

Modulation of the Innate and the Adaptive Immune Expression of THP1 Cell Line Following Infection with *Leishmania L. Major* and *L. Donovanii* Isolates from Sudanese Patients

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ABSTRACT

Leishmania, a protozoan parasite, is the causative agent of leishmaniasis. It lives and multiplies within the harsh environment of macrophages. Infection of macrophages by the intracellular protozoan *Leishman* leads to down-regulation of a number of macrophage innate host defense mechanisms, thereby allowing parasite survival and replication. In order to investigate how intracellular parasite manipulates the host cell environment, we undertook a quantitative study of human monocyte-derived macrophages (THP-1) following infection with *L. donovani* and *L. major*. In this study we aimed to measure the proliferation rate of human (THP1) macrophage cell line following infection with cutaneous and visceral leishmania isolates. We used *sysmex* to measure the proliferation rate of human (THP1) cells infected with cutaneous and visceral leishmania isolates after invitro infection of human (THP1) Macrophage cell line by leishmania isolates. We detected *L. major* significantly increased the proliferation of infected THP1 cell line compared with *L. donovani*. Our findings indicate that the infection of host cells human macrophage cell lines by *L. major* stimulates the replication of the cell as a mechanism to enhance the replication of the parasites.

Keywords

Leishmania donovani, *Leishmania major*, THP1 human Macrophage cell line.

Introduction

Leishmania parasites have a complex life cycle usually alternating between an insect vector and a vertebrate host, or between vertebrate hosts. The parasite is spread to humans through sandflies of the genus *Phlebotomus* or *Lutzomyia* during a blood meal [1]. Within the mammalian host, *Leishmania* infect macrophages, cells that play a critical role in regulation of immune system and in host defense [2]. Pivotal to cellular immune responses, macrophages function as antigen processing and presenting cells and produce a variety of cytokines that have pleiotropic effects within the host. *Leishmania* have evolved to evade the defense mechanism of these cells through inhibition of macrophage activation that enables pathogen replication and survival [3-5]. For example, essential

macrophage activation signaling molecules and pathways such as PKC, JAK/STAT, MAPK, NF- κ B as well as the transcription factor AP-1 are deactivated following infection with *Leishmania* [6]. The parasite is transmitted by phlebotomine sandflies to several mammals, including humans in whom it causes cutaneous, muco-cutaneous and visceral forms of disease. While cutaneous leishmaniasis caused by *L. major* is a self-healing disease, VL, also known as Kala-azar in India, is caused by *L. donovani* and represents the most severe form of the disease. An estimated 350-million populations are at risk of infection and approximately 100 000 cases of VL are estimated to occur annually in India alone [1,2].

The human promyelocytic THP-1 cell line has been found to support the growth of *Leishmania* parasites. Assays with macrophage-amastigotes models are considered closest to the pathophysiological conditions of leishmaniasis, and are therefore

the most appropriate for *in vitro* screening. Differentiated, non-dividing human acute monocytic leukemia cells (THP1) (make an attractive) alternative to isolated primary macrophages and can be used for assaying activity of different compounds against intracellular amastigotes.

Primary screens often target the parasite promastigote stage because of ease of culture and manipulation. Indeed, promastigotes from several *Leishmania* species are easily maintained as cell suspension *in vitro*. Culture conditions for promastigotes have been developed in order to facilitate the study of this stage of the parasite [4,7]. This has allowed promastigotes to be screened in a high-throughput manner [8]. These observations highlight the importance of the host macrophage in driving the parasite to specific adaptations.

Objective

To measure the proliferation rate of human (THP1) macrophage cell line following infection with cutaneous and visceral *leishmania* isolates.

Material & Methods

Study design

Laboratory based experimental study was conducted for analysis of the proliferations responses of human (THP1) macrophage infected by *Leishmania donovani* and *Leishmania major* parasites from CL and VL clinical forms.

Ethical clearance

The study was approved by the Institute of the Endemic Disease Ethics Committee - University of Khartoum.

Human macrophage THP1 cell line culture

Human macrophage (THP1) cell line was cultured from the cytobank at Institute of the Endemic Disease. Under sterile condition the contents of the thawed culture tubes were transferred into 15 ml sterile falcon tube and 2 ml of complete media was added to wash the cells from glycerol in the freezing media. The cells were centrifuged at 1200 rpm for 10 minutes at 4°C. The cells were transferred into 25 ml sterile tissue culture flask and about 10-15 ml of complete media was added and incubated at 37°C and 5% CO₂. The cells were examined over the inverted microscope every day to detect growth and contamination.

Parasite culture

Leishmania parasites from *L. donovani Archibald* and *L. major* were cultured using a modified biphasic NNN media, cultures were kept in a temperature range between 20-30°C in the field conditions and in a cold incubator (24-26°C) in the laboratory. Inoculated primary cultures were sub cultured every 3 days in freshly prepared biphasic media After growth, parasite isolates were sub cultured in a sterile 25 ml and 250 ml tissue culture flask using DMEM liquid media containing 25 mm Hepes pH 7.4, supplemented with 10% heat inactivated fetal calf serum (FCS) and Penicillin/ Streptomycin.

One ml of the live reference strain CL *L. donovani Archibald*, VL *L. Major* in stationary phase were centrifuged at 1200 rpm for 10 minutes, the pellets were washed three times using sterile phosphate buffer saline (PBS), then suspended in 1 ml of DMEM. (10 µL was added to 90µL) Equal volume of 2% formalin and parasite pellet suspension was mixed, loaded into a haemocytometer under the 40 X objective lens of a light microscope. The number of the parasites/ml was counted.

Invitro infection of the macrophage with the *Leishmania* parasite:

Cultures of well grown macrophages were transferred into 15 ml falcon tube and centrifuged at 1200 rpm for 10 minutes; the pellets were resuspended in 1.0 ml complete media. 10 µL was added to 90µL of trypan blue 0.4% for counting of live macrophages using haemocytometer. The growing macrophages Derived Monocytes THP1 cells were then exposed to metacyclic promastigotes of *L. donovani archibaldi* and *L. major* at ratio 1:5 in sterile tissue culture plates. The infected culture was incubated at 37°C.

Proliferation of the infected macrophage with the *Leishmania* parasites

The growing THP1 cells infected by *L. major* and *L. donovani donovani* at ratio 1:5 in sterile 15 ml flask to measure growth rate of infected macrophage in during 7 days using sysmex.

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 260 nm, and then was stored at -80°C 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 control 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 were 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, IL10 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'-TTCTTCACCTGCTCCACG-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'-TTCTTCACCTGCTCCACG-3'
<i>IFN-γ</i>	FW 5'-GAAGAATTGGAAAAGAGGAGAGTGA-3' RV 5'-GTATTGCTTTGCGTTGGAC-3'

Statistical analysis

Analysis of Real Time PCR results

A delta-delta cycle threshold ($\Delta\Delta Ct$) 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

Effect of *L. major* and *L. donovani* infection on proliferation of THP-1 cell:

Proliferation of THP1 macrophage cell lines infected with *L. major CL* and *L. donovani donovani VL* was measured by sysmex haematology analyser for seven days' post infection (Table 2), (Figure 1).

Counts			
Days	Non infected THP1 cell line (*10 ³ µl)	<i>L. major</i> infected THP1 cell line (*10 ³ µl)	<i>L. donovani</i> infected THP1 cell line (*10 ³ µl)
Day 0	0.3	0.3	0.3
Day one	0.4	0.8	0.5
Day two	0.6	1.1	0.7
Day three	0.6	1.2	0.7
Day four	0.7	1.3	0.9
Day Five	0.8	1	0.7
Day six	0.8	4.1	3.2

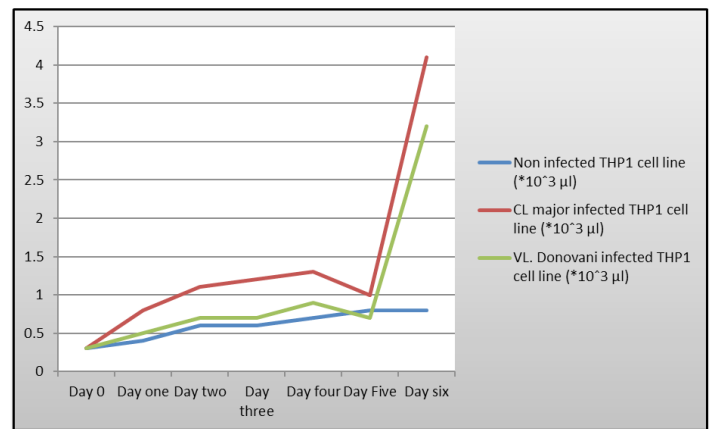


Figure 1: Proliferation of THP1 macrophage cell lines (*10³ µl) infected with *L. major CL* and *L. donovani VL* isolates.

Measurement of TLRs expression by THP-1 cell line infected with *L. major CL* and *L. donovani VL* isolates by Real-Time.

The frequencies of TLR2, TLR4 and TLR9, IL-10 & IFN-γ relative gene expression are shown in (Figure 2).

Expressions of TLR2

Low frequency of TLR2 gene expression was detected in cells infected by seven days infected cells by (VL) *L. donovani* (0.085) (CL) *L. major* (0.185) (Figure 2).

Expressions of TLR4

Low frequency of TLR4 gene expression was detected in cells infected with seven days infected cells by (VL) *L. donovani* (0.102) (CL) *L. major* (0.011) (Figure 2).

Expressions of TLR9

The frequency of TLR9 gene expression was significantly in THP-1 cell lines infected with seven days infected cells by (VL) and *L. donovani* (0.00) (CL) *L. major* (0.00) (Figure 2).

Expressions of IL-10

Low frequency of IL-10 gene expression was detected in stimulated cells of seven days infected cells by (VL) and *L. donovani* (0.01) (CL) *L. major* (0.01) (Figure 2).

Expression of IFN-γ

High frequency of IFN-γ gene expression was detected in stimulated cells of seven days infected cells by (VL) *L. donovani* (0.20) (Figure 2).

Cytokines measurement

Measurement of cytokines concentration in supernatants of infected THP1 cell lines:

Cytokines measurement in THP1 human macrophages infected with *Leishmania* parasites in day seven post infection:

Measurement of IL-6 concentration:

The highest concentration of IL-6 was detected in the supernatant of THP-1 infected with (CL) *L. major* parasites day seven post infection 755 pg/ml respectively compared with other isolates (Figure 3).

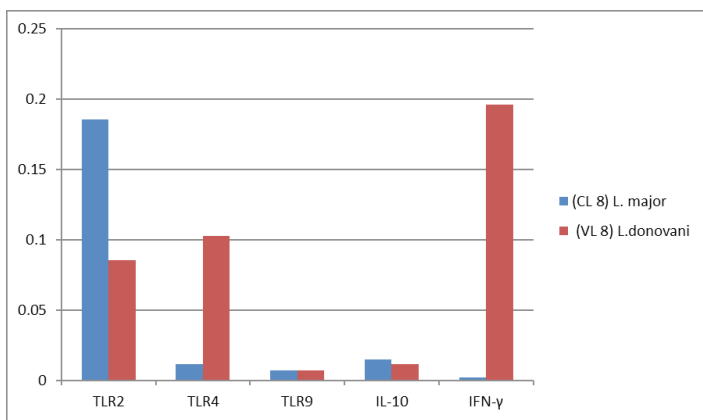


Figure 2: The frequencies of TLR2, TLR4, TLR9, IL-10& IFN- γ relative gene expression in human macrophage THP-1 cell lines infected with *L. major* CL and *L. donovani* VL isolates (1:5 ratio) at seven days. At seven days the frequency of TLR2 gene expression was more detected in THP-1 cell lines.

Measurement of TNF- α concentration

The low concentration of TNF- α was detected in the supernatant of THP-1 infected with (CL) *L. major* and (VL) *L. donovani* parasites 31 pg/ml, 53 pg/ml respectively (Figure 3).

Measurement of IL-1 β concentration:

The highest concentration of IL-1 β was detected in the supernatant of stimulated THP-1 infected with (CL) *L. major* and (VL) *L. donovani* parasites 289 pg/ml, 203 pg/ml (Figure 3).

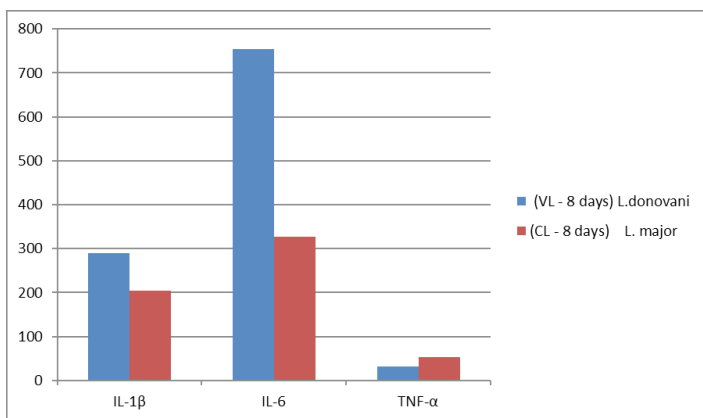


Figure 3: TNF- α IL-6 and IL-1 β measured in supernatant of THP1 human macrophages cell line infected with *Leishmania* parasites in day eight post infection.

Discussion

Protozoa of the genus *leishmania* cause extensive diversity pathogenesis ranging from self-healing skin lesion to lethal visceral disease, depending on the infecting species parasite and the adaptive immune response. Significant differences in host-parasite interactions have been found in cutaneous and visceral leishmaniasis [3]. The aim of this study was to quantify the multiplying frequency of human (THP1) macrophage cell line subsequent infection with cutaneous and visceral *leishmania* isolates.

The Proliferation of THP1 macrophage cell lines infected with *L. major* CL and *L. donovani* VL was measured by sysmex haematology analyzer for seven days' post infection. In this study major significantly increased the proliferation of infected THP1 cell line compared with *L. donovani*. It is known that *L. major* usually causes localized lesion that requires availability of macrophage to maintain the infection, the increased proliferation will provide more host cells to maintain infection.

This is the first data to show the effect of Leishmania infection in proliferation of THP-1. 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 2) a known Th1 inducer that generates protective immunity. Furthermore, significantly high expression of IFN- γ by human macrophage cell line following their infection by VL *L. donovani* isolates (Figures 2).

Conclusion

In additions to significant increase of proliferation of THP-1 cell line, the elevation of gene expression, IL- β , IL-6 and TNF, measurement my suggest that, the parasite try to develop a micro environment to maintain the infection and scape the immune system to confirm this suggestion we recommended more experimental trials to reveal the causative agents of this elevations.

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