Plasma levels of tumor necrosis factor-alpha (TNF-α), TNF-α soluble receptor type 1 (sTNFR I) and IL-22 in human leishmaniasis

Nateghi Rostami, M.1*, Seyyedan Jasbi, E.2, Khamesipour, A.3 and Miramin Mohammadi, A.3
1Department of Microbiology and Immunology, Faculty of Medicine, Qom University of Medical Sciences, Qom, Iran
2Department of Microbiology, Islamic Azad University, Qom Branch, Qom, Iran
3Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran
*Corresponding author e-mail: Rostami52@yahoo.com
Received 10 December 2014; received in revised form 29 December 2014; accepted 31 December 2014

Abstract. The role of pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) in human leishmaniasis is not fully understood. We analyzed the alterations in the plasma levels of TNF-α, soluble TNF receptor type 1 (sTNFR I), IL-17 and IL-22 in the volunteers with leishmaniasis. Blood samples were collected from patients with active cutaneous leishmaniasis (CL), the same CL patients after standard antimonial therapy as healed CL, active visceral leishmaniasis (VL) and healed VL volunteers. Levels of the cytokines were titrated on plasma samples by sandwich ELISA method. The mean level of TNF-α was significantly higher in active CL patients than healthy controls (P<0.001) and significantly reduced after treatment in the same volunteers (P<0.001). The mean level of sTNFR I was significantly higher in active CL patients than healthy controls (P<0.05). The mean level of IL-22 in plasma of the AVL patients was significantly higher than that of healthy control group (P<0.05). There is a negative correlation between the levels of TNF-α and sTNFR I and healing of CL. Measurement of cytokines in plasma samples is more feasible than cell culture in evaluation of immune response in human leishmaniasis.

INTRODUCTION

It is estimated that in 98 countries approximately 350 million people are at risk of acquiring protozoan parasitic infection, leishmaniasis (Alvar et al., 2012). Two most common clinical forms of the disease, Cutaneous Leishmaniasis (CL) and Visceral Leishmaniasis (VL), are mainly seen in 14 of the 22 countries of EMRO (Eastern Mediterranean Regional Office) region including Iran (Postigo, 2010). A dichotomy in immune response against Leishmania infection is well-defined in inbred mouse strains in which the outcome of L. major infection is dependent upon the effector functions of CD4+ Th1 and Th2 subsets (Heinzel et al., 1989; Mosmann & Coffman, 1989). The mechanisms of protection in human leishmaniasis have not been fully understood, while there is considerable data available on the contribution of Th1/Th2 cytokines to CL outcome the role of other productions of immune system, like proinflammatory cytokines has less been noticed (Nateghi Rostami et al., 2010; Khamesipour et al., 2012; Keshavarz Valian et al., 2013). TNF-α is produced mainly by macrophages and is involved in the innate immune response particularly in the defense against intracellular pathogens (Korner et al., 2010). Existing data on the role of TNF-α in human leishmaniasis development are controversial, but most of the reports implicate that unregulated production of TNF-α may contribute to the clinical outcome
of leishmaniasis early at infection and to the clinical severity of disease (Da-Cruz et al., 1996; Blackwell, 1999; D’oliveira et al., 2002; Antonelli et al., 2005). It was reported that percentage of Leishmania specific TNF-α producing T cells and the amount of TNF-α produced have a positive correlation with the lesion size (Antonelli et al., 2005; Oliveira et al., 2011), elevated levels of TNF-α production was shown in the lesions of CL patients nonresponsive to antimonial treatment and in skin lesions of ML patients (Bacellar et al., 2002; Antonelli et al., 2005). TNF-α exerts its various bioactivities through its type 1 and type 2 receptors (TNFR I and TNFR II) and soluble TNFRs modulate proinflammatory activities of TNF-α with competitive binding to this cytokine (Derouich-Guergour et al., 2001). The Th17 cells are a subset of CD4+ T cells which are characterized by their potential to produce cytokines in the IL-17 family including IL-17, IL-22, and IL-23 (Korn et al., 2009). Th17 cytokines exert antimicrobial immunity at epithelial and mucosal surfaces (Scriba et al., 2008; Kurebayashi et al., 2013), however, their functions are not elucidated in human leishmaniasis. In this study we have analyzed TNF-α, sTNFR I, IL-17 and IL-22 levels in leishmaniasis patients before and after healing.

MATERIALS AND METHODS

Volunteers and sampling
The study was approved by Ethical Committee of Qom University of Medical Sciences. Active CL (ACL) and healed CL (HCL) volunteers were recruited from Ghomrood and Ghanavat regions, Qom province, central Iran. Volunteers with active skin lesion caused by L. major whose diseases were confirmed using parasitological methods (microscopy and culture) and PCR analysis were included as ACL. After complete cure of the lesion(s) following standard course of antimonial (Glucantime) treatment, the same patients were included as HCL volunteers. Active VL (AVL) due to L. infantum and healed VL (HVL) were recruited from Meshkin-Shahr district, Ardabil province, northwest Iran. As control, healthy volunteers from non-endemic area with no response to leishmanin skin test (LST) were included. Twenty ml of heparinized blood samples were collected from each volunteer in a 15 ml Falcon tube and plasma was separated by centrifugation of tubes at 3000 xg for 10 min.

Parasite identification
Identification of the causative agent of the disease was performed using internal transcribed spacer 1 (ITS1) ribosomal DNA PCR. The target gene was amplified using primers Leish-F (5’-CCTCTCTTTTTTTCN
CTGTGC-3’) and Leish-R (5’-CAACACGCC GCCTCCTCCTCT-3’), yielding a 600-bp fragment for L. major and an 800-bp fragment for L. tropica. Amplification was carried out in a 25 µl reaction volume with conditions as previously described (Nateghi Rostami et al., 2013).

Plasma cytokine assay
The level of TNF-α, sTNFR I, IL-17, and IL-22 were measured in plasma using sandwich ELISA method according to the manufacturer’s instruction (R & D system, Minneapolis, MN, USA) as previously described (Nateghi Rostami et al., 2008). Briefly, the plates were coated with 100 µl of anti-TNF-α/sTNFR I/IL-17/IL-22 mAb in PBS, pH 7.2, and incubated at 4°C over night. After blocking the wells using buffer containing PBS plus 0.05% (v/v) Tween 20 and 0.1% (w/v) bovine serum albumin (BSA), 100 µl of culture supernatants were added to each well for 2 hrs at room temperature (RT). Next, 100 µl of 3,3’,5,5’-tetramethyl benzidine (TMB) substrate was added and plates were incubated 1 hr at RT and then the reaction was stopped with 1M H2SO4 solution. The plates were washed after each step of incubation using PBS+0.05% (v/v) Tween 20. The plates were read at 450 nm using a reader (BioTek, Winooski, VT, USA). The mean optical densities (ODs) of triplicate samples were compared with the
standard curves prepared using recombinant TNF-α, TNFR I, IL-17, and IL-22. The cytokine levels represent the differences between the ODs of test and background wells. The detection limit of the assays was 15 pg/ml for IL-17 and 5.8 pg/ml for IL-22, 1.2 pg/ml for sTNFR I and 5.5 pg/ml for TNF-α.

Statistical analysis
Non-parametric tests of Kruskal-Wallis and Dunn’s post-test were used to compare median of cytokine levels between different groups of the volunteers. Wilcoxon signed rank test for paired comparisons was used for comparison of the level of cytokine production before vs. after treatment. Statistical analysis of the data was done using SPSS version 18 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism version 5.01 (GraphPad Software Inc., La Jolla, CA, USA) softwares. P value of ≤0.05 was considered as significant.

RESULTS
The causative agent of each CL patients who were included in this study was identified as *L. major*. Demographic data is shown in Table 1. In ACL group, the number of lesion(s) ranged from 1 to 6, with the median of 2.5 and the mean±SD of the lesion size was 13.04±8.00 mm. In CL volunteers, most of the lesions were developed on hands (n=13; 54.2%) followed by feet (n=7; 29.2%), head & neck (n=3; 12.5%) and trunk (n=1; 4.1%). The age of all VL volunteers was less than 5 years; the mean age of CL volunteers was around 35 years.

The mean levels of cytokines in plasma samples of each group were shown in Figure 1. The mean level of sTNFR I in plasma of ACL patients was significantly higher than that of healthy controls (P<0.05). The mean level of TNF-α in plasma of ACL patients was significantly higher than that of healthy controls (P<0.001) and significantly reduced after treatment in HCL volunteers (P<0.001). In analysis between levels of TNF-α and sTNFR I in the same groups, the levels of sTNFR I were significantly higher than the levels of TNF-α in each group of the volunteers (P=0.001 for all groups). The mean level of IL-22 in plasma of AVL patients was significantly higher than that of healthy control group (P<0.05). No significant difference was seen in IL-17 levels between CL, VL and healthy control volunteers.

<table>
<thead>
<tr>
<th>Volunteers group</th>
<th>Gender n (%)</th>
<th>Age (year): mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M; F</td>
<td></td>
</tr>
<tr>
<td>ACL n=10</td>
<td>7 (25.9); 3 (20.0)</td>
<td>35.80±12.00</td>
</tr>
<tr>
<td>HCL n=10</td>
<td>7 (25.9); 3 (20.0)</td>
<td>35.80±12.00</td>
</tr>
<tr>
<td>AVL n=3</td>
<td>1 (3.7); 2 (13.3)</td>
<td>4.19±3.15</td>
</tr>
<tr>
<td>HVL n=6</td>
<td>4 (14.8); 2 (13.3)</td>
<td>3.52±3.01</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>8 (29.7); 5 (33.4)</td>
<td>35.50±11.00</td>
</tr>
<tr>
<td>TOTAL n=42</td>
<td>27 (100.0); 15 (100.0)</td>
<td>22.96±8.23</td>
</tr>
</tbody>
</table>

ACL=active cutaneous leishmaniasis; HCL=healed cutaneous leishmaniasis; AVL=active visceral leishmaniasis; HVL=healed visceral leishmaniasis.

Table 1. General and clinical characteristics of the volunteers included in the study.
Figure 1. Plasma was isolated from heparinized blood samples using centrifugation. The level of TNF-α, sTNFR I, IL-17 and IL-22 were titrated by sandwich ELISA method. The mean levels of cytokines were shown for both leishmaniasis and healthy control groups. *Significant difference $P \leq 0.05$. **Significant difference $P \leq 0.001$.

ACL=active cutaneous leishmaniasis; HCL=healed cutaneous leishmaniasis; AVL=active visceral leishmaniasis; HVL=healed visceral leishmaniasis

**DISCUSSION**

We measured cytokine levels in plasma samples of the same CL patients before and after treatment. This is important since it allows comparison of the cytokines alterations in a given patient during healing process from active phase to healed lesions. However, in most of the published data on human leishmaniasis, immune responses of subjects with active and with history of CL have been measured separately, and due to the broad variations of immune responses in every individual, the interpretation may not be always with precision.

Recently we have shown the modifications of TNF-α production in PBMCs culture supernatants in the same volunteers with leishmaniasis (Under review). PBMCs culture needs more blood samples, live cells, and antigen preparation for stimulation of the cells in culture. Also, the procedure is more complicated and time consuming. Measurement of cytokine secretions in plasma is rather a simple method and if the results are comparable to culture, it is convenient for patients. TNF-α levels were significantly reduced in all CL patients after standard treatment with antimony and clinical cure of the lesions. Therefore, clinical response to treatment and lesion healing in CL is conversely correlated to the amount of TNF-α productions in plasma. It was shown that in human cutaneous and mucosal leishmaniasis after treatment the level of TNF-α in PBMCs stimulated with Leishmania Ag was reduced (Oliveira et al., 2011; Brelaz-De-Castro et al., 2012), while high levels of TNF-α were detected in plasma and stimulated PBMCs culture during active disease (Castellano et al., 2009; Goto & Prianti, 2009). Studies on human CL before and after Sb(v) treatment suggested that antimonial treatment causes a significant increase in monocyte and neutrophil functions such as phagocytosis capability, TNF-α and TNF-mediated NO productions that all involved in the defense against Leishmania infection (Muniz-Junqueira & De Paula-Coelho, 2008; De Saldanha et al., 2012). There are evidences showing that TNF-α regulation is linked to leishmaniasis severity...
such as the size of the CL ulcer or severity of ML manifestations (Da-Cruz et al., 1996; Antonelli et al., 2005; Oliveira et al., 2011).

In the present study level of soluble TNFR I was higher in all ACL patients than healthy controls. One mechanism for regulating the function of TNF-α is interaction with the receptors, so sTNFR I might be utilized in binding with the TNF-α as an antagonist to modulate functions of the cytokine in CL after lesion healing (Derouich-Guergour et al., 2001). Significant down regulation of sTNFR I mRNA expression in PKDL and VL patients was demonstrated (Ansari et al., 2008). We found IL-22 is significantly higher in active VL comparing healthy controls. These results suggest that the level of IL-22 production conversely related to VL cure. However, it was claimed that IL-17 and IL-22 may have a synergistic role with Th1 cytokines in protection against human VL due to L. donovani (Pitta et al., 2009); but the situation is not clear in human VL due to L. infantum. Results of this study suggest the importance of cytokine TNF-α in concert with other Th1/Th2 cytokines in clinical outcome of human leishmaniasis. There is an association between the alterations in the level of TNF-α and sTNFR I and healing of CL. Since measurement of cytokines in plasma samples is more feasible than PBMC culture it might be used for analysis of cytokine levels in leishmaniasis subjects.

Acknowledgments. This work was funded by Cellular and Molecular Research Center, Qom University of Medical Sciences and Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Iran.

Disclosure
The authors declare that they have no conflict of interest.

REFERENCES


