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Brief communication

Leishmania donovani infection activates Toll-like receptor 2, 4 expressions and Transforming growth factor-beta mediated apoptosis in renal tissues

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ABSTRACT

The present study was aimed to identify the underlying mechanisms of improper renal function in *Leishmania donovani* infection that causes VL. Mice (BALB/c) were infected with *L. donovani* and different parameters for proteinuria were assessed. The levels of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), lipid peroxidation (MDA), inflammatory cytokines, and toll-like receptor (TLR) 2 and 4 expression were found significantly elevated at 60th day in these animals and declined at 90th day post infection. However, TGF-β and caspase 3 activities were higher at 90th day in comparison to 60th day post infection. These findings suggested that exacerbated inflammatory conditions correlate with abnormal renal functions in *L. donovani* infection, which is further augmented by activated TLRs expressions by circulating leishmanial antigens. Further, the increased levels of TGF-β and caspase 3 at 90th day suggested TGF-β mediated apoptotic cell death of renal and other cells during later stages of disease that may eventually result in release of host and parasitic factors in urine during visceral leishmaniasis.

The global annual burden of all forms of leishmaniasis is approximately 12 million per year and approximately 350 million people are at risk; however, exact statistical data are lacking.[1] The disease is characterized by three clinical forms, i.e. visceral (VL), cutaneous (CL) and mucocutaneous (MCL) leishmaniasis. Out of these the visceral disease is almost always fatal, if left untreated. An estimated annual global burden of VL is about 0.2–0.4 and CL is approximately 0.7–1.2 million.[1,2] Out of the total VL cases globally, about 90% occurs in India, Nepal, Bangladesh, Sudan, and Brazil.

Along with poor establishment of immune response, host kidneys are also affected in leishmaniasis. In chronic leishmanial infection the renal abnormalities are clinically characterized by impaired renal function, i.e. proteinuria and hematuria along with morphological changes such as crescent formation.[3] However, the exact cause of mechanisms of

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renal damage and abnormal renal function is still unknown in visceral leishmaniasis. Oxidative stress is major determinant of various renal diseases and also contributes to the manifestation of glomerulonephritis. Immune response in renal cells can also be augmented by toll-like receptors (TLRs) in response to circulating antigens, which activate production of various inflammatory cytokines. TLRs have also been shown to be involved in the abnormal renal function in several diseases like tuberculosis, salmonellosis, and rheumatoid arthritis, but their role is largely unknown in visceral leishmaniasis.[4] TLRs signaling occur via MyD88 dependent or independent pathways that lead to the activation of either p38 or ERK1/2 related mitogen activated protein kinases (MAPK) and subsequent production of either inflammatory cytokines such as TNF-α, IFN-γ, and IL-12 or anti-inflammatory cytokines such as IL-4, IL-10.[5] Many studies also suggest the importance of TLRs signaling in the onset of leishmanial pathogenesis, resistance and susceptibility; however, limited data exist on their expression in the kidney and their role in renal damage in *L. donovani* infection.[6]

Proteinuria is also associated with apoptotic death of renal cells.[7] TNF-α induced TGF-β mediated apoptotic cascade has been found as one of the major pathway that leads to renal cells death.[7] TGF-β is also shown to be involved in pathogenesis of renal diseases via stimulating the synthesis of extracellular matrix components and decreasing collagenase production but its role is yet not examined in *L. donovani* induced renal abnormality.[8] In this study, we measured the levels of oxidative stress markers, TNF-α, IFN-γ, IL-12, TGF-β, and expression levels of TLR 2, 4, caspase 3 in renal tissues of *L. donovani* infected mice with the symptoms of proteinuria.

Ten-week-old female BALB/c mice were used in this study. This study was conducted following principles of laboratory animal care guidelines and approved by institutional animal ethical committee. The animals were kept in polypropylene cages with chopped wheat straw as the bedding material and temperature around 20–30 °C. They were fed with a standard chow and water *ad libidium*. *L. donovani* promastigotes were cultured to obtain metacyclic forms in Schneider's insect media (pH – 5.5) supplemented with 20% fetal calf serum for 24 h in a BOD incubator as previously described.[9] Animals (8, in each group) were infected with metacyclic promastigotes (2×10^7 per ml), intravenously for the development of VL pathogenesis. After three weeks, confirmation of leishmanial pathogenesis was done in infected animals by rk39 strip test and demonstration of parasites in splenic aspirates by Giemsa staining.

For histopathological observations, the kidney sections were hematoxylin–eosin stained and observed under bright field microscope. For estimations of urinary proteins, the urine samples were collected using clear plastic wrap method described by Kurien and Scofield for further analysis.[10] As soon as the animal urinated, the animal was removed and the urine was collected. Protein concentrations were determined by a modified Bradford method, adapted to a microtiter plate assay as previously described.[11] The 24 h urinary protein excretion was calculated from the 24 h volume and urinary protein concentration. Serum and urine creatinine concentrations were measured by the alkaline picric acid method using an autoanalyzer.

All other biochemicals were estimated in kidney tissue lysates in all experimental animals comprising eight mice in each group (control, 60th and 90th day post infection). All parameters were measured at 60th and 90th day post infection. The kidneys of mice without infection were used as control. To prepare tissue lysate, kidney tissue (~50 mg) were homogenized in Tris–HCl buffer (pH – 8.0) containing leupeptin (10 μg/ml), aprotinin (10 μg/ml), Triton X-100 (1%), PMSF (1 mM), EGTA (1 mM), NaF (5 mM), and sodium orthovanadate (10 mM). The homogenized samples were centrifuged at 10,000 rpm for 10 min at 4 °C. The protein content in supernatant was measured by Bradford method[11] and stored at −80 °C for measurements of various oxidants, cytokines, and TLRs. The superoxide anion content was estimated by the method described elsewhere[12] in 100 μl of kidney lysate supernatant and expressed as nmoles of O_2^- liberated per mg of protein. The production of hydrogen peroxide (in 100 μl lysate) was measured fluoremetrically according to the method described elsewhere[13] represented in nmole/mg of protein. LPO was measured according to standard protocol as described previously in 500 μl of lysate[14] and lipid peroxidation (LPO) was expressed as nmoles of MDA/mg of protein. The levels of TNF-α, IL-12, IFN-γ, and TGF-β were estimated by ELISA MAX^{TM} standard set enzyme-linked immunosorbent assay kit as per manufacturer's instructions (Biolegend, USA). The renal cytokine levels were normalized to protein concentration of lysate and expressed in terms of pg of cytokines/mg of protein. The caspase 3 activity in renal tissue was measured by caspase 3 fluorimetric assay kit (Sigma Chemicals, USA) as per manufacturer's instruction. The caspase 3 activity was and expressed in picomoles per minute per μg of protein.

TLR 2, 4, and caspase 3 mRNAs were quantified by real time PCR on ABI700Fast cycler. Total RNA from kidney tissues (~50 mg) was extracted by RNeasy Mini kit (Quaigen Cat. No-74104) following manufacturer's instructions. RNA pellets were washed thrice with 70% DEPC ethanol and digested with RNAse free DNase (Fermantas, Germany). For cDNA preparation, 1 μg total RNA (kept equal for each amplification) was subjected to reverse transcription using 20 U M-MLV reverse transcriptase Fermantas, Germany), 1× RT buffer, 20 mM dNTPs (New England Biolabs, USA), 20 U RNasin (Fermentas, Germany), 0.1 M DTT with DEPC treated water, and 100 ng of random hexamers (Fermentas, Germany). The expression levels were quantified on ABI7500Fast system as per manufacturer instructions (Applied Biosystem) using mice mRNA specific forward (TLR 2: 5′-TCTGGCTCAAATCCTGGTTG-3′, TLR 4: 5′-TGGGTGAGAAATGAGCTGGT-3′, caspase 3: 5-GAGCAGCTTTG-TGTGTGTGA-3′) and reverse (TLR 2: 5′-GCACCTACGAGCAAGA-TCAA-3′, TLR 4: 5′ACCACAATAACCTTCCGGCT-3′, caspase 3: 5′-TTCGGCTTTCCAGTCAGACT-3′) primers (5 pmol/μl). The β-actin gene (F: 5′-AACCGCGAGAAGATGACCCAGATCATGTTT-3, R: 5′-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3′) was used as housekeeping control. Briefly, 20 μl of real time mix contained 10 μl of Power SYBER green master mix (Applied Biosystem), 1 μl cDNA, 1.5 μl each, forward and reverse primers, 6 μl MilliQ water. PCR conditions were set with an initial incubation of 50° C for 2 min, followed by denaturation at 95° C for 10 min, and 40 cycles at 95° C for 15 s, 60° C for 1 min, and 72° C for 40 s. The abundance of mRNA was normalized