

Modulation of the Expression of TLR2, TLR4, TLR9, IFN γ and IL-10 in THP1 Cell Line Following Infection with Leishmania Donovanii Isolates from Different Clinical forms of Leishmaniasis

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ABSTRACT

Introduction: Protozoa of the genus *Leishmania* cause a wide range of pathologies from self-healing skin lesions to visceral pathology. The outcome of infection depends on the species of the infecting *Leishmania* parasite, the quality and quantity of the host immune response and the host genetic background.

Objective: This study aimed to determine the differential expression of TLR2, TLR4, TLR9, IFN- γ and IL-10 cytokine genes of Human THP1 cell line following in vitro infection with *Leishmania donovani* isolates from different clinical forms.

Methods: Human THP1 cells were infected by live promastigotes of *leishmania donovani* isolates from visceral (VL), cutaneous (CL), Post Kala-Azar dermal Leishmaniasis (PKDL) and mucosal Leishmaniasis (ML) Patients. The expression of Tolls like receptor TLR2, TLR4 and TLR9 and the expression of IFN- γ and IL-10 cytokine were measured using Real Time PCR.

Results: A significant increase in the expression of TLR 2, TLR4 and TLR9 by THP-1 was detected following infection of THP-1 cells with *L. donovani* isolates from ML and PKDL patients. A high expression IL-10 mRNA by THP-1 cells was detected in cells infected by *Leishmania donovani* isolates from mucosal lesions.

Conclusion: *Leishmania donovani* isolates from different clinical forms of Leishmaniasis induce different cytokine responses.

Keywords

Leishmania donovani, Toll Like receptors (TLR2 - TLR4 – TLR9), cytokines (IL-1 β , IL-6 and TNF- α).

Introduction

Leishmaniasis is a group of diseases that presents in different clinical forms. Depending on the infecting leishmania species, the quality of the host immune response and the genetic predisposition of the host, infection can present in a wide range of clinical forms

ranging from simple self-curing cutaneous ulcer, to a more serious mucocutaneous form to a potentially lethal visceral form [1].

Sudan is one of the six countries where 90% of the global VL cases due to *L. donovani* occur. *Leishmania donovani* in Sudan is the cause of VL and the dermal sequel of VL clinical form the Post Kala Azar Dermal Leishmaniasis. Cases of mucosal leishmania were reported in Sudan mostly in VL endemic areas and they were caused by *L. donovani*. Elamin *et al* (2008) reported cases

of cutaneous leishmania due to *L. donovani*. Possible factors associated with the development of the different clinical forms caused by *L. donovani* are not known [2].

In this manuscript we describe the response of human THP1 cell to infection by live promastigote of *L. donovani* isolates from CL, VL, PKDL and ML patients to determine the immunomodulation of infection on THP1 cells with *L. donovani* isolates from different clinical forms of leishmaniasis.

Material & Methods

Ethical consideration:

The study was approved by the ethics committee of the Institute of the Endemic Disease.

Human macrophage THP1 cell line culture

Human Macrophage (THP1) cell line was maintained at 37 °C in a cell-free DMEM medium supplemented with 10% heat-inactivated(30 minutes at 56°C) fetal calf serum(FCS),100IU/ml Penicillin and 100µg/ml Streptomycin or 25µg/ml Gentamicin, 2mM L-glutamine and 25mM HEPES pH 7.4 (GIBCO BRL). This medium was used for subsequent cultures and termed complete medium. The cells were examined over the inverted microscope every day for confluent growth. Cultures which exhibited any signs of contamination were discarded. The cells were examined over inverted microscope every day to detect growth and contamination.

Parasite culture

Leishmania L.donovani isolates from VL, PKDL, CL and ML patients were cultured using a modified biphasic NNN media. promastigotes were initially cultivated at 26°C in a modified biphasic NNN medium and subsequently maintained in complete medium until stationary phase of the culture was reached. The number of viable parasite was counted.

In vitro infection of the macrophage with the *Leishmania* parasite

The growing THP-1 macrophages were infected with promastigotes of *L. donovani* From VL, PKDL, CL and ML clinical isolates at ratio 1:5 in sterile tissue 24-well culture plates. The infected cultures were incubated a 37°C, 5% CO₂ for 48 after which the cells were harvested for RNA extraction.

RNA extraction

Easy-BLUE™ Total RNA Extraction Kit (iNtRON biotechnology / catalog No. 17061) Used for extraction of RNA from the cells those were harvested and frozen in -80°C.

The procedure was done according to the manufacture instructions (iNtRON biotechnology, Inc.).

RNA concentration and purity was measured by Nano drop spectrophotometer at 260nm, and then was stored at -80 till used for cDNA synthesis.

cDNA synthesis

cDNA was synthesized using power cDNA synthesis kit (iNtRON

biotechnology / catalog No. 25011).

15ul mix was prepared by mixing, 7ul water, 4ul buffer, 3ul MgCl₂, and 1ul dNTPs, without adding reverse transcriptase enzyme, then the mixture was added to primer/RNase-free water mix.

PCR cycles

All samples in addition to a positive and a negative controls were denatured at 75°C using PCR machine (Techni) for 5 minutes, extended at 42° C for 60 minutes, heat to 70°C for 5 minutes and finally cooled at 4° C, then the amplified cDNA was stored at -20°C till used for Real-Time PCR.

Quantitative Real time-PCR assays

Primers used in this study, were listed in (Table 1.) Rt PCR was done by using a 2x Real Mod™ Green Real Time PCR Master Mix kit (iNtRON biotechnology / catalog No. 25344), Standard curves were done from 1.2 kb kanamycin positive control RNA for all primers, a tenfold serial dilution was used to test the efficiency of β Actin housekeeping gene, TLR2, TLR4 and TLR9, IL-10 and IFN-g primers. Two microliters from each dilution was added to 18 µl real-time PCR master mix in each tube, five points was used to construct the curve, and finally run into the real-time PCR machine (Rotor-Gene Q).

The cycling condition were 95°C for 15 minutes pre-incubation, followed by 40 cycles of 95°C for 20seconds, 60°C for 20 seconds and 72°C for 20 seconds, for β Actin, TLR2, TLR4 ,TLR9, IL-10 and IFN-g. Dissociation curve run followed as 95°C for 1min then 55°C to 95°C generating the melting curve. None template controls (NTC) amplified in each run.

Table 1: β Actin, TLR2, TLR4, TLR9, IL-10 and IFN-g oligonucleotides primers.

<i>Actin β</i>	FW 5'-CTG TGG CAT CCA CGA AAC TA-3' RV 5'-AGT ACT TGC GCT CAG GAG GA-3'
<i>TLR2</i>	FW 5'-CGA TAT GCT AAA CAC AAT GAC -3' RV 5'- CAA ATG ACG GTA CAT CCA CGT -3'
<i>TLR4</i>	FW 5'CAGACATCAAGGCGCAT-3' RV 5'TTCTTACCTGCTCCACG-3'
<i>TLR9</i>	FW 5'- GGG TTG GAA GAT GCT AGAAGA -3' RV 5'- CGA GCA GGG GAG GGT CAG ACC -3'
<i>IL-10</i>	FW: 5'CAGACATCAAGGCGCAT-3' RV: 5'TTCTTACCTGCTCCACG-3'
<i>IFN-γ</i>	FW: 5'- GAAGAATTGAAAGAGGAGAGTGA-3' RV: 5'-GTATTGCTTTGCGTTGGAC-3'.

Statistical analysis

Analysis of Real Time PCR results:

A delta-delta cycle threshold (ΔΔCt) method was used to quantify a relative gene expression of TLR2, TLR4 and TLR9, comparing with housekeeping gene β Actin, against non-stimulated sample, the number of copies of each gene was measured in all clinical forms, and controls, entered to Excel, statistical analysis was done by sigma blot 11, the mean frequency of each gene was compared in all stimulated cells, and controls. The delta delta CT method required that the efficiency of the two genes must be at equal level approximately, it was described by Schmittgen and Livak (2008).

Results

Measurement of TLRs expression by THP-1 cell line infected with VL, PKDL, CL and mucosal isolates by Real-Time

The frequencies of *TLR2*, *TLR4* and *TLR9* relative gene expression are shown in (Figure 1 and 2).

Expressions of *TLR2*

The frequency of *TLR2* gene expression was significantly expressed in THP-1 cell lines infected with (ML) *L.donovani* (121.09), (CL) (66.717) reference strains compared with other strains and non-infected (0.16) cells. Low frequency of *TLR2* gene expression was detected in cells infected by (PKDL) (3.052), and seven days infected cells by (VL) *L.donovani* (0.085) (CL) *L. major* (0.185).

Expressions of *TLR4*

The frequency of *TLR4* gene expression was detected higher in THP-1 cell infected with (ML) *L.donovani* (58.485), (CL) (42.517) reference strains compared with other strains and non-infected (0.04) cells. Low frequency of *TLR4* gene expression was detected in cells infected with (PKDL) (3.052), (VL) *L.donovani* (0.3842) and seven days infected cells by (VL) *L.donovani* (0.102) (CL) *L. major* (0.011) (Figure 1 and 2).

Expressions of *TLR9*

The frequency of *TLR9* gene expression was significantly in THP-1 cell lines infected with (ML) *L.donovani* (300.25) reference strains compared with other strains and non-stimulated (VL) *L.archibaldi* (0.673), and seven days infected cells by (VL) and *L.donovani* (0.00) (CL) *L. major* (0.00) (Figure 1 and 2).

Expression of TLRs by THP-1 cell line infected with *L. donovani* isolates

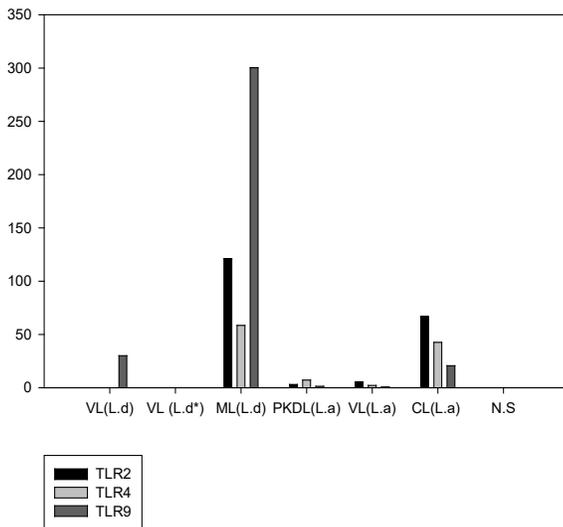


Figure 1: The frequencies of *TLR2*, *TLR4* and *TLR9* relative gene expression in human macrophage THP-1 cell lines infected with *CL*, *VL*, and *PKDL* & mucosal leishmania isolates (1:5 ratio) at 48 hrs. At 48hrs the frequency of *TLR2* gene expression was more detected in THP-1 cell lines.

The frequencies of *TLR2*, *TLR4* and *TLR9* relative gene expression

in human macrophage THP-1 cell lines infected with *CL*, *VL*, and *PKDL* & mucosal leishmania isolates (1:5 ratio) at 48 hrs.

At 48 hrs the frequency of *TLR2* gene expression was more detected in THP-1 cell lines Infected with (ML) *L.donovani* (121.09), (CL) *L.archibaldi* (66.717), the frequency of *TLR4* gene expression was more detected in Stimulated THP-1 cell lines infected with (ML) *L.donovani* (58.485), (CL) (42.517) and the frequency of *TLR9* gene expression was more detected in THP-1 cell lines infected with (ML) *L.donovani* (300.25) compared with other strains and non-stimulated control cells.

(VL) *L.donovani* (VL) *L.d*, (VL) *L.donovani** Seven day post infected cells: (VL) *L.d**, (ML) *L.donovani* (ML) *L.d*, (PKDL) *L.archibaldi*: (PKDL) *L.a*, (VL) : (VL) *L.a*, (CL) (CL) *L.a*, (N.S): non-infected cells.

Expression of TLRs by THP-1 cell line infected with *L. donovani* isolates

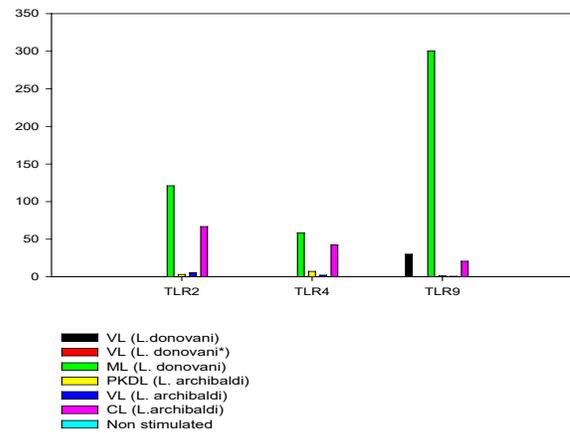


Figure 2: The frequencies of *TLR2*, *TLR4* and *TLR9* relative gene expression in human macrophage THP-1 cell lines.

Infected with *CL*, *VL*, and *PKDL* & mucosal leishmania isolates (1:5 ratio) at 48 hrs.

VL (*L.donovani*): (VL) *L.d*, (VL) *L.donovani**,(Seven day post infected cells: (VL) *L.d**, (ML) *L.donovani*: (ML) *L.d*,(PKDL) (PKDL) *L.VL*) (VL) *L.a*, (CL) (CL) *L.a*, (N.S): non-infected control cells.

Real-Time PCR cytokines measurement

The frequencies of *IL-10* and *IFN-γ* relative gene expression were shown in (figure 3).

Expressions of *IL-10*

The mean frequency of *IL-10* gene expression was detected in stimulated THP-1 infected with (ML) *L.donovani* (206.50), (CL) (127.12) infected strains compared with other strains and uninfected (0.97) control cells. Low frequency of *IL-10* gene expression was detected in stimulated cells of (PKDL) (0.69), and eight days infected cells by (VL) and *L.donovani* (0.01) (CL) *L. major* (0.01) (Figure 3).

Expression of *IFN-γ*

The mean frequency of *IFN-γ* gene expression was more detected in stimulated THP-1 infected with (*ML*) *L.donovani* (477.71), (*CL*) (105.42) infected strains compared with other strains and non-stimulated (0.34) control cells. Low frequency of *IFN-γ* gene expression was detected in stimulated cells by (*PKDL*) (0.05), (*VL*) *L.donovani* (0.03), and seven days infected cells by (*VL*) and *L.donovani* (0.20) (*CL*) *L. major* (0.00) (Figure 3).

Expression of cytokines by THP-1 cell line infected with *L. donovani* isolates

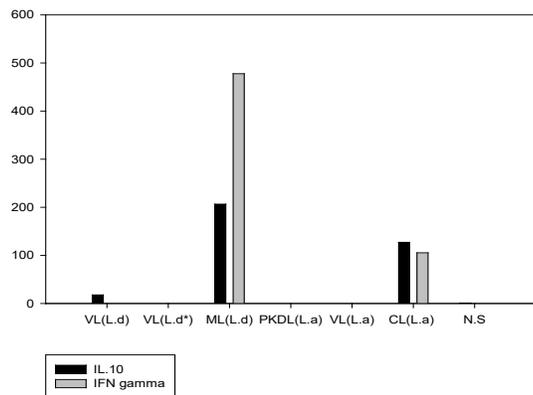


Figure 3: The frequencies of IL-10& *IFN-γ* relative gene expression in human macrophage THP-1 cell lines infected with *CL*, *VL* and *PKDL* & *Mucosal leishmania* isolates (1:5 ratio) at 48 hrs. At 48 hrs the frequency of IL-10 gene expression was detected in stimulated THP-1 infected with (*ML*) *L.donovani* (206.50), (*CL*) *L.archibaldi* (127.12) infected strains; Low frequency of IL-10 gene expression was detected in.

Stimulated cells of (*PKDL*) *L.archibaldi* (0.69), and eight days infected cells by (*VL*) and *L.donovani* (0.01) compared with other strains and Non stimulated control cells and the mean frequency of *IFN-γ* gene expression was more detected in stimulated THP-1 infected with (*ML*) *L.donovani* (477.71), (*CL*) (105.42) infected strains compared with other strains and non-stimulated control cells. Low frequency of *IFN-γ* gene expression was detected in stimulated cells by (*PKDL*) (0.05), (*VL*) *L.donovani* (0.03), and seven days infected cells by (*VL*) *L.donovani*.

(*VL*) *L.donovani*: (*VL*) L.d, (*VL*) *L.donovani** Seven day post infected cells: (*VL*) L.d*, (*ML*) *L.donovani*: (*ML*) L.d, (*PKDL*) *L.archibaldi*: (*PKDL*) L.a, (*VL*) *L.archibaldi*: (*VL*) L.a, (*CL*) *L.archibaldi*: (*CL*) L.a, (N.S): non stimulated control cells.

Discussion

The host immune response to *Leishmania* infection is complex and varies depending on the infecting species. Significant differences in host–parasite interactions have been found in cutaneous and visceral leishmaniasis [3]. Toll like receptors were shown to play a significant role in the parasite host cell interaction [4].

This study showed a significant increase in the expression of TLR 2 by THP-1 cell line infected with *L.donovani* isolate (Figures 1 and 2) from mucosal patients. TLR 2 is known to recognize the

lipophosphoglycan (LPG) antigen of the *Leishmania* inducing NK cell activation and promoting Th1 response [5]. Up-regulation of several TLRs was reported during macrophage infection by *leishmania* [6]. It was shown that *L. (Viannia) panamensis* infection results in up-regulation of TLR1, TLR2, TLR3, and TLR4 expression [7], inducing activation of infected macrophages, whereas infection with *L. donovani* suppresses the TLR2–NF-κB–mediated pro-inflammatory cytokine response [8].

Interestingly TLR4 expression was up regulated in THP-1 cell line infected with live *Leishmania donovani* mucosal isolates and *CL* isolates (Figures 1, 2) compared with infection by parasites from *VL*. This finding suggests a common patho-biological mechanism shared by the two parasite populations. TLR4 associated with an increase in IL-10 production.

Furthermore, infection of THP-1 by live *Leishmania donovani* mucosal isolates also increased the expression of TLR9 (Figures 1 and 2), a known Th1 inducer that generates protective immunity. The induction of proinflammatory cytokines, in particular of IL-12, by antigen-presenting cells was found to be dependent on TLR9 activation by (LPG), indicating that TLR9 stimulation could lead to protective immunity against the parasite [9].

In this study we found significantly high expression of IL-10 by human macrophage cell line following their infection by *ML L. donovani* and *CL* isolates (Figures 3 and 4), which may indicate macrophage-deactivating cytokine production. Increased IL-10 production was reported in patients suffering from *VL* [10]. IL-10 levels were shown to decline upon resolution of *VL* following chemotherapy indicating that IL-10 is a predisposing factor in *VL* [11,12].

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