (Biolegend, cat. 135205), PD-L1 (Biolegend, cat. 124311), PD-L2 (Biolegend, cat. 107205) or appropriate isotype controls, for 20 min on ice. Cells were then washed twice, fixed in 2\% paraformaldehyde and analyzed using a FACScanto II flow cytometer equipped with DIVA software (BD Biosciences, USA).

2.8. Statistical analysis

All data are expressed as mean \(\pm\) SD (standard deviation of the mean). Comparisons between experimental groups were performed using Mann–Whitney \textit{U}-test. A value of \(p<0.05\) was considered statistically significant, using Prism 5 for Mac OS X\textsuperscript{\textregistered}. Three or more independent experiments were analyzed for three mice per group.

3. Results

3.1. Vaccination with LPG induces exacerbation and progression of \textit{L. mexicana} infection

Our group previously demonstrated that LPG exerts an immunomodulatory effect on different cells of the immune response\cite{1–3}. We were therefore interested in analyzing whether this molecule could confer protection against \textit{L. mexicana} infections. BALB/c mice were vaccinated with 10 \(\mu\)g \textit{L. mexicana} LPG.
Twenty days after the third immunization, mice were challenged in ear dermis with \(1 \times 10^5\) Leishmania promastigotes and the infection was followed throughout 8 weeks. Once the inflammation was detectable, the lesion was measured weekly with a Vernier. Control mice were injected with 10 µL PBS. The ear dermal lesions appeared first in non-vaccinated mice around the third week. Lesions of mice vaccinated with LPG appeared around the fourth week. Throughout the course of the infections, both groups of mice showed similar inflammatory lesions (Fig. 1). After 6 weeks, only the vaccinated mice began to show dissemination of the parasite, forming nodules in the contralateral earlobe, paws and nose, simulating diffuse cutaneous leishmaniasis found in humans (data not shown). Once the disease disseminated in vaccinated mice, the inflammatory lesions in their earlobes tended to evolve slower after 6–7 weeks of infection, as compared to non-vaccinated mice (Fig. 1). It remains to be analyzed whether dissemination increases overall Leishmania numbers that possibly induce inhibitory molecules on inflammatory cells, thereby diminishing the inflammation yet not the disease progression. These data show that vaccination with LPG induces a more rapid dissemination of the parasites.

3.2. Macrophages infected in vitro with L. mexicana and stimulated with LPG over-express PD-L2 but show no changes in PD-L1 expression

We studied the modulation exerted by in vitro stimulation of macrophages from healthy mice with LPG (1, 5 or 10 µg) and analyzed the ligands of regulatory molecules of T cells in macrophages. Stimulation with 1 µg LPG led to an increased PD-L2 expression, yet when the challenge was augmented to 5 µg, the PD-L2 expression significantly increased (3-fold) whereas stimulation with 10 µg only slightly enhanced the expression (2-fold), which was not different from non-stimulated controls (Fig. 2A). These results suggest that LPG is capable of regulating the interaction between T lymphocytes and macrophages by inducing PD-L2 in a dose-dependent fashion.

Furthermore, we analyzed whether in vitro infection of macrophages could regulate the expression of these inhibitory molecules. Peritoneal macrophages were infected with L. mexicana promastigotes in a ratio 1:10 (cells:parasites). In one group, Leishmania promastigotes combined with 5 µg LPG were used to infect macrophages. The cells were stained with antibodies against F4/80, PD-L1 and PD-L2. PD-L1 expression decreased slightly in macrophages infected with Leishmania promastigotes (Fig. 2B). In contrast, PD-L2 was up-regulated (2.4-fold) in macrophages infected with Leishmania combined with LPG, as compared to non-infected cells (Fig. 2B). In conclusion, LPG stimulation seems to have a more potent effect to induce PD-L2 in peritoneal macrophages, as compared to the infection with L. mexicana alone.

3.3. L. mexicana LPG induces PD-1 expression in CD8+ T cells of vaccinated mice

After finding that LPG exacerbated disease progression and modulated the PD-L2 expression in macrophages, we were interested in analyzing the effect exerted by LPG on spleen CD8+ and CD4+ T lymphocytes of mice immunized with two different doses of LPG. Vaccination with 10 or 100 µg LPG increased PD-1 expression in CD8+ T cells. Re-stimulation of these cells in vitro with 1, 5 or 10 µg LPG maintained their elevated expression of PD-1 (Fig. 3A).

LPG had an opposite effect on CD137 expression in CD8+ T cells. Mice vaccinated with 10 µg down-regulated their CD 137 expression by 20%, whereas vaccination with 100 µg decreased CD137 expression by 25% (Fig. 3B). Re-stimulation with 5 or 10 µg LPG
During further re-stimulation, re-stimulation CD4+ T lymphocytes were obtained and re-stimulated in vitro with 1, 5, or 10 μg LPG during 24 h, fixed with paraformaldehyde and stained with anti CD3, CD4, CD8, PD-1 and CD137 antibodies. (A) PD-1 expression in CD8 T cells, (B) CD137 expression in CD8 T cells, (C) PD-1 expression in CD4 T cells, and (D) CD137 expression in CD4 T cells. The bars represent normalized data of three separate experiments. Mean ± SD is shown. *p ≤ 0.05 was considered significant.

Further reduced CD137 in mice vaccinated with 10 μg, as compared to non-vaccinated controls (Fig. 3B).

The analysis of CD4+ T cells of mice vaccinated with 10 or 100 μg LPG showed no modification in the PD-1 expression. Yet in vitro re-stimulation with 5 or 10 μg LPG reduced PD-1 expression in CD4+ cells of mice vaccinated with 10 μg, as compared to non-vaccinated controls (Fig. 3C). When analyzing the expression of CD137 in CD4+ T cells, mice vaccinated with 10 μg LPG showed a reduced expression, which diminished even more after these cells were re-stimulated in vitro with 10 μg LPG (Fig. 3D).

Together these data show that L. mexicana LPG negatively regulates CD8+ cell activation by enhancing PD-1 expression and concomitantly reducing CD137 expressions, where the degree of the modulation depends upon the dose of LPG used for immunization as well as the dose of the subsequent stimulus. In contrast to CD8+ T cells, vaccination with LPG had no inhibitory effect on CD4+ T cells, since it did not modify their PD-1 expression and re-stimulation with LPG reduced their PD-1 expression. Thus, LPG vaccination seems to exert the inhibitory effect only on CD8+ T cells, in a dose dependent fashion.

3.4. The expression of PD-1 in CD8+ T lymphocytes of mice infected with L. mexicana is related to parasite load

To analyze whether parasite infection modulates PD-1 expression in T lymphocytes, BALB/c mice were infected in the earlobe dermis with 1 × 10^4 or 1 × 10^5 L. mexicana promastigotes. Mice were sacrificed prior to ulceration of the lesions. Splenocytes were isolated and re-stimulated in vitro with 1, 5 or 10 μg LPG during 24 h and PD-1 as well as CD137 were analyzed. We found that PD-1 expression is enhanced in CD8+ T cells of mice infected with 1 × 10^4 (0.5-fold) or 1 × 10^5 (3.6-fold) parasites, as compared to CD8+ T cells from non-infected mice (Fig. 4A). In vitro stimulation with all three doses of LPG showed the same high expression of PD-1.

The analysis of CD137 in CD8 T cells showed a 40% down-regulation in mice infected with 1 × 10^4 promastigotes, whereas mice infected with 1 × 10^5 promastigotes showed a similar expression as non-infected mice. In vitro re-stimulation with LPG did not alter CD137 expression (Fig. 4B).

CD4+ lymphocytes showed a minimal increase in PD-1 expression after infections with either number L. mexicana parasites, and
Fig. 4. L. mexicana infection promotes the PD-1 expression in CD8 T cells. Mice were infected in the ear dermis ear $1 \times 10^4$ (stripped bars) or $10^5$ (black bars) L. mexicana promastigotes. Healthy BALB/c mice were used as a control group (white bars). Mice were euthanized before the lesion ulcerated. T cells from spleen were re-stimulated with 1, 5 or 10 µg LPG during 24 h, fixed with paraformaldehyde and stained with anti CD3, CD4, CD8, PD-1 and CD137 antibodies. (A) PD-1 expression in CD8 T cells, (B) CD137 expression in CD8 T cells, (C) PD-1 expression in CD4 T cells and (D) CD137 expression in CD4 T cells. The bars represent normalized data of three separate experiments. Mean ± SD is shown. * $p \leq 0.05$ was considered significant.

showed no changes despite secondary stimuli with LPG (Fig. 4C). Furthermore, the expression of CD137 in CD4+ T cells of infected mice also remained unaltered. The only up-regulation of this activation marker was observed in CD4+ T cells of mice infected with $1 \times 10^6$ parasites after they were re-stimulated in vitro with 5 µg LPG (Fig. 4D).

In conclusion these results show that L. mexicana infection induces significantly enhanced PD-1 expression only in CD8+ T cells, in a dose-dependent fashion. The reduced expression of CD137 in association with the increased levels of PD-1 in these CD8+ T cells seems to indicate that they resemble an exhausted phenotype. PD-1 is minimally expressed in CD4+ cells during L. mexicana infections and not altered by in vitro LPG stimuli, showing that L. mexicana exerts a stronger inhibitory effect on CD8+ T cells, as compared to CD4+ T cells.

3.5. Vaccination with LPG or L. mexicana infection of mice regulates PD-L2 expression in spleen macrophages

Since vaccination with LPG immunomodulated CD8+ T lymphocytes toward inhibition, we analyzed if immunization with different LPG concentrations or infection with different parasite numbers also modulated the expression of PD-L2 on spleen macrophages. Macrophages from mice vaccinated with 10 µg LPG and re-stimulated in vitro with 1 µg LPG, showed diminished expression of PD-L2 whereas vaccination with 100 µg LPG tended to increase the expression of PD-L2 in macrophages after receiving secondary stimuli with LPG (Fig. 5A).

Mice infected with $1 \times 10^6$ or $10^5$ parasites down-regulated PD-L2 expression by 50% (Fig. 5B). Re-stimulation of macrophages from mice infected with $1 \times 10^4$ parasites with LPG always showed diminished expressions of this inhibitory marker, whereas those from mice infected with $10^3$ parasites slightly increase their PD-L2 expression, albeit never reaching the levels expressed in cells of non-infected mice (Fig. 5B).

Together, these data show that Leishmania infections reduce PD-L2 expression in spleen macrophages and that this down-regulation persists despite secondary in vitro stimulation with LPG.

4. Discussion

Our data shed new light on the cause of enhanced disease progression after immunization with Leishmania LPG that has also been reported in the literature [16]. In an attempt to understand the underlying cause of this unsuccessful vaccination with LPG, we immunized mice with different concentrations of LPG and thereafter stimulated their spleen cells with various doses of LPG in vitro in an attempt to simulate a secondary exposure to LPG antigen, as
would occur during a natural infection. Additionally, we infected mice with different *L. mexicana* numbers and also re-exposed their lymphocytes to a secondary challenge with LPG. We here show that immunization of BALB/c mice with LPG or infections with *L. mexicana* promastigotes enhances the expression of the inhibitory receptor PD-1 in CD8⁺, whereas CD4⁺ T cells remain unaltered. The increase of these inhibitory molecules in CD8⁺ T cells acts in concert with their reduction of the activating molecule CD137, when these cells are confronted with a new challenge of LPG. These changes vary according to the amount of the LPG used for the vaccination and the parasite load during infection and they also vary according to the amount of parasite antigen (LPG) encountered by these cells after renewed exposure. The combination of these events possibly leads to a severe down-regulation of the functional capacity of CD8⁺ T cells in controlling the parasite infection. The response of CD4⁺ T cells was less clear.

PD-1 (programmed-death 1) receptor is related to CD28 and CTLA-4. It is inducible after T cell activation and down-regulates activated T cells [11]. Its ligands, PD-L1 and PD-L2, are up-regulated in APCs following activation [8]. PD-1 and PD-L2 may have distinctive roles in regulating Th-1 and Th-2 responses and reducing T cell proliferation by arresting the cell cycle [17,18]. This inhibitory receptor and its ligands have been studied in tumors, showing that the engagement of PD-1 with PD-L1 and PD-L2 attenuates T cell responses and help tumor cells escape immunosurveillance [19].

In chronic viral infections, suppressed CD8⁺ T cell responses have been attributed to PD-1/PD-L1 interactions [20].

To the best of our knowledge, we here describe for the first time that suppressor receptor PD-1 is induced after vaccination with elevated doses of *Leishmania* LPG or with the infection with elevated amounts of *L. mexicana* promastigotes. This expression is specifically dominant on CD8⁺ T lymphocytes possibly leading to a suppression of these cells that are critical in the control of leishmaniasis, both through IFN-γ production, as well as in their cytotoxic effect against autologous *Leishmania*-infected macrophages [5,6]. These results call for a careful pre-immunization evaluation of potential vaccine candidates against *Leishmania*, since the induction of a suppressive effect can lead to detrimental blockage of the immune response, favoring a more virulent disease progression. These data open a new field of research in vaccine developments and provide a novel strategy for therapeutic intervention in leishmaniasis, where the blockade of PD-1 could represent a valuable approach for anti-Leishmania immunotherapy.

Our data also yield information on novel parasite evasion strategies, achieving CD8⁺ T cell suppression, thereby eliminating one of the more powerful defense mechanisms against *L. mexicana* [13].

We conclude that vaccination models should assess whether PD-1 and/or PD-L2 are induced, that, far from activating CD8⁺ T cells, it could lead to their inhibition. Additionally, during experimental models of *L. mexicana* infections, the parasite load must be taken into account, since it can have opposing effects on PD-1 expression in lymphocytes. This study provides insight into the regulatory pathways elicited in vaccine models using different antigen concentrations or during *Leishmania* infections with different parasite loads, showing that the outcome can be polarly opposed, leading to contradictory results.

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