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Enhanced expression of Toll Like Receptors (TLRs) 2 & 4, but not 9, in spleen tissue from visceral leishmaniasis patients

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Abstract

Toll like receptor (TLR) signaling is involved in first line defense against *Leishmania* parasites by triggering NF-κB activation and down-stream production of pro-inflammatory cytokines. Experimental models of visceral leishmaniasis (VL) support a protective role for TLRs 2, 4 and 9 in host immune responses to *Leishmania* infection. There is limited data available on expression of these TLRs in human VL, particularly in sites of infection, such as the spleen. This study aimed to determine if the expression of mRNA encoding the expression of TLRs 2, 4 and 9 were altered in VL and compare expression patterns in splenic biopsies and peripheral blood mononuclear cells.

Keywords

Visceral Leishmaniasis; Toll like Receptors; Splenic Aspirate

Introduction

Visceral leishmaniasis (VL) is a fatal, chronic infectious disease caused by the intra cellular protozoan parasite *L. donovani*. VL remains a public health problem in the Indian sub-continent and East Africa (1) and is associated with high-grade fever, hepato-splenomegaly, pancytopenia and hypergammaglobulemia. The drugs available to treat patients are expensive and associated with toxicity. In addition, parasite drug resistance is an increasing concern and there is no licensed vaccine (2). While our understanding of the immune response following *Leishmania* infection in humans is increasing, knowledge for the rational design of an effective vaccine is still insufficient (3).

Toll Like Receptors (TLRs) are type I transmembrane proteins, expressed primarily by innate immune cells, including monocytes, macrophages, neutrophils, dendritic cells,

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Conflict of Interest

We declare no conflict of interest.

mucosal epithelial cells, and dermal endothelial cells (4). This class of receptors, also called pattern recognition receptors (PRR's), is capable of sensing organisms such as protozoa, bacteria, fungi and viruses via pathogen associated molecular patterns (PAMPs). In contrast to the significant body of knowledge on bacterial PAMP's, relatively little is known about the role of TLRs in the host response to eukaryotic parasites. TLR signaling is thought to trigger NF- κ B pathway signaling pathways after interactions with *Leishmania* parasites (5, 6). Studies using experimental models revealed that TLR 2, 4 and 9 contribute to the recognition of *Leishmania major* and to the development of subsequent immune responses. Mice lacking the TLR signaling pathway adaptor protein MyD88 show enhanced susceptibility to *L. major* infection (7), and a TH2 polarized response (8), suggesting the presence of TLR ligands on *L. major* and the importance of this innate immune recognition pathway in control of infection. *Leishmania* lipophosphoglycan (LPG), a TLR-2 agonist, has been shown to enhance anti-parasitic immunity in a MyD88 dependent manner during *L. major* infection (7). Using TLR-competent and TLR-deficient mice, it was also shown that TLR4 contributes to both innate and acquired immune responses during *L. major* infection (9). In addition, TLR4 was shown to control parasite replication by early induction of inducible nitric oxide synthase and lowering arginase levels, an enzyme known to promote parasite replication (10). In another study, TLR9 signaling was found to be essential for control of lesion development and parasite replication, as well as NK cell responses in murine cutaneous leishmaniasis and VL (11, 12). It has also been suggested that TLR2 and TLR 3 play an important role in recognition of *L. donovani* promastigote by IFN γ -primed macrophages by using RNA interference (13).

The above studies on TLR's were all restricted to animal model of *Leishmania* infection. Recent studies on samples from patients with tegumentary and cutaneous leishmaniasis indicate differential expression and function of TLRs in different forms of human disease (14, 15). Down-regulation of TLR-4 expression has been reported *in-vitro* using monocytes derived macrophages from active VL patients. (16). However, no study has yet addressed the *ex-vivo* expression of TLRs in VL patients. This study aimed to complement existing data by measuring the expression of mRNA encoding the expression of TLRs 2, 4 and 9 in pre and post treatment splenic biopsies (site of infection), as well as in peripheral blood mononuclear cells (PBMC) from VL patients.

Materials and Methods

All patients presented with symptoms of VL at the Kala-azar Research Center, Muzaffarpur, Bihar, India. Their diagnosis was confirmed by detection of amastigotes in splenic aspirate smears. In total, 33 patients were enrolled in the study with their prior consent and ethical clearance from the Institutional Ethic committee of Banaras Hindu University. The number of samples analyzed varied for the various gene expressions measured depending upon availability of mRNA. Splenic needle aspirates were collected for diagnostic purposes before treatment and 3–4 weeks after initiation of anti-leishmanial treatment to evaluate parasitologic cure. Heparinized blood was also collected from patients ($n=9$), as well as endemic controls ($n=7$), to isolate PBMC's. Control spleen cells ($n=8$), were obtained from healthy organ donors (HODs), as described elsewhere (17). The clinical data relating to VL patients are given in Table 1.

The left over splenic aspirates from diagnostic procedures (100–150 µl after smear preparation) were collected directly into RNeasy lysis buffer at pre- and post-treatment and stored at –70 until mRNA isolation and analysis. PBMCs were isolated by ficoll hypaque (GE Healthcare, NJ, USA) gradient centrifugation and collected in the same way as spleen cells. Total RNA was isolated using RNeasy minikit and Qiashredder homogenizers (Qiagen) according to the manufacturer's protocol. The quality of RNA was assessed by denaturing agarose gel electrophoresis. cDNA synthesis was performed in 20 µl reactions on 0.5–1.0 µg RNA using High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA).

RNA was prepared from enriched fractions of splenic aspirate cells, isolated using a modification of the sequential selection protocol described previously (17). Briefly, cell subsets were enriched by sequential positive selection of CD19+ (B cells), followed by CD3+ (T cells) cell selection from the CD19-depleted fraction. CD14+ (monocytes/macrophages) cells were selected from the CD19- and CD3-depleted fraction. All selections were carried out using MACS beads (Miltenyi Biotec, Auburn, CA) and Magnetic LS columns (Miltenyi Biotec), according to the manufacturer's instructions. The positively selected subsets were re-suspended in 200–300 µl RNA later and stored at –70°C until use. RNA isolation and cDNA preparation were carried out as described above. Real-time PCR was performed on an ABI Prism 7500 sequence detection system (Applied Biosystems) using cDNA specific FAM-MGB-labeled primer/probe for IL-10, IFN-γ, TLR2, TLR4, TLR9 and MyD88. The relative quantification of products was determined by the number of cycles over 18S mRNA endogenous control required to detect the gene expression of interest. Statistical analysis was performed using Prism 5 (Graph-Pad prism software, La Jolla, CA, USA), and employing either a paired t-test or Mann-Whitney U test. A p value < 0.05 was considered statistically significant.

Results and Discussion

Analysis of mRNA expression revealed significantly elevated levels of mRNA encoding TLR2 ($p = 0.0006$) and TLR4 ($p = 0.0084$) in pre-treatment splenic aspirate samples, compared to post treatment samples, but no change in expression of TLR 9 ($p = 0.43$) in these paired samples (Fig. 1A). As a control for tissue integrity, and in accordance with previous findings (i.e. enhanced IL-10 and IFN-γ mRNA in splenic biopsies from VL patients (17)), we measured IL-10 and IFN-γ mRNA accumulation in the samples included in this study. We confirmed the elevated mRNA IL-10 ($p = 0.0005$) and IFN-γ ($p = 0.0043$) in pre-treatment compared to post-treatment splenic biopsies, (Figure 1A), indicating the tissue used was displaying similar immune characteristics as previously reported. We observed a range of one to three PCR cycle differences among the paired spleen samples used in this experiment. Given the fact that this is an *ex-vivo* mRNA expression profile of the genes used, we believe that these differences are of relevance. To identify which splenic cell population was responsible for the higher expression of TLR 2&TLR 4, mRNA, we assessed mRNA from enriched fractions of splenic aspirate cells, as described above. We found elevated mRNA expression of TLR-2 and -4, as well as their downstream signaling molecule MyD88, in CD14+ splenic cells, compared to CD19+ and CD3+ cells (Figure 1B). *Leishmania* parasites reside in CD14+ cells in spleen tissue, and result indicated

involvement of TLR signaling during active disease. Our previous analysis of splenic material estimated the frequency of CD14+ cells to be around 7%, with no significant changes pre and post treatment (17). To further address TLR expression during active disease, we analyzed mRNA encoding TLR-2, -4 and -9, as well as IFN- γ and IL-10, in PBMC from patients before and after drug treatment. As expected, levels of IFN- γ and IL-10 mRNA was significantly elevated in pre-treatment PBMC's, compared to post treatment and healthy endemic controls (figure 1C). However, no difference in TLR-2,-4,-9 mRNAs was observed between PBMCs from pre-treatment subjects, compared to post-treatment group or that of healthy endemic controls (figure 1C), suggesting site and cell specific up regulation of mRNA expression for these TLRs during ongoing disease.

Significant progress has been made in understanding the role of host innate immune responses in shaping adaptive immune responses following infection. TLRs are important molecules for innate immune cell sensing and play an important roles in linking innate and adaptive immune responses (18). Little is known about TLR- responses during *L. donovani* infection in humans. As a first step to address the role of TLR's in VL, we measured the expression of TLR-2, -4, and-9 mRNA in VL patients. Our data showed enhanced tissue-specific TLR 2 and 4 mRNA accumulations in spleen biopsies from patients prior to treatment, compared to post treatment and HOD. However, no change was observed in PBMC's from these patients. A possible explanation for this tissue-specific expression of TLR- 2 and -4 could be the higher prevalence of parasites in the spleen, compared to the blood. We found that splenic CD14+ cells had the highest expression mRNA encoding for the TLRs studied. As monocytes/macrophages are host cells for the parasite this further support that the observed up-regulation is directly driven by the presence of parasite. The implications of these findings remain to be elucidated.

Since elevated expressions of TLR-2 and-4 mRNAs were restricted to the spleen, further assessment using splenic tissue was not feasible due to the very restricted amount of splenic tissue available following diagnosis.

Experimentally in mice, it was shown that interactions between LPG and TLR-2 reduced anti-leishmanial responses via a cytokine-mediated decrease of TLR-9 expression (19). In line with findings in mice, we found more TLR-2 mRNA in spleen tissue from VL patients pre- compared to post-treatment, while TLR-9 mRNA expression remained unchanged in both spleen and PBMC, suggesting that TLR9 activation may be suppressed.

Thus, it is not unreasonable to suggest that the elevated TLR-2 and -4 mRNA expression observed in spleen during *L. donovani* infection has a role in shaping the immune response of CD14+ cells.

In conclusion, this is the first report on the *ex-vivo* expression of TLR-2, -4 and -9 in the spleen and PBMC's of VL patients. These finding may help to identify suitable TLR targets for TLR-based immune-therapy or TLR agonist-based *Leishmania* vaccines in the future.

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References

- Alvar J, Velez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*. 2012; 7:e35671. [PubMed: 22693548]
- Sundar S, Chakravarty J. Leishmaniasis: an update of current pharmacotherapy. *Expert Opin Pharmacother*. 2013; 14:53–63. [PubMed: 23256501]
- Singh RK, Srivastava A, Singh N. Toll-like receptor signaling: a perspective to develop vaccine against leishmaniasis. *Microbiological research*. 2012; 167:445–451. [PubMed: 22326459]
- Imler JL, Hoffmann JA. Toll receptors in innate immunity. *Trends in cell biology*. 2001; 11:304–311. [PubMed: 11413042]
- Tuon FF, Amato VS, Bacha HA, Almusawi T, Duarte MI, Amato Neto V. Toll-like receptors and leishmaniasis. *Infect Immun*. 2008; 76:866–872. [PubMed: 18070909]
- Barton GM, Medzhitov R. Toll-like receptor signaling pathways. *Science*. 2003; 300:1524–1525. [PubMed: 12791976]
- de Veer MJ, Curtis JM, Baldwin TM, et al. MyD88 is essential for clearance of *Leishmania major*: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. *Eur J Immunol*. 2003; 33:2822–2831. [PubMed: 14515266]
- Muraille E, De Trez C, Brait M, De Baetselier P, Leo O, Carlier Y. Genetically resistant mice lacking MyD88-adaptor protein display a high susceptibility to *Leishmania major* infection associated with a polarized Th2 response. *J Immunol*. 2003; 170:4237–4241. [PubMed: 12682257]
- Kropf P, Freudenberg N, Kalis C, et al. Infection of C57BL/10ScCr and C57BL/10ScNcr mice with *Leishmania major* reveals a role for Toll-like receptor 4 in the control of parasite replication. *Journal of leukocyte biology*. 2004; 76:48–57. [PubMed: 15039466]
- Kropf P, Freudenberg MA, Modolell M, et al. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite *Leishmania major*. *Infect Immun*. 2004; 72:1920–1928. [PubMed: 15039311]
- Weinkopff T, Mariotto A, Simon G, et al. Role of Toll-like receptor 9 signaling in experimental *Leishmania braziliensis* infection. *Infect Immun*. 2013; 81:1575–1584. [PubMed: 23439309]
- Liese J, Schleicher U, Bogdan C. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. *Eur J Immunol*. 2007; 37:3424–3434. [PubMed: 18034422]
- Flandin JF, Chano F, Descoteaux A. RNA interference reveals a role for TLR2 and TLR3 in the recognition of *Leishmania donovani* promastigotes by interferon-gamma-primed macrophages. *Eur J Immunol*. 2006; 36:411–420. [PubMed: 16369915]
- Tuon FF, Fernandes ER, Pagliari C, Duarte MI, Amato VS. The expression of TLR9 in human cutaneous leishmaniasis is associated with granuloma. *Parasite Immunol*. 2010; 32:769–772. [PubMed: 21086718]
- Tuon FF, Fernandes ER, Duarte MI, Amato VS. Expression of TLR2 and TLR4 in lesions of patients with tegumentary American leishmaniasis. *Revista do Instituto de Medicina Tropical de Sao Paulo*. 2012; 54:159–163. [PubMed: 22634888]
- Das S, Pandey K, Kumar A, et al. TGF-beta1 re-programs TLR4 signaling in *L. donovani* infection: enhancement of SHP-1 and ubiquitin-editing enzyme A20. *Immunology and cell biology*. 2012; 90:640–654. [PubMed: 21968712]
- Nylen S, Maurya R, Eidsmo L, Manandhar KD, Sundar S, Sacks D. Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis. *J Exp Med*. 2007

18. Tipping PG. Toll-like receptors: the interface between innate and adaptive immunity. *Journal of the American Society of Nephrology: JASN*. 2006; 17:1769–1771. [PubMed: 16775026]
19. Srivastava S, Pandey SP, Jha MK, Chandel HS, Saha B. Leishmania expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. *Clinical and experimental immunology*. 2013; 172:403–409. [PubMed: 23600828]
20. Srivastava A, Singh N, Mishra M, et al. Identification of TLR inducing Th1-responsive *Leishmania donovani* amastigote-specific antigens. *Molecular and cellular biochemistry*. 2012; 359:359–368. [PubMed: 21858498]

Figure: 1a

Figure 1b

Figure 1c

Figure 1.

mRNA expression of IL-10, IFN γ , TLR-2, TLR-4 & TLR-9 in spleen tissue and PBMC's of VL patients and healthy controls. A) mRNA expression of IL-10, IFN γ , TLR-2, TLR-4 & TLR-9 in splenic aspirates from VL patients and HOD. B) mRNA expression of TLR-2, TLR-4 and MyD88 in enriched splenic subset of CD3+, CD14+ and CD19+ cells. C) mRNA expression of IFN γ , IL-10, TLR-2, TLR-4 & TLR-9 in PBMC' from VL patients endemic control samples.

Table I

Aggregate clinical data for VL patients

N	33
Age (years)	25.17 ± 16.51 (23)
Sex (M/F)	16/17
Duration of illness (days)	33.5 ± 25.61 (25)
WBC counts, pre treatment	4027 ± 1994 (3700)
WBC counts, post treatment ^a	8600 ± 2800 (7900)
Spleen Size (cm), pre treatment	3.65 ± 2.39 (3.00)
Spleen Size (cm), post treatment ^a	0.138 ± 0.58 (0)
Splenic Score ^b	2.58 ± 1.35 (3)

Median values are given within bracket.

^aPost treatment values are from day 15 or day 30 post treatment.

^bScoring of parasite load is on a logarithmic scale from 1 to 6, where 0 is no parasites per 1000 microscopic fields (1000X), 1 is 1–10 parasites per 1000 fields, and 6 is >100 parasites per field.