**Leishmania donovani** influenced cytokines and Toll-like receptors expression among Sudanese visceral leishmaniasis patients

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**SUMMARY**

Leishmaniasis remains a serious health problem. The outcome of Leishmania infection depends on the early innate response. In this study, whole blood samples of 40 patients with visceral leishmaniasis (VL), 10 leishmanin skin test-negative (LST–ve) controls and 10 leishmanin skin test-positive (LST+ve) controls were stimulated by live L. donovani promastigotes. Also, THP1 human cell line was infected with L. donovani. The production of interleukin 10 (IL-10), tumour necrosis factor alpha (TNF) and interferon gamma (IFNG) cytokines was measured, and the expression of Toll-like receptors (TLR2, TLR4 and TLR9) was done in the blood samples and also in the THP1 cell line. IL-10 was found to be higher in LST+ve controls compared with VL patients. TNF was moderately produced with no variation between patients, controls and THP1 cells. IFNG was higher in LST+ve controls also in THP1 cells. TLR4 and TLR9 were found to be highly expressed in patients with VL. L. donovani increases the expression of TLR4 and TLR9 in patients with VL and TLR2 in THP1 cells, suggesting a TLRs relation in induction of a mixed cytokine response. TLR9 was markedly recognized by L. donovani DNA.

**Keywords** Leishmania, TLR, cytokine

**INTRODUCTION**

Leishmaniasis remains a serious health problem in many countries. Visceral leishmaniasis (VL) is the most severe clinical form and it is fatal, when left untreated (1). More than 90% of global VL cases occur in six countries India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (2).

The outcome of Leishmania infection depends on the infecting Leishmania species, the host genetics and immune response to infection. The early innate immune responses by the host, including macrophage–parasite interaction, are crucial in determining the persistence or clearance of the parasites (3).

Susceptibility to VL correlates with the presence of a Th2 response (4). In the murine VL model, interleukin 10 (IL-10) directly inhibits the antimicrobial machinery of macrophages by modulating normal signal transduction mechanisms (5). In addition, tumour necrosis factor alpha (TNF) was also shown to stimulate the action of interferon gamma (IFNG) in the induction of nitric oxide production in macrophages to kill the parasite (6).

Experimental studies revealed a diverse repertoire of receptors that are involved in Leishmania–macrophage interactions (7–10); Toll-like receptors (TLRs) are hallmarks of cellular receptors that recognize pathogen-associated molecules and participate in innate responses to infections (11).

Several Leishmania-derived molecules were reported to be recognized by TLRs, and the majority of the studies to date were focused on the activation of TLR2, TLR4 and TLR9, mostly in the skin of patients affected by cutaneous leishmaniasis (12).

Toll-like receptors initiate innate responses in a variety of ways, leading to the production of inflammatory cytokines by macrophages (13, 14). The TLR signalling activates macrophage effector functions and could lead to either Th1 or Th2 response depending upon the nature of parasitic molecules (15, 16). Leishmania employs various survival strategies to subvert the proinflammatory response generated by the host macrophages (17). The leishmanial entry reduces normal effector functions of macrophages, viz. oxygen free radical generation, major histocompatibility class II expression and production of inflammatory cytokines. In addition, leishmanial antigens modulate various signal transduction pathways such as
protein kinase-C (PKC) and mitogen-activated protein kinase (MAPK) signalling (18).

Toll-like receptor-inducing ligands that favour the production of proinflammatory cytokines and increased Th1 response will be better candidates for future vaccine development. However, the *Leishmania*-derived antigens that can regulate T helper 1/T helper 2 dichotomy via TLRs are not yet well identified. There are limited data available on expression of TLRs in human VL and also in healed leishmaniasis and in healthy endemic controls.

We use in this study a whole blood model of stimulation to mimic the real infection scene and also a THP-1 cells model to detect the influence of the macrophage cell alone without the presence of the rest of other immune cells. We compare some of the outcome of these experiments in each model to detect whether there is a similarity in stimulation and infection of these cells of both models which can give us a guide for forthcoming experiments and can help in understanding of the interplay between *Leishmania* and its hosts that leads to resistance or susceptibility. We measure the transcript expression of TLR 2, TLR4 and TLR9 in whole blood samples of patients with VL and in the human macrophage cell line THP1 following stimulation with live *L. donovani* promastigotes and correlate these findings with the production of IFNG, TNF and IL-10 cytokines.

**MATERIALS AND METHODS**

**Ethical consideration**

The study was approved by the ethics committee of the Institute of Endemic Diseases, University of Khartoum. Written consents were obtained from all participants before their enrolment.

**Parasite culture**

*Leishmania donovani* parasite was isolated from patients with VL as described elsewhere (19) using a modified biphasic NNN media. Subcultures were grown in sterile DMEM complete media supplemented with 10% heat-inactivated foetal calf serum and 1% penicillin–streptomycin antibiotics. One millilitre of the live stationary phase *L. donovani* was centrifuged at 241 g for 10 min, the pellets were washed, and the number of the parasites/mL was counted using light microscope.

**Patients and controls**

Forty patients with VL were recruited from Gedarif state (eastern Sudan) and White Nile state (south west of Khartoum State). The age range of the patients was from 1 to 70 years. The male-to-female ratio was 3 : 1. The duration of the clinical signs ranged between 2 and 4 months. Inclusion of VL patients after confirmation of the disease and exclusion of coinfection or any secondary immunosuppression. VL diagnosis was based on detection of antileishmanial antibodies using direct agglutination test and confirmed by identification of *Leishmania* amastigotes in lymph node aspirates. Ten leishmanin skin test-negative healthy individuals living in VL endemic region (endemic controls) and 10 leishmanin skin test positives (healed CL patients) were included in the study as controls. LST was performed by intradermal injection of 0·1 mL of leishmanin suspension containing 5 × 10⁶/mL *L. major* promastigotes in 0·5% phenol saline. The diameter of the skin indurations was measured after 72 h. Indurations with a diameter of 5 mm or more were considered positive based on previous reports (20).

**Blood sample**

Two millilitres of heparinized venous blood samples was collected from every patient and from controls under a septic condition. The samples were diluted 1 : 1 with DMEM complete medium. One millilitre of each diluted sample was pipetted into every well of a sterile 24-well flat-bottomed cell culture plates. Stimulation of whole blood was performed by addition of 5 × 10⁶ live *L. donovani* promastigotes/well, while cells without parasites were kept as nonstimulated controls. The cultures were incubated at 37°C under 5% CO₂. After 48 h, the supernatants of live *L. donovani* stimulated whole blood cultures were collected and frozen in –20°C for cytokines measurement, and the cells were harvested in PAXgene for RNA extraction.

**In vitro infection of the human macrophage THP1 cell line with the *Leishmania* parasite**

Human (THP1) cell line was cultured in 10–15 mL of sterile DMEM complete media and incubated in 5% CO₂ at 37°C. Growing THP1 cells were examined for the morphology and adherence and then infected by *L. donovani* stationary phase promastigotes at ratio 5 : 1 in sterile tissue culture plates. The infected and noninfected cultures were incubated in 5% CO₂ at 37°C for 48 h. Percentages of infected macrophages and numbers of amastigotes per macrophage were determined on Giemsa-stained preparations after counting about 500 cells per sample. The supernatants were then collected and frozen in –20°C for cytokines measurement, and the cells were harvested in Paxgene and frozen at –80°C.
Cytokines measurement

Concentration of IL-10, TNF and IFNG cytokines produced by patients, controls and THP1 cell line was measured in aliquots of cell-free supernatants by a sandwich ELISA using BD OptEIA™ ELISA Set B (Catalog Number: 550534; BD Biosciences, San Diego, CA, USA). The ELISA procedure was carried out according to the manufacture instructions.

RNA extraction and cDNA synthesis

Total RNA was extracted from Paxgene stored cells using Easy-BLUE™ Total RNA Extraction Kits (iNtRON Biotechnology, DCC-BIONET, 358-11, Sangdaewon-Dong, Joongwon-Ku, Sungnam, Kyungki-Do, 462-120, KOREA) according to the manufacture instructions. RNA concentration and purity were measured by Nanodrop spectrophotometer at 260 nm. cDNA was synthesized using power cDNA synthesis kit (iNtRON biotechnology). cDNA synthesis was made by denaturing at 75°C for 5 min, extended at 42°C for 60 min, heat to 70°C for 5 min and finally cooled at 4°C, and the amplified cDNA was stored at −20°C till used for real-time PCR.

Quantitative real-time PCR assays

Real-time quantitative PCR amplification was used to estimate the copy number of TLR2, TLR4 and TLR9 relative to a housekeeping gene beta actin (ACTB) in L. donovani-infected THP1 human macrophages cell line and in L. donovani stimulated whole blood cells of patients with VL and controls. Primers used in this study are listed in Table 1. The amplification was performed using a 2× Real Mod™ Green Real Time PCR Master Mix kit (iNtRON biotechnology).

Standard curves were created from 1-2 kb kanamycin-positive control RNA for all primers. A tenfold serial dilution was used to test the efficiency of the ACTB housekeeping gene, TLR2, TLR4 and TLR9 primers. The cycling condition was 95°C for 15 min pre-incubation, followed by 40 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 20 s, for ACTB, TLR2, TLR4 and TLR9. None template controls (NTC) were amplified in each run to validate the amplification (Table 2).

Statistical analysis

A delta–delta cycle threshold (ΔΔCt) method (21) was used to quantify the relative gene expression of TLR2, TLR4 and TLR9, comparing with housekeeping gene ACTB, against nonstimulated sample. The number of copies of each gene was measured in patients and controls, and statistic analysis was performed using SigmaPlot software, version 12.0 (Systat Software, Inc, Chicago, Illinois, USA). Because the cytokine levels did not show a normal distribution, nonparametric tests were used. The Kruskal–Wallis test was first applied to compare three or more independent samples, and the Dunn’s post-test was used to compare the mean ranks. Differences were considered to be statistically significant when \( P \leq 0.05 \).

RESULTS

The patients with VL varied in disease severity, most severe form of the disease noticed clearly in children <5 years, and the delayed diagnosis in some cases worsened the magnitude of the symptoms. The whole blood samples of patients with VL and controls and the THP1 cells showed a marked increase in the proliferation of cells after stimulation and infection by L. donovani.

Cytokines measurement in patients and controls

IL10 was significantly produced in LST+ve controls more than in the patients with VL, and when we compared IL-10 production in LST+ve and LST–ve controls, we found

Table 2 β-Actin, TLR2, TLR4 and TLR9 oligonucleotide primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin-β</td>
<td>FW 5′-CTG TGG CAT CCA CGA AAC TA-3′</td>
<td>RV 5′-AGT ACT TGC GCT CAG GAG GA-3′</td>
</tr>
<tr>
<td>TLR2</td>
<td>FW 5′-CGA TAT GCT AAA CAC AAT GAC-3′</td>
<td>RV 5′-CAA ATG GTA CAT CCA CGT-3′</td>
</tr>
<tr>
<td>TLR4</td>
<td>FW 5′CAGACATCAAGGCGCAT-3′</td>
<td>RV 5′TTCTTCACGGCTCATCAGG-3′</td>
</tr>
<tr>
<td>TLR9</td>
<td>FW 5′-GGG TTG GAA GAT GCT AGA AGA-3′</td>
<td>RV 5′-CGA GCA GGG GAG GGT CAG ACC-3′</td>
</tr>
</tbody>
</table>

Table 1 The number, age, gender and residence of participants of the study

<table>
<thead>
<tr>
<th>Participants</th>
<th>Number</th>
<th>Average age/years ± SD</th>
<th>Gender (Male : Female)</th>
<th>Residence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visceral leishmaniasis patients with symptoms 2-4 months</td>
<td>40</td>
<td>22.2 ± 20.7</td>
<td>29 : 11</td>
<td>White Nile/Gedarif State</td>
</tr>
<tr>
<td>Leishmanin skin test nonreactive controls (LST-ve)</td>
<td>10</td>
<td>34.6 ± 7.7</td>
<td>7 : 3</td>
<td>Khartoum State</td>
</tr>
<tr>
<td>Leishmanin skin test reactive controls (LST+ve)</td>
<td>10</td>
<td>38.9 ± 10.4</td>
<td>5 : 5</td>
<td>Khartoum State</td>
</tr>
</tbody>
</table>

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the difference between them was insignificant as well as between the patients with VL and LST–ve controls. TNF production was noticeably produced after stimulation of whole blood of the patients with VL, LST+ve and LST–ve controls, but the difference between them did not surpass the statistical significances. IFNG concentration was found to be higher in LST+ve controls significantly when compared with the LST–ve controls and patients with VL, while the difference between LST–ve controls and patients with VL was insignificant (Figure 1a).

TLR expression in patients and controls

The stimulated whole blood cells of patients with VL, LST+ve and LST–ve controls showed a distinct detectable expression of TLR 2, TLR4 and TLR9 which significantly found to be higher when compared with the expression of these TLRs in nonstimulated cells. A significant huge expression in TLR2 was detected in patients with VL when compared to LST–ve and LST+ve controls and significantly the latter was the lowest. The expression of TLR4 and TLR9 resembles the TLR2 in being in their maximal expression in patients with VL then followed by LST–ve controls which were found to be significantly less than in the patients with VL and more higher in the LST+ve controls (Figure 1b).

Cytokines measurement in THP1 cells

Infected THP-1 cells produced a detectable amount of IL10, TNF and very high amount of IFNG significantly more than noninfected THP-1 cells (Figure 1c). TNF that observed in THP1 cells when compared to the whole blood cells revealed insignificant difference in this production. When we compared the production of IFNG between the stimulated whole blood of patients and controls and infected THP-1 cells, we found a significant difference in IFNG concentration between the infected THP-1 cells and the stimulated whole blood of the patients with VL and LST–ve controls. The THP-1 cells were surprisingly the predominant IFNG producer in this study, however, no significant difference in IFNG concentration between LST+ve controls and THP-1 cells (Figure 1a,c).

TLR expression in THP1 cells

TLR2, TLR4 and TLR9 expressions were tangible in infected THP-1 cells in comparison with the noninfected THP-1 cells (Figure 1d), when we compared that noteworthy expression of the infected THP-1 cells with the whole blood stimulated cells of patient and controls, we found a significant difference in TLR2 expression between THP-1 cells on one side and on the flip side LST+ve and LST–ve controls, the expression of TLR2 in LST+ve and LST–ve controls was less than the expression of TLR2 in THP-1 cells. THP-1 cells were the prominent producer of TLR2 transcripts, although the difference in expression of TLR2 between these cells and the expression in the patients with VL was insignificant. In contrast to TLR2 expression, TLR4 and TLR9 were significantly at the minimal expression in THP1 cells when compared to the patients with VL. LST–ve controls were a producer of a significant moderate transcripts of TLR4 and TLR9 which was tend to be more higher than the expression of these TLRs in THP-1 cells; furthermore, the expression of TLR4 and TLR9 in THP-1 cells is in parallel to the expression of these TLRs in LST+ve with the latter being at its minimal expression without reaching the statistical significance and diminished in both of them (Figure 1b,d).

DISCUSSION

The early interaction between *Leishmania* parasites and the host innate immune cells is an important phases that determines the quality and the quantity of the subsequently induced adaptive immune response leading to the persistence or cure of the parasite (22). Analysis of the immune responses conducted in this study reflected a more complex cell interaction, and a diverse cytokine production following live *Leishmania* parasites stimulation of whole blood samples of active patients with VL compared with healthy endemic LST–ve controls and leishmaniasis cured individuals with LST+ve reaction also treated as controls in this study. Despite infection and stimulation with one parasite strain within these study groups, different immune responses were elicited (Figure 1). Considering the magnitude of the immunological events triggered during active VL, the relevance of separating the immune responses into Th1 and Th2 in human VL has been debated (21). Our data revealed that there is a shared pattern of cytokines production between LST–ve and patients with VL, and we found no significant difference in production of IL10, TNF and IFNG in both groups (Figure 1a), which is in agree with a previous data represented the shared IL10 production between the same two groups (23). Moreover, our data showed an increase in IL-10 and IFNG levels in LST+ve controls more than in active VL patients, which reflected the mixed Th1 and Th2 pattern in our study groups, and this is in consistent with many previous reports (3, 24–28). IL-10 is an important regulatory cytokine that suppresses potentially damaging inflammatory immune responses; however, these immunosuppressive properties of IL-10 can also target antigen presentation pathways in macrophages and DCs, thereby affecting T-cell activation and cytokine production during
chronic infection, potentially promoting parasite persistence (29).

In VL, it has been reported previously that the production of IL10 inhibit the production of IFNG (30). Resolution of infection depends on the production of Th1 cytokines mainly IFNG (31–35), which is proportionate with our data concerning IFNG production in the LST+ve controls, the major producer of the IFNG reflected in that, the protective immunity in LST+ve controls. There seems to be a dichotomy in the role of IL10 in VL. On one side, IL10 suppresses host immunity and helps parasite survival, and on the flip side, IL10 also protects the host from tissue damage by exaggerated inflammation (36). Recent findings suggest that some IFNG-producing cells are a crucial source of IL10, which act as a negative feedback mechanism to control tissue damage (37, 38). The IL10 production in THP1 cells was significantly reduced when compared to IL10 in LST–ve and LST+ve controls with no difference in production compared to patients with VL. A study with patients with VL splenic T cells showed elevated expression of IFNG and IL10 suggesting that many cells could be the producer of IL10 and IL10 implicated in the pathogenesis of human VL (39), which with our findings we need to investigate the exact cellular source of these cytokines to trace this dichotomy. Other IL-10 neutralizing studies also showed enhanced IFNG production by antigen-activated whole blood cells taken from patients with VL (40). Here, we could not find any discrimination between our study groups in TNF production although it was significant in production when compared to nonstimulated whole blood samples (data not shown); it was secreted in a moderate level in patients and controls suggesting that its secretion did not alter or contribute to clinical situation of the host and it reflected the proinflammatory action owing to Leishmania infection and suggestive of groups symmetrical immunological attempts to resolve the infection. It has been documented the role of TNF in control of leishmaniasis (41–43) TNF also shares a significant role in resistance because TNF−/− mice readily succumb to infection with Leishmania species (44), although recently it has been observed the susceptibility to leishmaniasis in patients receiving TNF antagonists drugs (45, 46). Moreover, THP1 cells showed the same quality and quantity of the moderate secretion of TNF similar to that in whole blood samples of patients with VL and controls. Another interested finding in the innate immune response in this study is the significantly high IFNG production by THP1 cells following their infection by Leishmania parasites. A few studies reported production of IFNG by human and murine macrophage after Leishmania infection (47, 48). The same cells also secreted a significant concentration of TNF, another inflammatory cytokine associated with cure from Leishmania infection.

The pre-exposure THP1 human macrophages produced IFNG concentration more than all other clinical groups (Figure 1c), and it has been reported previously the auto-activation of macrophages (48). The major biological function of IFNG is to activate macrophages and enhance the microbicidal activity of these cells to kill intracellular pathogens (49). IFNG induces iNOS (inducible nitric oxide synthase) expression and NO production by phagocytes harbouring intracellular parasites and is required for activating macrophages to eliminate parasites and resolve Leishmania infection (50). These finding suggests that the presence of other cells in whole blood modulates the chemokine and cytokine responses of macrophages to Leishmania infections.

In this study, in vitro stimulation of whole blood samples of patients with VL by live L. donovani promastigotes resulted in a significant increase in the transcript expression of TLR4 (Figure 1b), a receptor known to bind to the proteoglycolipid complex ligand on Leishmania parasite leading to the production of IL-12p40, favouring a Th1 response and increasing the production of inducible nitric oxide synthase (17, 51). Furthermore, neutrophil elastase had been shown to bind to TLR4 on macrophages leading to macrophage activation and killing of Leishmania. Similarly, the same VL samples showed a significant increase in the expression of TLR9, a known Th1 inducer leading to protective immunity. TLR9 activates bone marrow-derived dendritic cells (DC) leading to favouring TH1 induction. Furthermore, TLR9 activation in NK cells is known to induce IFNG production and enhance the clearance of the parasite (52). Although the increased expression of TLR4 and TLR9 was expected to favour TH1 response, stimulated whole blood samples of patients with VL showed a mixed Th1 and TH2 cytokines production which need to be confirmed whether it is under influence of TLRs expression or not.

In agreement with previous reports on TLR expression in patients with VL, this study showed a low expression of TLR2 in VL whole blood samples. TLR2 is known to bind to lipophosphoglycan (LPG) on Leishmania parasites leading to production of IFNG and TNF proinflammatory cytokines production (53, 54). The interactions between LPG and TLR2 have been reported to reduce antileishmanial responses via cytokine-mediated decrease of TLR9 expression (55). Leishmania parasites were shown to evade the induction of proinflammatory cytokines by reducing the expression of TLR2 by suppressing MAPK P38 phosphorylation and activating extracellular regulated kinase (ERK)1/2 phosphorylation (52). Interestingly, whole blood samples from healthy endemic controls living in VL endemic area – with no clinical signs of VL – showed similar TLR expression profiles as the confirmed patients with
VL. The fact that those individuals tested negative with Leishmanin skin test (LST−ve) suggest their susceptibility to clinical VL. On the other hand, Leishmanin skin test reactive (LST+ve) individuals who cured previous cutaneous diseases showed low expression of TLR2, TLR4 and TLR9. Further studies are on going to investigate the expression of these TLRs and to correlate it with cytokine production.

In contrast to whole blood samples of patients with VL, stimulation of cultured THP1 macrophage cell line with live L. donovani promastigotes resulted in significant expression of TLR2 and low expression of TLR4 and TLR9 (Figure 1d). The significant expression of TLR4 and TLR9 in whole blood samples of patients with VL compared with stimulated THP1 cell line suggests the participation of other cells, for example natural killer cells (NK) or DC, and neutrophils in VL patient’s responses. Furthermore, Leishmania parasites might have downregulated TLR2 in patients with VL to evade the induction of proinflammatory response as reported by Chandra et al. (17). In this context, it has been reported the role of TLRs in cytokine production in L. mexicana LPG stimulated human macrophages, LPG binds TLR2 and TLR4 receptors in human macrophages and specifically induced the production of TNF-α, IL-1β, IL-12p40, IL-12p70 and IL-10 cytokines and using of TLRs inhibitors suppressed these proinflammatory cytokines production (56). Although the high production of IL-10 and IFNG in LST+ve controls (Figure 1a) could reflect the TLRs expression, surprisingly the TLRs expression showed very low transcripts frequency (Figure 1b). However, as described above, a mixed type 1 and type 2 immune profiles demonstrated the ability of patients with VL to respond to Leishmania differently to LST+ve controls. TLRs in THP1 cells showed TLR2 predominance that need to be correlated with the high amount of IFNG, in addition to this, the similarity between LST−ve and patients with VL in the low or moderate cytokines production, suggesting the role of the high expressed TLR2, TLR4 and TLR9. Moreover, the minimal expression of these TLRs in LST+ve controls also may be lead to the high amount of IFNG and IL10.

In conclusion, these findings are relevant to understanding of the dynamic of immunological events associated with the different clinical status of human VL. We have demonstrated here the cytokine profiles, driven by IL10, TNF, and IFNG, are important for the clinical status changes observed. The regulatory activity rather than the pathogenesis model of IL10 was clearer in our study groups. Live Leishmania promastigotes increased the expression of TLR4 and TLR9 in peripheral blood samples of patients with VL leading to the induction of aTh1 and Th2 mixed cytokine response, TLR9 seems to be recognizing L. donovani DNA. The increasing expression of TLR2 in THP1 cell line may be the cause of high IFNG-γ production. On the other hand, our data support the hypothesis that the TLRs activation seems to be the major mechanism associated with active disease. In this context also the protective function of IFNG was demonstrated among the LST+ve controls. THP1 cells produced huge amount of IFNG more than whole blood samples suggesting the presence of other factors in whole blood samples that affect the IFNG production. Despite infection and stimulation with one parasite strain within these study groups, different immune responses were elicited. Macrophage infection reveals a varied immune response distinct of that of whole blood infection scenario.

CONFLICT OF INTEREST
I declare that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
41. Murray HW, Jungbluth A, Ritter E, Monte-libano C & Marino MW. Visceral Leishmaniasis in mice devoid of tumor necrosis factor