

CTL Responses to DCs Stimulated with Leishmania Antigens Detected by DCs Expressing Leishmania gp63

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ABSTRACT

Background: *Leishmania* is a pathogenic parasite which infects mononuclear cells in vertebrate hosts. Different strategies have been taken to develop immunity against *Leishmania*. DCs loaded with immunogenic antigen have resulted in different levels of Th1-type immune response and cytotoxic T lymphocytes (CTL) activity. **Objective:** To evaluate the potency of DCs primed with soluble *Leishmania mexicana* antigens (SLA) in developing CTL activity. **Methods:** DCs were loaded with SLA and injected to *Balb/c* mice. After two weeks the mice were sacrificed and their splenocytes were used as effector cells in a standard 4-hour cytotoxicity assay against DCs transfected with pcDNA3 containing *L. mexicana* gp63 gene. **Results:** Immunization of *Balb/c* mice with DCs loaded with SLA resulted in high levels of CTL activity against DCs transfected with pcDNA3 containing *L. mexicana* gp63 gene. **Conclusions:** The results indicate a high potency for DCs primed with *Leishmania* antigens in inducing CTL activity, which can be used for developing an immunogenic vaccine against *Leishmania*.

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Keywords: *Balb/c*, Cytotoxicity, DCs, gp63, *Leishmania*

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INTRODUCTION

Leishmaniasis is a worldwide disease prevalent in tropical and sub tropical countries. According to WHO, leishmaniasis is now considered as a neglected disease, which is transferred mainly by travelers and those with human immunodeficiency virus (HIV) infection (www.who.int/tdr). Due to difficulties associated with treatment of leishmaniasis (1), developing an effective vaccine is considered as the best way to control the disease. Previous studies showed that some subsets of DCs are biased to Th1 immune response and activate cytotoxic T lymphocytes (CTL) through MHC class I (2). It is now believed that the immunity against *Leishmania* parasites relies on a Th1 response, which is associated with IL-12 and IFN- γ production. However, the species of the parasite is important in this phenomenon (3). Although the exact role of CD8+T cells in resistance to *Leishmania* infection is not fully understood (4-5), some recent studies emphasize on the role of these cells where they showed CD8+ T-cell-depleted C57BL/6 mice failed to control *L. major* infection after the inoculation of metacyclic promastigotes into the ear dermis (6). These results confirmed by others where IFN- γ was detected in CD8+ T cells derived from immune *Balb/c* mice rechallenged with *L. major* (7).

Dendritic cells, as professional antigen presenting cells, have crucial function in directing CTL responses (8). Therefore, DCs have been used as potent candidates for immunotherapy of Leishmaniasis suggesting a new approach in *Leishmania* vaccination. In recent studies, DCs, first primed with relevant antigens *in vitro* and then injected into the animals, elicited Ag-specific protective immune responses (9).

In present study, we have developed a model to evaluate CTL activity in mice immunized with DCs loaded with soluble leishmania antigen (SLA). To generate a suitable target for detecting CTL activity against specific *Leishmania* antigen, bone marrow-derived dendritic cells (BM-DCs) were transfected with *L. mexicana* gp63 cDNA and used to measure cytotoxic activity of T cells in a standard 4-h ⁵¹Cr-release cytotoxicity assay.

MATERIALS AND METHODS

Parasite. *L. mexicana* strain M379 was obtained from Dr. V. Yardley, the London School of Hygiene and Tropical Medicine (LSHTM), and cultured in Schneider culture media (Sigma) supplemented with 10% FCS at 25°C as described by Ali *et al.* (11).

Animals. *Balb/c* mice were purchased from the Harlan Olac (Oxon, UK) housed and bred at the Nottingham Trent University. All animals were handled in accordance with the Home Office Codes of Practice for the housing and care of animals.

Preparation of SLA. The *L. mexicana* SLA was prepared according to procedure previously described by Ali *et al.* (11). Briefly, after four times wash with PBS (PBS tablets pH 7.2-7.4, OXOID), *L. mexicana* promastigotes were resuspended in 100 mM Tris (Fisher Scientific Ltd) buffer (pH 7.3) containing 1 mM EDTA (Bio Whittaker, Europe), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) and 2.5 μ g/ml Leupeptin (Sigma), and lysed by sonication. The lysate was centrifuged at 780g for 20 min and the supernatant was centrifuged again for 4 h at 18700g. The SLA was dialysed overnight against 5 liters of cold PBS with continuous agitation and sterilised by passing through 22 μ m filters (Sartorius).

Cloning gp63 into pcDNA3. *L. mexicana* gp63 cDNA was cloned into pcDNA3 as previously described by Ali *et al.* (11). In brief, to successfully transfect DCs with *L. mexicana* gp63 and due to the need for a mammalian antibiotic resistant gene for selection, *L. mexicana* gp63 cDNA (kindly gifted by Dumonteil E. University of Autónoma de Yucatán, Mexico) was inserted into pcDNA3 expression vector (Invitrogen, Paisley, UK), which contained Geneticin or g418 (resistant gene. For that the gene was first cut by EcoRI restriction enzyme off the original construct. pcDNA3 vector was also digested using the same restriction enzyme. The cut *L. mexicana* gp63 gene and the digested vector were then ligated using a DNA ligase enzyme (T4 Ligase Enzyme, Promega). The presence of the *L. mexicana* gp63 gene in pcDNA3 vector was first determined by restriction enzyme digestion (data not shown) and by PCR amplification using 5'-ACATCCTCACCGACGAGAAG- 3' (forward) and 5'-CTTGAAGTCGCCACAGATCA-3' (reverse) primers. The sequence of the sub-cloned gene was also checked and aligned with that of the original construct.

Generation of DCs. Dendritic cells from Balb/c mice were cultured *in vitro* and their phenotype was analysed as previously described by Rezvan (12). Briefly, hind limb bone marrow cells were cultured at 1×10^6 cells per ml in BM-DC media [complete RPMI (Bio Whittaker, Europe), 5% by volume FCS (Autogen Bioclear, UK), 1% by volume glutamine (IRH Biosciences), 20 mM HEPES (Bio Whittaker, Europe), 50 μ M 2-mercaptoethanol (Bio Whittaker, Europe), 50 U/ml penicillin/streptomycin (Bio Whittaker, Europe), 0.25 μ g/ml fungizone (Bio Whittaker, Europe)] containing 100 ng/ml of murine GM-CSF (produced by x63 cells cultured in the lab) incubating overnight at 37°C and 5% CO₂. On days 2 and 4, 700 ml of media as well as non-adherent cells were gently removed and replaced with fresh DC media containing GM-CSF. On day 7, BM-DCs were split into three groups. Flowcytometry and antibodies against CD11c, CD80, CD40, CD45R, CD4, CD8, MHC class II, and macrophage/monocyte Ags were used for phenotyping of the DCs. DCs were then split into three groups. The first group was used for phenotyping by flowcytometry, the second group (test) was pulsed with 10 μ g/ml SLA and the third group was used as control. Control and test groups were pulsed 4-6 hours later by 1 μ g/ml LPS to induce maturation and used for immunisation. One injection of 2×10^6 the matured DCs of test and control groups in serum free RPMI 1640 media was applied per mouse intera-dermally for inducing CTL activity in the following day.

Flowcytometry. To assess the phenotyping of DCs, 5×10^5 DCs in each tubewere washed twice in PBS + 0.1% BSA + 0.02% NaN₃ and a number of antibodies including rat anti-mouse CD80, macrophage/monocyte marker (F4/80), I-A (murine class II and CD45), DEC205 (all from Serotec, Oxford, UK), and hamster anti-mouse CD11c (N418 hamster hybridoma; American Type Culture Collection, Manassas, VA, USA) monoclonal antibodies were added. Appropriate isotype controls were also used in each experiment. The cells were incubated on ice for 30 minutes with primary antibodies and then washed twice in PBS + 0.1% BSA + 0.02% NaN₃ incubating for 30 minutes on ice with FITC coupled goat anti-rat IgG or goat anti-hamster IgG as secondary antibodies when appropriate. Finally the cells were washed in PBS + 0.1% BSA + 0.02% NaN₃ and resuspended in 500 μ l of sheath fluid, and analysed by FACS.

Transfection of DCs with pcDNA3 *L. mexicana* gp63. DCs were transfected with pcDNA3 *L. mexicana* gp63 using lipofectamine 2000 (Invitrogen) according to the manufacture's instruction with slight modifications. Briefly, DCs were cultured at 1×10^6 per well in 24-well plates incubating at 37°C, 0.5% CO₂ to produce 90% confluence

on the day of transfection. Lipofectamine 2000 and pcDNA3 *L. mexicana* gp63 were diluted in serum free DMEM media (Bio Whittaker, Europe) at 2/50 μ l and 0.8 μ g in 50 μ l, respectively, and incubated at room temperature for 5 minutes. The diluted lipofectamine 2000 and DNA were mixed together and incubated again for 20-30 minutes at room temperature. The DC culture supernatant was gently removed and the DNA-lipofectamine mixture was gently added followed by 4-6 hours incubation at 37°C with 0.5% CO₂. 1 ml/well DMEM media complemented with 10% FCS was then added. To select the stable transfected cells, the media was replaced 16-24 hours later with a fresh medium containing 500 μ g/ml G418 (Bio Whittaker, Europe).

CTL Assay. Standard 4-hour chromium release cytotoxicity assay was applied as previously described by Rezvan *et al.* (13) with slight modification. In brief, LPS blast cells, which are used as potent APCs for in vitro restimulation of CTLs in cytotoxicity assay, were prepared by in vitro restimulation of naive splenocytes cultured at 1.5×10^6 in T-cell media [complete RPMI, 10% FCS (by volume), 1% glutamine (by volume), 20 mM HEPES, 50 μ M 2-mercaptoethanol, 50 U/ml Penicillin/streptomycin, 0.25 μ g/ml fungizone] containing dextran sulphate 7 μ g/ml and LPS (Sigma) 25 μ g/ml for 3 days. On day 2, cells were pulsed with 10 μ g/ml SLA. After 24 hours the cells were irradiated at 3000 rads for 4 min. The cells were then washed and pulsed again with 100 μ g/ml of SLA for at least 1h. After washing, the cells were counted and added to culture plates containing splenocytes of immunized mice at 5×10^5 per well and incubated at 37°C and 5% CO₂.

For in vitro generation of CTLs, *Balb/c* mice were immunized intradermally with DCs loaded with 10 μ g/ml of SLA or PBS (for controls) at a dose of 2×10^6 cells per mouse. After two weeks, immunized mice were sacrificed and their splenocytes were plated out in a 24-well plate at 2.5×10^6 cells per well. 5×10^5 SLA-pulsed LPS blasts were irradiated and added to each well followed by incubation for 5 days at 37°C and 5% CO₂. On day 5 after stimulation, splenocytes were harvested, washed twice in serum-free medium, counted and resuspended in T-cell media and used as effector cells. In certain control experiments to confirm the role of CD8+ T cells in inducing CTL activity, CD8+ cells were depleted by using CD8 beads (Dyna, Wirral, UK) according to manufacturer's instructions. DCs used as targets in cytotoxicity assay were first transfected (the test group with pcDNA3 *L. mexicana* gp63 and the control group with pcDNA3 alone), harvested, washed and then labeled with ⁵¹chromium (Amersham, Buckinghamshire, UK) followed by 1h incubation at 37°C and 5% CO₂. A standard 4-h ⁵¹Cr release assay was performed and the specific cytotoxicity was determined using the following formula.

$$\text{percentage cytotoxicity} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})} \times 100$$

RESULTS

BM-DC phenotyping. To assess the phenotype of BM-DCs, the cells were washed twice in PBS + 0.1% BSA + 0.02% NaN₃ and then appropriate antibodies for murine CD80, F4/80, DEC205, class II, CD45 and CD11c monoclonal antibodies were added. Appropriate isotype controls were also used in each experiment (see materials and methods). Results showed expression of aforementioned markers in the cultured DCs as shown in Table 1.

Table 1. DC phenotypic analysis.

Markers	DEC205	F4/80	MHC Class II	CD40	CD11c	CD45R	CD80
Expression	+	-	+	+	+	-	+

Detection of *L. mexicana* gp63 in transfected DCs. Presence of the *L. mexicana* gp63 gene was first determined in the transfected cells by RT-PCR and mouse GAPDH gene was also detected as control (Figure 1).

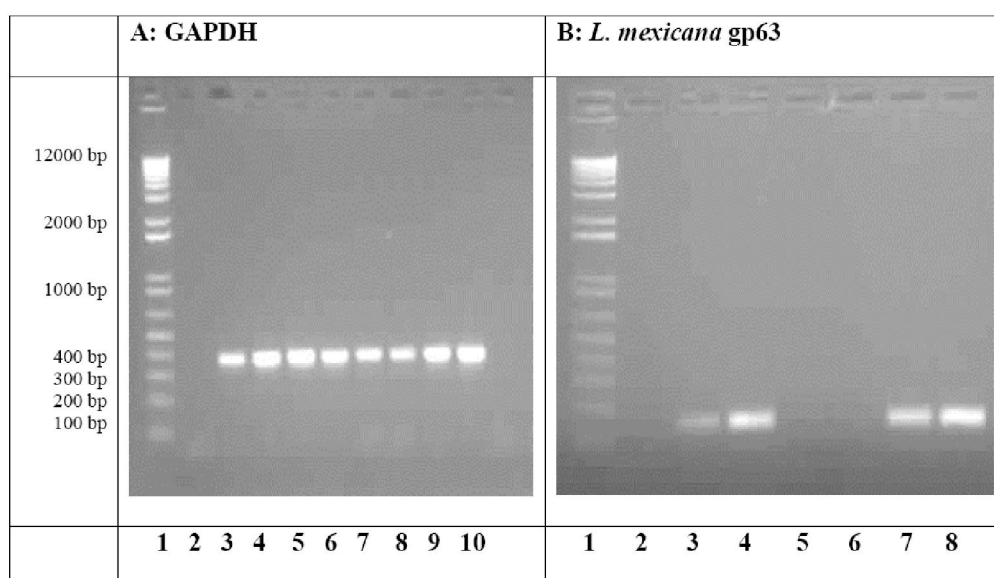


Figure 1. Expression of *L. mexicana* gp63 gene in transfected DCs detected by RT-PCR.

A: Expression of mouse GAPDH (primers are expected to produce 400bp) 1: standard DNA 2: PCR negative control 3-10: transfected DCs 11: non-transfected DCs (control) **B:** expression of *L. mexicana* gp63 in DCs (primers are expected to produce 180bp) 1: standard DNA 2: PCR negative control 3,4: pcDNA3 *L. mexicana* gp63 (control) 5,6: non-transfected CT26 (control) 7,8: transfected DCs.

The expression of gp63 protein was also determined by FACS analysis using anti *L. mexicana* gp63 antibodies (Figure 2).

CTL activity in mice immunised with SLA loaded DCs against DCs transfected with pcDNA3 *L. mexicana* gp63 construct. To evaluate the CTL response to immunization with DCs loaded with SLA a standard 4-h ⁵¹Cr-release cytotoxicity assay was used. DCs were loaded with SLA and injected into *Balb/c* mice intradermally at a dose of 2×10^6 cells per mouse and mice were sacrificed two weeks later (see materials and methods).

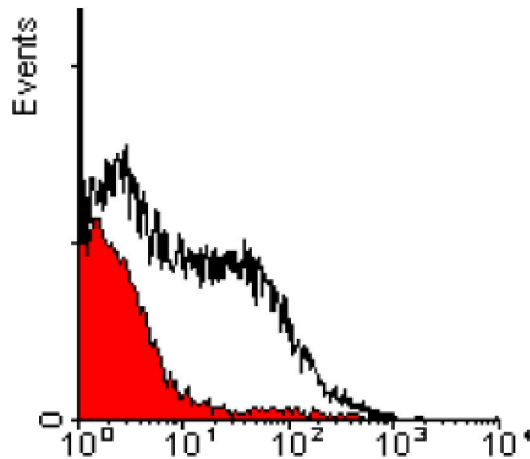


Figure 2. expression of gp63 on transfected DCs Mouse bone marrow cells were cultured in presence of GM-CSF. DCs were then split into two groups. Both groups were transfected; the first group with pcDNA3 containing *L. mexicana* gp63 and the second group with pcDNA3 alone (see materials and methods). After pulse with LPS, the expression of *L. mexicana* gp63 was determined with anti- *L. mexicana* gp63 Ab and FACS analysis. Red line control, black line test.

The splenocytes were harvested and cultured *in vitro* for 5 days together with blast cells pulsed with LPS and SLA. On day 5, the cultured splenocytes were used as effectors in ⁵¹Cr-release cytotoxicity assays against DCs transfected with pcDNA3 *L. mexicana* gp63 construct as targets.

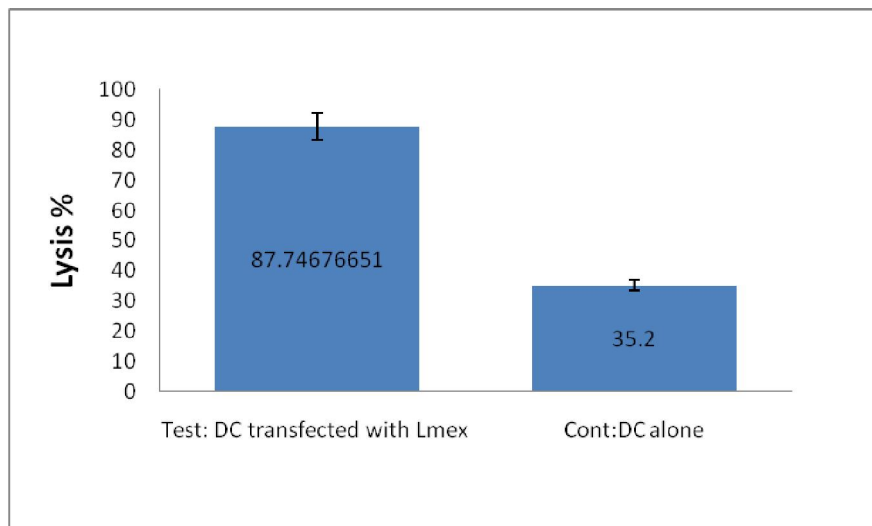


Figure 3. CTL activity in mice immunised with DCs pulsed with SLA against DCs expressing *L. mexicana* gp63 gene. *Balb/c* mice were immunised I.D. with SLA loaded DCs 2×10^6 per mouse. After two weeks the mice were sacrificed and their splenocytes were cultured *in vitro* for 5 days together with blast cells pulsed with LPS and SLA. On day 5 they were used as effector cells in a standard 4-hour cytotoxicity assay against DCs transfected with pcDNA3 containing *L. mexicana* gp63 gene. $p=0.003$

The results clearly showed that immunization of mice with SLA pulsed DCs induced significant CTL activity against DCs expressing *L. mexicana* gp63 protein (Figure 3).

DISCUSSION

The potency of DC-based vaccines has been shown in both immunotherapy and chemotherapy of leishmaniasis (14-16). In the present study the potency of DCs to induce cytotoxic immune response against Leishmania was evaluated. Hence, DCs were loaded with Leishmania crude antigens and injected to Leishmania susceptible mice to check whether or not they can elicit induction of cytotoxic T cells against Leishmania antigens. The capability of DCs in cross presentation of intracellular microbial antigens through MHC class I is already discussed by other researchers (2). Besides, there is convincing evidence to show that CD8⁺ T cells play a crucial role in immunity against Leishmania (6,17). Our results clearly showed that DCs primed with Leishmania antigens have a high potency in presenting these antigens through MHC class I and are capable of directing the immune system toward T cell-mediated cytotoxicity. However, protection induced by DCs loaded with Leishmania lysate is not clearly defined. In our previous study, no protection was observed in mice immunised with DCs loaded with *Leishmania mexicana* lysate (unpublished data) where other researchers reported a complete protection induced by immunisation of mice with pDC loaded with *L. major* lysate (18). It has also been shown that induction of protection to Leishmania by DCs, in addition to CTL activity, depends on expression of other factors such as CXCL10 and IL-12 (15,19) and correlates with the amounts of IFN- γ produced by CD8⁺ T cells (20). The results also indicated a high capacity of DCs in expression of Leishmania genes transfected into these cells and presenting their antigens through MHC class I, which made them potent antigen delivering targets in cytotoxicity assay. This is in line with the results of other studies showing that injection of DCs primed with relevant antigens elicits Ag-specific immune responses, which protects mice from tumor growth (21). Similar results were also obtained when DCs were co-transfected with plasmids coding CCR7 and gp100 a tumor suppressor (22). Our results confirm findings of other studies reporting a high efficiency, long-term viability, and strong protein expression for DCs after transfection (23).

In conclusion, the potency of DCs primed with Leishmania antigens in inducing CTL immune response confirms the feasibility of DC-based vaccine approaches for Leishmania and may pave the way for developing a potent immunogenic vaccine against this parasite. However, this approach for Leishmania vaccine still requires further investigation.

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