International Immunopharmacology 69 (2019) 321–327

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Host's immune response in unresponsive and responsive patients with anthroponotic cutaneous leishmaniasis treated by meglumine antimoniate: A case-control study of Th1 and Th2 pathways

Mehdi Bamorovat[a], Iraj Sharifi[b,*], Mohammad Reza Aflatoonian[a], Balal Sadeghi[c], Alireza Shafiian[d], Razieh Tavakoli Oliaee[b], Alireza Keyhani[b], Abbas Aghaei Afshar[b], Ahmad Khosravi[b], Mahshid Mostafavi[b], Maryam Hakimi Parizi[b], Mehrdad Khatami[e], Nasir Arefinia[f]

[a] Research Center of Tropical and Infectious Diseases, Kerman University of Medical Sciences, Kerman, Iran
[b] Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran
[c] Food Hygiene and Public Health Dept., Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran
[d] Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran
[e] School of Medicine, Bam University of Medical Sciences, Bam, Iran
[f] Dadbin Health Clinic, Kerman University of Medical Sciences, Kerman, Iran

ARTICLE INFO

Keywords:
Immune response
Unresponsive patients
Anthroponotic cutaneous leishmaniasis
Case-control study
Th1 and Th2

ABSTRACT

The objective of the present study was to compare the host's immune responses between unresponsive and responsive patients with anthroponotic cutaneous leishmaniasis (ACL) treated by meglumine antimoniate.

A case-control study was carried out in an endemic focus in Iran. Blood samples were taken from patients and peripheral blood mononuclear cells (PBMCs) were isolated. Two wells were considered for each isolate of unresponsive and responsive patients; one was exposed to *L. tropica* (*Lt*-stimulated cells) and the other remained non-exposed (non-stimulated cells). After 24 h of incubation, whole RNA was extracted from each sample. Real-time quantitative PCR was carried out to confirm the differences in expression levels of IL-12 P40, IFN-γ, IL-1β, IL-4 and IL-10 among isolates. Data were analyzed and $P < 0.05$ was considered to be statistically significant.

In our study, *Lt*-stimulated cells and non-stimulated cells in unresponsive groups demonstrated significantly lower expression levels of IL-1β, IL-12 P40 and IFN-γ genes and higher expression levels of IL-4 and IL-10 genes, compared to *Lt*-stimulated cells and non-stimulated cells in responsive groups. There was a negative correlation between IL-12 P40 with IL-10 and IL-1β with IL-10 in ACL *Lt*-stimulated cells in unresponsive group, while a positive correlation between IL-12 P40 with IL-1β and IL-12 P40 with IFN-γ in ACL *Lt*-stimulated cells in responsive group.

Probably, different immune responses caused by various factors play a major role in the pathogenesis and development of unresponsiveness in ACL patients. The profile and timing of cytokine production correlated well with the treatment outcome of *Leishmania* infection.

1. Introduction

Leishmaniasis, a clinically heterogeneous disease, caused by the protozoan parasites of the genus *Leishmania*, is one of the most important infectious diseases in the world [1]. This disease represents itself in several diverse clinical forms in endemic areas. Cutaneous leishmaniasis (CL) is the most widespread form and remains a global and major public health problem. CL causes a range of diseases comprising of self-healing infections to chronic and disfiguring disease with serious social and political consequences [2–4]. Both forms of rural (zoonotic CL; ZCL) and urban CL (anthroponotic CL; ACL) are highly prevalent in Iran and 34,351,077 (46%) people out of 75,156,975 are at high risk of contracting CL [5].

There is currently no efficacious and safe vaccine against various species causing leishmaniasis [6]. Among different drugs, pentavalent antimonial compounds such as Glucantime® and Pentostam® have widely been used as the standard recommended treatment for CL [7,8]. Glucantime (meglumine antimoniate) is the primary treatment for CL in

* Corresponding author at: Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman 76169-14115, Iran.
E-mail address: sharifi@kmu.ac.ir (I. Sharifi).

https://doi.org/10.1016/j.intimp.2019.02.008
Received 15 December 2018; Received in revised form 12 January 2019; Accepted 4 February 2019
Available online 14 February 2019
1567-5769/

Fig. 1. Representative pictures of active lesions taken from patients with anthroponotic cutaneous leishmaniasis caused by *Leishmania tropica* in Kerman, southeastern Iran. Unresponsive (A, B, C and D) and responsive patients (E, F, G and H).

Iran; however, new pentavalent (SbV)-resistant *Leishmania tropica* isolates have recently emerged from unresponsive patients. Various factors including geographic and social variances, type of disease, parasite species and host immune system influence leishmaniasis treatment outcome [9–11].

The insufficient immunity could be the main factor responsible for unresponsive forms of leishmaniasis [10,12,13]. Host genetic factors are likely to play a key role in the disease [14]. It is worth mentioning that drug resistance in ACL, which is frequently caused by human-to-human transmission, is more prevalent than that of ZCL. The control of leishmaniasis is eminent by cellular immune responses leading to activation of macrophages and other immune cells and parasite killing. Although humoral immunity is present during the infection, antibodies show no role in protection and are associated with the unresponsive forms [15,16].

Research in animal models has established the critical role of CD4 + T helper (Th) cells demonstrating that cure is associated with strong *interferon-gamma* (IFN-γ), interleukin-2 (IL-2) and IL-12 responses in the absence of classical Th2 cytokines such as IL-10 [17]. The immunological outcome of *Leishmania* infections in human is functionally specified by two distinct T-helper (Th) cell populations, namely Th1 (IL-1β, IFN-γ and IL-12) and Th2 (IL-4, IL-10 and IL-13). Generally, the disease susceptibility in uncontrolled or non-healed infections has been related to Th2 proliferation and production of IL-4, IL-5 and IL-10. In contrast, healing responses and resistance to infection have been associated with expansion of IFN-γ-producing Th1 cells [18–21]. T-cell responses in individuals with mild infection involve a mixture of Th1 and Th2, whereas patients with exacerbating disease show an absence of Th1 response to specific *Leishmania* antigens. Failure to cure is associated with low levels of IFN-γ produced by *Leishmania*-specific CD4 + T and high levels of IL-4 [22]. Increased levels of IL-10 expression were found in *Leishmania major* lesions and were associated with progressive disease [23,24]. Furthermore, IL-10 has been shown to block the induction of Th1 cells and consequently diminishes a cytotoxic response by down-regulation of IL-12 and IFN-γ production [24].

The present study was aimed to compare the host's immune responses between unresponsive and responsive patients with ACL caused by *L. tropica* in an endemic focus in southeastern Iran. These analyses can help improve our understanding of host's immune response which could tightly be linked to their different treatment outcome.

2. Materials and methods

2.1. Study design

A case-control study was conducted from December 2015 to November 2017 in unresponsive (case group) and responsive (control group) patients with ACL in Kerman and Bam Counties, in southeast of Iran. Clinical isolates were randomly selected from patients with ACL referred to the health centers in Kerman and Bam Counties. In Kerman province, CL is a common disease and dates back decades [25].

2.2. Ethical statement

Research ethical approval was obtained from the Ethical Committee of Kerman University of Medical Sciences (Ethics no. IR.KMU.REC.1394.115 and contract no. 94.621). Initially, face-to-face meetings and interviews were held with patients. The main purposes and potential benefits of the study and work processes were clearly described in the meetings. All subjects participated in the survey voluntarily and willingly provided written informed consent before participation. Moreover, parents provided informed consent on behalf of the children participated in the study. All patients' data were considered to be confidential.

2.3. Unresponsive and responsive patients

All the unresponsive and responsive patients participated in the present research were selected from Kerman and Bam Counties, southeastern Iran, where *L. tropica* has been endemic. Unresponsive patient is defined as one who is not healed and remains with an active lesion, despite receiving > 3 courses of intra-lesional meglumine antimoniate (20 mg/kg/weekly for 12 weeks) along with cryotherapy or systemic meglumine antimoniate alone (20 mg/kg/daily for 3 weeks). Responsive patient is one whose lesion heals by one treatment course with intramuscular administration of meglumine antimoniate or intralesional meglumine antimoniate together with cryotherapy after a follow-up period of 10 months (Fig. 1). Totally, 20 patients with active

Table 1
Specific primers and reference gene sequences.

Primer	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	PCR Product (bp)
IL-12 P40	CTGGAGCACTCCCCATTCCTA	GCAGACATTCCCGCCTTTG	160
IL-10	CTTACTGACTGGCATGAGGATCA	GCAGCTCTAGGAGCATGTGC	101
IFN-γ	TCGGTAACTGACTTGAATGTCCA	TCGCTTCCCTGTTTTAGCTGC	93
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA	132
IL-4	CGGCAACTTTGTCCACGGA	TCTGTTACGGTCAACTCGGTG	111
HPRT	CCTGGCGTCGTGATTAGTGAT	AGACGTTCAGTCCTGTCCATAA	131

lesions were participated in this study and assigned to one of the two treatment arms (10 patients each).

2.4. Parasite species

Samples were taken from 20 patients with active lesion, including 10 unresponsive and 10 responsive to meglumine antimoniate treatment that had a positive direct microscopic examination and PCR confirmation. Based on recent studies performed in Kerman and Bam Counties, all explored CL cases (responsive and unresponsive) were infected with *L. tropica* species [10,11].

2.5. Isolation of PBMCs

Fifteen milliliters blood samples were taken from unresponsive (case) and responsive (control) patients. Samples were collected from under treatment patients of both sexes. From whole blood using a density gradient method (Histopaque-1077, Biosera, France), peripheral blood mononuclear cells (PBMCs) were isolated. Concisely, the ring rich in lymphocytes and monocytes was removed with Ficoll-Hypaque gradient at 400xgand 18 °C for 20 min, washed twice with RPMI-1640 and afterwards re-suspended in complete medium of RPMI-1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 25 mM HEPES, 100 mg/ml penicillin-streptomycin (Biosera, France) and mixed well by gradual pipetting. Assessment of cells' viability was performed using 0.4% Trypan Blue staining. PBMCs in complete medium (1×10^6 cells/ml) from unresponsive and responsive patients with ACL were seeded in 24-well culture plates and motivated with *L. tropica* strain MHOM/IR/10/175 (S175) promastigotes at a ratio of 10:1 cells at 37 °C for 24 h in 5% CO_2. Moreover, PBMCs were incubated with 100 ng/ml lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO) as the positive control under similar condition as previously mentioned (PBMCs of responsive and unresponsive patients were stimulated with LPS). Subsequently, the supernatants with extracellular parasites were removed and the cells were placed in new complete RPMI-1640 media and incubated for 24 h. Two wells were considered for each isolate of unresponsive and responsive; one was exposed with *L. tropica* (*Lt*-stimulated cells) and the other remained non-exposed (non-stimulated cells). After 24 h of incubation, each well was scraped, centrifuged and cell lysates were aliquoted and stored at −70 °C for Th1 and Th2 cytokine genes expression levels.

2.6. RNA isolation and cDNA synthesis

Whole RNA was extracted from PBMCs infected with unresponsive or responsive isolates using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the guidelines recommended by the manufacturer. Nanodrop (ND-2000, Thermo Scientific Fisher, US) and 2% agarose gel electrophoresis were applied for analyzing the quality and quantity of the extracted RNA, respectively. Complementary DNA (cDNA) synthesis was performed using the Prime Script RT reagent Kit (Takara, Tokyo, Japan) according to the instructions of its protocol. Concisely, cDNA was synthesized by 100 ng of extracted RNA, 50 pmol/μl random hexamer primer, 25 pmol/μl oligo-dT, RT Enzyme Mix I (each in 0.5 μl) and 2 μl of 5X Prime Script Buffer and then it was incubated at 37 °C for 15 min followed by incubation at 85 °C for 5 s to inactivate reverse transcriptase. The obtained cDNA was diluted in DNase/RNase-free distilled water and was stored at −70 °C for later examination.

2.7. Quantitative real-time PCR (qPCR) analysis

Quantitative real-time PCR was carried out to confirm the differences in expression level of IL-12 P40, IFN-γ, IL-1β, IL-4 and IL-10 among the PBMCs unresponsive and responsive groups. Hypoxanthine phosphoribosyltransferase (HPRT) primer was used as the housekeeping gene for normalization purpose. The sequences of each gene amplified in this study are given in Table 1. Quantitative real-time PCR was performed by SYBR® Premix Ex Taq™ II (Takara, Japan) on real-time PCR cycler, Rotor-Gene Q (Corbett, Qiagen). Each reaction had a total volume of 15 μl, containing 2 μl of cDNA target, 7 μl SYBR® Premix, and primers at a final concentration of 50 nM. PCR amplification was run in duplicate by the thermal profile of initial denaturation at 95 °C for 30 s followed by 40 cycles, each consisting of denaturation at 94 °C for 10 s, annealing at 60 °C for 18 s and extension at 72 °C for 20 s. The specificity of reaction was determined by a melt curve analysis. A no-cDNA sample was included as the negative control in each run for each gene.

2.8. Gene expression analysis

To calculate the relative value of the expression level, $2^{-\Delta\Delta Ct}$ method was used as a relative quantification approach for qPCR data analysis [26]. The relative expression level of cytokine genes was determined based on the cycle threshold (Ct) value of the target gene with that of the reference one and HPRT to normalize the results.

2.9. Statistical analysis

Data were analyzed using independent sample *t*-test and one-way ANOVA. Also, correlation analysis was performed by Pearson test. *P* values for significant differences in the expression levels of unresponsive and responsive groups of different genes were calculated with GraphPad Prism 7.01 (GraphPad Software, Inc., San Diego, CA, USA). A $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Studied groups

In general, a total of 20 patients with active ACL lesions, including 10 unresponsive and 10 responsive patients were recruited and studied in six groups (*Lt*-stimulated cells in responsive (LR), non-stimulated cells in responsive (NR), LPS-stimulated cells in responsive (LPS-R), *Lt*-stimulated cells in unresponsive (LU), non-stimulated cells in unresponsive (NU) and LPS-stimulated cells in unresponsive (LPS-U) groups). Fig. 2 displays the studied groups. The number of male and female patients in both unresponsive and responsive cases was equal (5 males and 5 females).

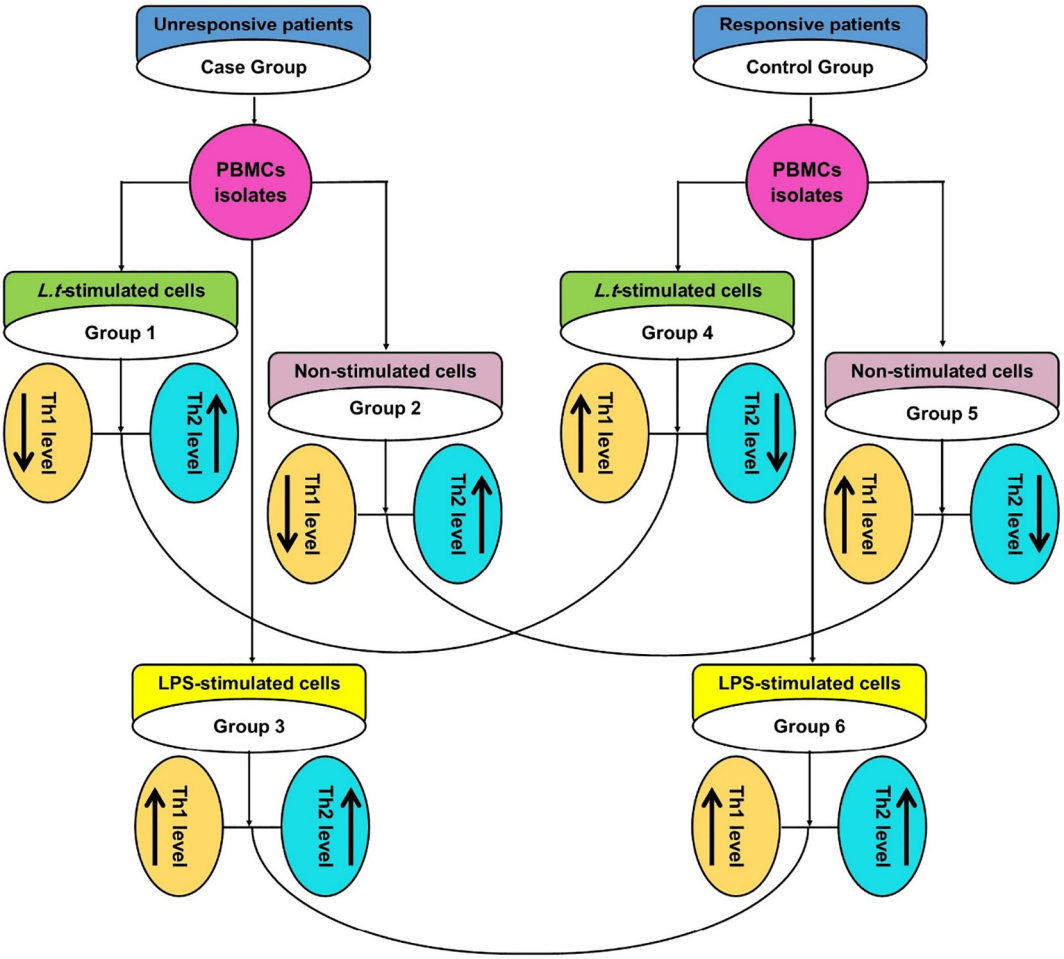

Fig. 2. Schematic profile and comparison of results in unresponsive and responsive groups.

3.2. Gene expression level of Th1 cytokines

3.2.1. Inter group comparisons

The results showed that gene expression level of IL-1β, IL-12 P40 and IFN-γ, as the measure of Th-1, significantly increased in both *Lt*-stimulated cells and non-stimulated cells responsive groups, compared to the corresponding groups in unresponsive arm ($P < 0.05$) (Fig. 3a, b and c). In responsive and unresponsive groups, LPS-stimulated cells, represented significantly higher expression of IL-1β, IL-12 P40 and IFN –γ ($P < 0.05$) (Fig. 3a, b and c).

3.2.2. Intra group comparisons

Significant differences were observed in *Lt*-stimulated cells in unresponsive group, compared to the non-stimulated cells in unresponsive group and *Lt*-stimulated cells in responsive group, compared to the non-stimulated cells in responsive group ($P < 0.05$) (Fig. 3a, b and c).

3.3. Gene expression level of Th2 cytokines

3.3.1. Inter group comparisons

Our results demonstrated that the gene expression level of IL-4 and IL-10, as the measure of Th-2, significantly increased in *Lt*-stimulated cells and non-stimulated cells in unresponsive groups, compared to the corresponding groups in responsive arm ($P < 0.05$) (Fig. 3d and f). LPS-stimulated cells in responsive and unresponsive groups, represented significantly higher expression of IL-4 and IL-10 ($P < 0.05$) (Fig. 3d and f).

3.3.2. Intra group comparisons

There were significant differences in *Lt*-stimulated cells in unresponsive group, compared to the non-stimulated cells in unresponsive group and *Lt*-stimulated cells in responsive group, compared to the non-stimulated cells in responsive group ($P < 0.05$) (Fig. 3d and f).

3.4. Correlation analysis in ACL Lt-stimulated cells in unresponsive and responsive groups

As shown in Fig. 3A and D, the increased expression level of IL-12 P40 and IL-1β are associated to decreased level of IL-10 with a significant negative correlation in ACL *Lt*-stimulated cells in unresponsive group ($P = 0.002$, $r = 0.83$ and $P = 0.008$, $r = 0.7$, respectively). Also in Fig. 3B and C, the increased expression level of IL-12 P40 is associated to increased level of IFN-γ and the increased expression level of IL-1β is associated to increased level of IL-12 P40 with a significant positive correlation in ACL *Lt*-stimulated cells in responsive group ($P = 0.004$, $r = 0.9$ and $P = 0.004$, r = 0.8, respectively).

4. Discussion

Cutaneous leishmaniasis is one of the most serious health risks and major health problems among neglected diseases in Iran, particularly in Kerman province in southeastern Iran [27]. Treatment failures for CL with pentavalent antimonials, notably meglumine antimonite, are increasingly reported in many endemic areas [10,28,29].

Most of the information on the spectrum of clinical presentations, immunologic mechanisms upon infection and protection against the

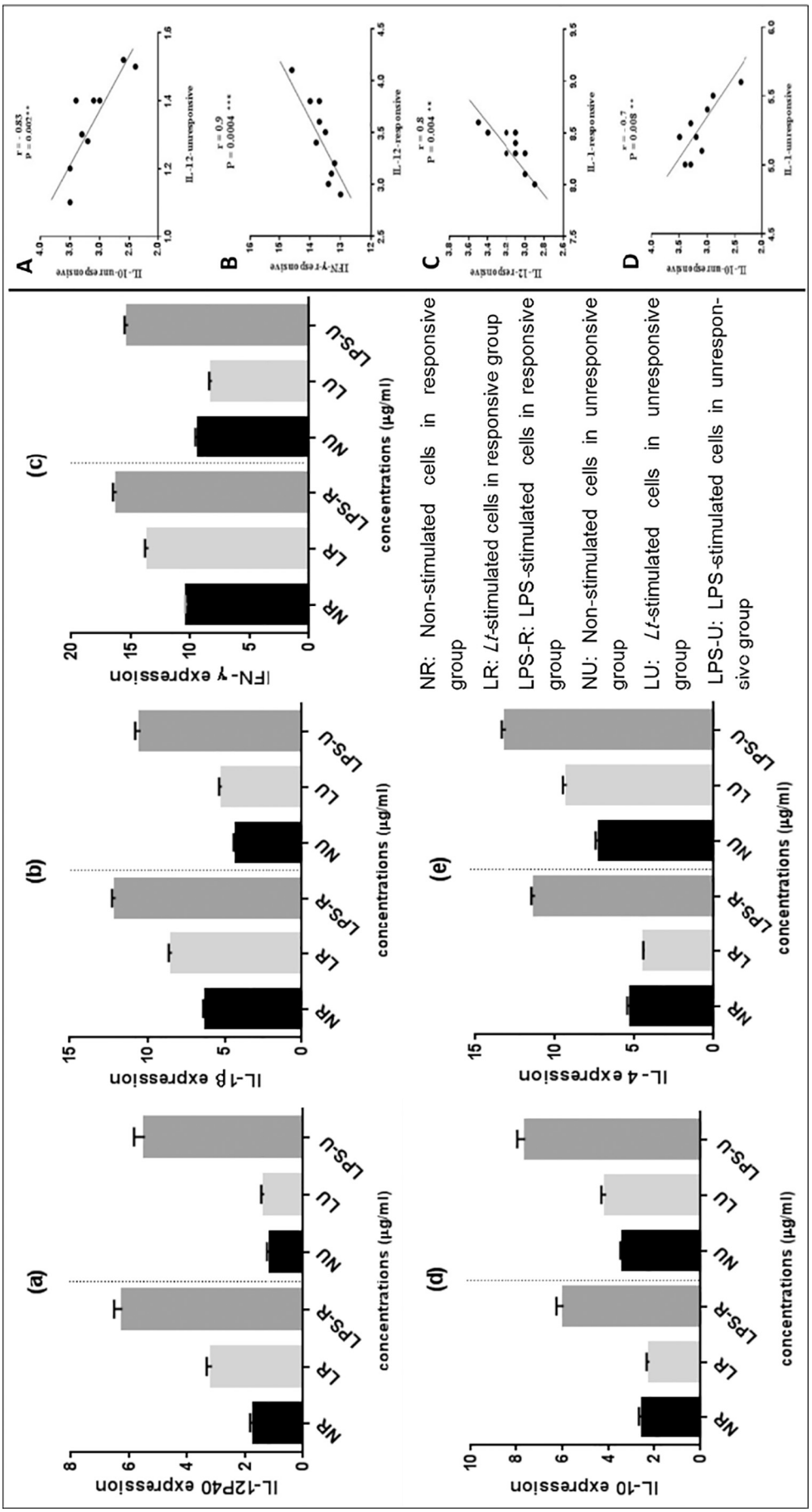

Fig. 3. Gene expression levels of IL-1β, IL-12 P40 and IFN-γ, as the measure of Th-1 ($P < 0.05$) (a, b and c) and IL-4 and IL-10, as the measure of Th-2, in various groups of the study ($P < 0.05$) (d and e). Also, a negative correlation between IL-12 P40 with IL-10 and IL-1β with IL-10 in ACL Lt-stimulated cells unresponsive group (A and D) and a positive correlation between IL-12 P40 with IL-1β and IL-12 P40 with IFN-γ in ACL Lt-stimulated cells responsive group (B and C).

Leishmania parasites are derived from studies performed in mice; however, some crucial findings from murine leishmaniasis have recently been confirmed in humans [30].

Although the majority of differential regulation of Th1 and Th2 type responses was initially observed in humans with clinical manifestations of the disease [3]. The two principal immunological mechanisms responsible for the control of *Leishmania* parasites are the generation of reactive oxygen species (ROS), produced by the respiratory stress that occurs during phagocytosis, and nitric oxide (NO) generated by inducible NO synthase (iNOS) following activation of cells by IFN-γ [31].

Further comprehensive and thorough studies on the immune modulation in subclinical individuals are required to understand the nature of infection with *Leishmania* parasite and to develop effective treatment and control strategies. Genetic diversity among different human population exposed to the parasite makes it difficult to understand the parameters of resistance and control mechanism in humans [30].

In our study, *Lt*-stimulated cells and non-stimulated cells in unresponsive groups exhibited significantly lower expression levels of IL-1β, IL-12 P40 and IFN-γ genes, compared to the *Lt*-stimulated cells and non-stimulated cells in responsive groups. As expected, the expression levels of IL-4 and IL-10 genes were higher in the *Lt*-stimulated cells and non-stimulated cells in unresponsive groups, compared to responsive ones. Also, there was a negative correlation between IL-12 P40 with IL-10 and IL-1β with IL-10 in *Lt*-stimulated cells in unresponsive group with ACL; in contrast, there was a positive correlation between IL-12 P40 with IL-1β and IL-12 P40 with IFN-γ in ACL *Lt*-stimulated cells in responsive group.

The typical Th2 cytokine in leishmaniasis is IL-4. IL-4 drives Th2 response and promotes susceptibility through inhibition of macrophage activation and abrogation of IL-12 expression [32]. IL-10 is a major immunosuppressive cytokine in leishmaniasis and is necessary for parasite persistence during the exacerbation of the disease. It is a potent suppressor of macrophage activation and is produced by a plethora of immune cells [33,34]. Also, It has been revealed that IL-10 differentially affects the quality, magnitude and defensive efficacy of Th1 cells [35].

Cytokines with the ability to influence Th1 development, such as IL-12 and IFN-γ, play a protective role in leishmaniasis. IL-12 promotes resistance through macrophage activation and NO production and is essential for the priming of naïve T cells toward the Th1 pathway [15].

Moreover, reports show that IL-1β is a key interleukin for host resistance to infection, especially intracellular parasites which contributes to induce NOS2- mediated production of NO as a main host resistance mechanism against leishmaniasis [36,37].

The clinical outcome of the disease seems to be mainly determined by *Leishmania* species and the host's immunological response. It is necessary to mention that the host's immunological response itself depends on several factors such as clinical, demographic and socio-economic variables [10,17].

In a recent study performed in Iran, patients with chronic diseases such as diabetes, opium addiction, cardiovascular problems, and tuberculosis indicated no response to basic treatment, compared to those without chronic diseases. Development of unresponsive forms in the patients with chronic complications is probably due to defects in cellular innate immunity which results from various factors [10]. Moreover, it has been mentioned that unresponsiveness in patients with ACL was significantly associated with increased age and various factors including chronic diseases which contribute to the pathogenesis of unresponsive forms in patients of older age groups [10].

Although a multifactorial etiology is proposed for unresponsive cases, the role of immune response appears to be a major factor in determining the fate of recovery in such patients. In a study performed in Iran, there was a positive association between the rate of unresponsiveness in ACL and poor interior housing conditions [10]. Generally, most individuals with ACL live in low-income families belonging to lower social classes. Therefore, they have miserable lives with variable stresses that hardly meet their basic needs. It seems that life problems and resultant stressful events, can probably influence the immune response and eventually increase the risk of developing unresponsive forms in these patients [10]. The aforementioned factors presumably play an important role in immune response and treatment outcome of patients with ACL. According to the WHO report, poverty-stricken individuals are commonly affected by this disease, which is due to their malnutrition, nomadic life, weak immune system and poor living standards [4]. Following infection with *Leishmania*, multiple pathways influence the pathogenesis and the type of immune response which play a crucial role in determining disease outcome to become self-healing or exacerbating chronic disease [3].

Finally, it should be noted that a whole range of cytokines are involved in the immune response to *Leishmania*; therefore, they are not limited to IL-4, IL-10, IL-12, IL-1β and IFN-γ. The profile and timing of cytokine production correlate with the clinical outcome of *Leishmania* infection. Additional studies need to be carried out in this regard in the future investigations.

5. Conclusion

The results of the present study clearly showed that the expression levels of IL-1β, IL-12 P40, and IFN-γ genes in *Lt*-stimulated cells and non-stimulated cells in unresponsive groups were lower than *Lt*-stimulated cells and non-stimulated cells in responsive groups. In contrast, the expression levels of IL-10 and IL-4 genes were higher in unresponsive ones, compared to responsive groups. Probably, different immune responses caused by various factors play a major role in the pathogenesis and development of unresponsiveness in patients with ACL.

Financial support

This work was supported by Vice-Chancellor of Research, Kerman University of Medical Sciences Kerman, Iran (grant number 94.161) for financial support. The funder had no role in the study design, data collection, data analysis and manuscript preparation. We would like to thank the patients for their cooperation and the health personnel in Dadbin Health Clinic for their help in conducting this study.

Author disclosure statement

The authors declare that this article content has no conflict of interest.

References

[1] J. Kaur, T. Kaur, S. Kaur, Studies on the protective efficacy and immunogenicity of Hsp70 and Hsp83 based vaccine formulations in Leishmania donovani infected BALB/c mice, Acta Trop. 119 (2011) 50–56.
[2] P. Desjeux, The increase in risk factors for leishmaniasis worldwide., Trans. R. Soc. Trop. Med. Hyg.. 95 (2004) 239–43.
[3] P. Scott, F.O. Novais, Cutaneous leishmaniasis: immune responses in protection and pathogenesis, Nat. Rev. Immunol. (2016).
[4] F. Bailey, K. Mondragon-Shem, P. Hotez, J.A. Ruiz-Postigo, W. Al-Salem, A. Acosta-Serrano, D.H. Molyneux, A new perspective on cutaneous leishmaniasis-Implication for global prevalence and burden of diseases estimates, PLoS Negl. Trop. Dis. 11 (2017) e0005739.
[5] T. Chaiworapongsa, R. Romero, A.E. Whitten, S.J. Korzeniewski, P. Chaemsaithong, E. Hernandez-Andrade, L. Yeo, S.S. Hassan, The use of angiogenic biomarkers in maternal blood to identify which SGA fetuses will require a preterm delivery and mothers who will develop pre-eclampsia, J. Matern. Fetal Neonatal Med. 29 (2016) 1214–1228.
[6] R.O. Dillman, Cancer immunotherapy, Cancer Biother. Radiopharm.. 26 (2011) 1–64.
[7] A.S. Bourinbaiar, V. Jirathitikal, Low-cost anti-HIV compounds: potential application for AIDS therapy in developing countries, Curr. Pharm. Des. 9 (2003) 1419–1431.
[8] WHO, Control of the leishmaniases: Report of a meeting of the WHO expert committee on the control of leishmaniases, Control of the Leishmaniases: Report of a Meeting of the WHO Expert Committee on the Control of Leishmaniases, World

Health Organization, Geneva, 2010, pp. 1–187.
[9] Y. Dowlati, Cutaneous leishmaniasis: clinical aspect, Clin. Dermatol.. 14 (1996) 425–431.
[10] M. Bamorovat, I. Sharifi, M.R. Aflatoonian, H. Sharifi, A. Karamoozian, F. Sharifi, A. Khosravi, S. Hassanzadeh, Risk factors for anthroponotic cutaneous leishmaniasis in unresponsive and responsive patients in a major focus, southeast of Iran, PLoS One. 13 (2018) e0192236.
[11] M. Bamorovat, I. Sharifi, M.A. Mohammadi, S. Eybpoosh, S. Nasibi, M.R. Aflatoonian, A. Khosravi, Leishmania tropica isolates from non-healed and healed patients in Iran: a molecular typing and phylogenetic analysis, Microb. Pathog. 116 (2018) 124–129.
[12] J.P. Sison, C.A. Kemper, M. Loveless, D. McShane, G.S. Visvesvara, S.C. Deresinski, Disseminated Acanthamoeba infection in patients with AIDS: case reports and review, Clin. Infect. Dis. 20 (1995) 1207–1216.
[13] S.E. Geerlings, A.I.M. Hoepelman, Immune dysfunction in patients with diabetes mellitus (DM), FEMS Immunol. Med. Microbiol. 26 (1999) 259–265.
[14] J.M. Blackwell, Genetic susceptibility to leishmanial infections: studies in mice and man, Parasitology. 112 (1996) S67–S74.
[15] A. Ponte-Sucre, E. Diaz, M. Padrón-Nieves, Drug Resistance in Leishmania Parasites: Consequences, Molecular Mechanisms and Possible Treatments, Springer Science & Business Media, 2012.
[16] A. Ponte-Sucre, F. Gamarro, J.-C. Dujardin, M.P. Barrett, R. López-Vélez, R. García-Hernández, A.W. Pountain, R. Mwenechanya, B. Papadopoulou, Drug resistance and treatment failure in leishmaniasis: a 21st century challenge, PLoS Negl. Trop. Dis.. 11 (2017) e0006052.
[17] M.T.M. Roberts, Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment, Br. Med. Bull.. 75 (2005) 115–130.
[18] Y.R. Díaz, R. Rojas, L. Valderrama, N.G. Saravia, T-bet, GATA-3, and Foxp3 expression and Th1/Th2 cytokine production in the clinical outcome of human infection with Leishmania (Viannia) species, J. Infect. Dis. 202 (2010) 406–415.
[19] P. Saha, D. Mukhopadhyay, M. Chatterjee, Immunomodulation by chemotherapeutic agents against Leishmaniasis, Int. Immunopharmacol. 11 (2011) 1668–1679.
[20] K.J. Gollob, L.R.V. Antonelli, D.R. Faria, T.S.L. Keesen, W.O. Dutra, Immunoregulatory mechanisms and CD4− CD8−(double negative) T cell subpopulations in human cutaneous leishmaniasis: a balancing act between protection and pathology, Int. Immunopharmacol. 8 (2008) 1338–1343.
[21] M.I. Muniz-Junqueira, V.N. de Paula-Coelho, Meglumine antimonate directly increases phagocytosis, superoxide anion and TNF-α production, but only via TNF-α it indirectly increases nitric oxide production by phagocytes of healthy individuals, in vitro, Int. Immunopharmacol. 8 (2008) 1633–1638.
[22] S. Ajdary, M.H. Alimohammadian, M.B. Eslami, K. Kemp, A. Kharazmi, Comparison of the immune profile of nonhealing cutaneous leishmaniasis patients with those with active lesions and those who have recovered from infection, Infect. Immun. 68 (2000) 1760–1764.
[23] H. Louzir, P.C. Melby, A. Ben Salah, H. Marrakchi, K. Aoun, R. Ben Ismail, K. Dellagi, Immunologic determinants of disease evolution in localized cutaneous leishmaniasis due to Leishmania major, J. Infect. Dis.. 177 (1998) 1687–1695.
[24] A. Ribeiro-de-Jesus, R.P. de Almeida, H. Lessa, O. Bacellar, E.M. Carvalho, Cytokine profile and pathology in human leishmaniasis, Braz. J. Med. Biol. Res.. 31 (1998) 143–148.
[25] M.R. Aflatoonian, I. Sharifi, B. Aflatoonian, M.R. Shirzadi, M.M. Gouya, A. Kermanizadeh, A review of impact of bam earthquake on cutaneous leishmaniasis and status: epidemic of old foci, emergence of new foci and changes in features of the disease, J. Arthropod. Borne Dis.. 10 (2016) 271.
[26] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method, Methods 25 (2001) 402–408.
[27] I. Sharifi, M.R. Aflatoonian, A.R. Fekri, M. Hakimi Parizi, A. Aghaei Afshar, A. Khosravi, F. Sharifi, B. Aflatoonian, A. Khamesipour, Y. Dowlati, F. Modabber, A. Nadim, A comprehensive review of cutaneous leishmaniasis in Kerman province, southeastern Iran-narrative review article., Iran. J. Public Health. 44 (2015) 299–307.
[28] R. Hadighi, M. Mohebali, P. Boucher, H. Hajjaran, A. Khamesipour, M. Ouellette, Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant Leishmania tropica parasites, PLoS Med. 3 (2006) e162.
[29] R. Rojas, L. Valderrama, M. Valderrama, M.X. Varona, M. Ouellette, N.G. Saravia, Resistance to antimony and treatment failure in human Leishmania (Viannia) infection, J. Infect. Dis. 193 (2006) 1375–1383.
[30] J.M. Mutiso, J.C. Macharia, M.M. Gicheru, H. Ozwara, B. Moodi, M. Aliabadian, A. Moshaverinia, O.M. Kakhki, A. Chaudhary, C. Verma, Immunology of leishmaniasis, Sci. Parasitol.. 14 (2013) 51–61.
[31] C.A. Nacy, M.S. Meltzer, E.J. Leonard, D.J. Wyler, Intracellular replication and lymphokine-induced destruction of Leishmania tropica in C3H/HeN mouse macrophages., J. Immunol.. 127 (1981) 2381–2386.
[32] M. Kopf, F. Brombacher, G. Köhler, G. Kienzle, K. Widmann, K. Lefrang, C. Humborg, B. Ledermann, W. Solbach, IL-4-deficient Balb/c mice resist infection with Leishmania major., J. Exp. Med.. 184 (1996) 1127–1136.
[33] Y. Belkaid, C.A. Piccirillo, S. Mendez, E.M. Shevach, D.L. Sacks, CD4+ CD25+ regulatory T cells control Leishmania major persistence and immunity, Nature. 420 (2002) 502.
[34] A. O'Garra, P. Vieira, T H 1 cells control themselves by producing interleukin-10, Nat. Rev. Immunol.. 7 (2007) 425.
[35] P.A. Darrah, S.T. Hegde, D.T. Patel, R.W.B. Lindsay, L. Chen, M. Roederer, R.A. Seder, IL-10 production differentially influences the magnitude, quality, and protective capacity of Th1 responses depending on the vaccine platform, J. Exp. Med. 207 (2010) 1421–1433.
[36] R.M. Mukbel, C. Patten Jr., K. Gibson, M. Ghosh, C. Petersen, D.E. Jones, Macrophage killing of Leishmania amazonensis amastigotes requires both nitric oxide and superoxide, Am. J. Trop. Med. Hyg. 76 (2007) 669–675.
[37] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nat. Rev. Immunol.. 8 (2008) 958.