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Evaluation of Th17 associated antigen in Old World Cutaneous Leishmaniasis: A comparative study in acute versus chronic human cutaneous Leishmaniasis using immunohistochemistry

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Abstract. There are little information about Th17 cells and cutaneous Leishmaniasis (CL), due to an important effect of Th17 cells on immune response, it is worth to explore the role of Th17 on CL. The purpose of this study was to assess Th17 population in patients with acute *vs*. chronic CL lesions in comparison with skin samples collected from healthy volunteers in an endemic region of Old World CL. A total of 49 patients with clinical manifestations of chronic (n=16) and acute (n=33) CL lesions were recruited. The clinical diagnosis of CL was confirmed by direct smear or PCR. Biopsy specimens from prelesional skin of non-infectious lesions of 30 healthy individuals were used as control. Tissue sections of 3µm thickness were prepared and used for immunohistochemistry (IHC) analysis with primary antibody specific for Th17 associated antigen (CD161). For IHC, Envision+ (DakoCytomation) system was used and developed by using diaminobenzidine (DakoCytomation). The mean age of 33 patients with acute CL and the mean age of 16 patients with chronic CL were accordingly 45.24±16.43 and 33.56±15.87. In acute and chronic CL the mean (±standard deviation) and median (±interquartile range) were accordingly 2.92±2.21, 2.56±2.9 and 2.1±1.99, 1.54±2.81. In healthy controls the mean (±standard deviation) and median (±interquartile range) were 0.72±0.41 and 0.61±0.58 respectively. With pairwise comparison of acute, chronic and control groups, there were significant difference between acute and control (P value < 0.001), chronic and control (P value = 0.043). The results showed that there was an increasing cellular response of Th17 in both acute and chronic CL patients. Th17 was significantly higher in patients with acute and chronic CL lesions in comparison with healthy control group. However, there was no significant difference between acute and chronic infection concerning to Th17 cells.

INTRODUCTION

The worldwide prevalence of Cutaneous Leishmaniasis (CL) is increasing (Murray *et al.* 2005). The occurrence of 0.2–0.4 million new annual visceral cases of the disease and 0.7 to 1.2 million CL cases and overall prevalence of 12 million cases globally have

been previously reported (Alvar *et al.*, 2012). CL in Iran is caused by *Leishmania*, *L. major*, *L. tropica* and rarely by *L. infantum* (Shirian *et al.*, 2013). The clinical feature of CL depends on both the host immune response and the causative species induced the lesion (Mahmoodi *et al.*, 2003; Perez-Franco *et al.*, 2016). The clinical appearance of CL caused by different *Leishmania* species ranges from self-healing to non-healing (chronic) cutaneous lesions (Mera-Ramírez *et al.*, 2017). Clinical stages of CL are also classified as localized CL (LCL), mucocutaneous leishmaniasis (MCL), and diffuse CL (DCL) (WHO 2010; Shirian *et al.*, 2014). Self-healing of CL is mostly occurred after several weeks or months, it has been reported that *L. tropica* is associated with longer disease duration and low tendency for self-healing, however, information in regard to differences in healing process in CL caused by various species of *Leishmania* is not available (Kroid *et al.*, 2014). In chronic or even non-healing lesions, the infection last several years and often not responding to conventional treatments. The cellular immune response has an important role in progression or regression of the disease. Paradigm of T helper 1 (Th1)/Th2 responses has been defined to determine susceptibility/resistance of *Leishmania major* infection and the role of interleukin (IL) 4 and IL-12 respectively in driving Th2 and Th1 cell development in murine models (Gumy *et al.*, 2004).

It has been reported that IL-1β induces protective or pathogenic effects during visceral leishmaniasis by affecting on the development of Th17 cells (Ghosh *et al.*, 2013). Th17 cells are characterized by IL-17 production (Korn *et al.*, 2009). The significant role of IL-17 in the migration, recruitment, and activation of neutrophils is important effector and play regulatory function during *Leishmania* infection (Charmoy *et al.*, 2010). Although, Th17 cells expected to play a significant role during *Leishmania* infection but little is known about Th17 cells in the context of CL.

In CL, 5 to 10% of patients get a chronic or non-healing course (Akilov *et al.*, 2007). According to Akilov *et al.* (2007) CL lesions with duration more than one year are called non-healing, others believe that CL lesions with duration more than 2 years are called non-healing (Dowlati 1996; Akilov *et al.*, 2007; Mortazavi *et al.*, 2016).

To the best of our knowledge, immune cells play a significant role in healing or non-healing process of CL lesions. In this regard, most of previous studies have been performed on blood samples (Gaafar *et al.*, 1995; Ajdary *et al.*, 2000). Studies using immunohistochemistry (IHC) method in Old World CL are rare and most of previous studies by IHC are about New World CL. Although *in situ* cellular immune response approching T cell has been widely discussed in the literature but there are limited studies on Th17 cells evaluation (Barral *et al.*, 1987; Rahman & Bari 2006; Boaventura *et al.*, 2010).

The aim of this study was to compare the rate of Th17 populations in acute (less than one year duration) versus chronic (more than three years duration or non-healing) lesions of CL patients in comparison with normal skin of control group using IHC in this endemic region of Old World CL.

Patients and methods

A total of 49 patients with various clinical manifestations of the chronic (n=16) and acute (n=33) CL forms were included this study (Fig. 1, A to D). The clinical diagnosis of CL was confirmed by direct microscopy and PCR. The species of *Leishmania* was detected by nested-PCR as well. Biopsy specimens from pre-lesional skin of non-infectious lesions of 30 healthy individuals referred to department of plastic surgery of Razi Hospital for removal of their benign lesions were used as control group. Demographic information of the patients including sex, age, history of drug consumption, history of concurrent disease, and residential address were recorded. The patients entered the study were from various rural and urban areas of several provinces of Iran, who were referred to referral dermatology Razi Hospital, the affiliated hospital to Tehran University of Medical Sciences Tehran Iran, between April 2013 and March 2016.

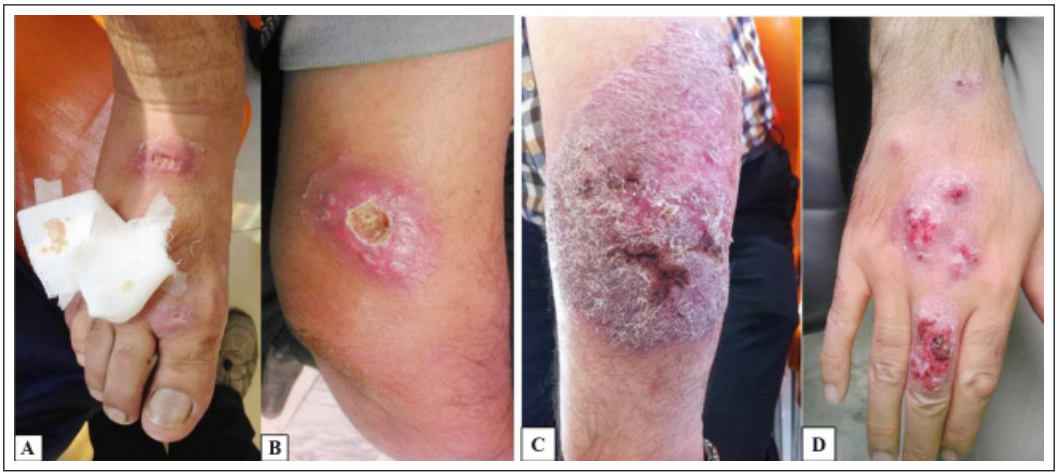

Figure 1. Clinical pictures of acute cutaneous leishmaniasis: (A) sporotrichoid (B) ulcerated plaque, Clinical pictures of chronic cutaneous leishmaniasis: (C) tumoral and ulcerated (D) sporotrichoid.

Ethical Consideration

The Ethics Committee of Deputy of Research, Tehran University of Medical Sciences (TUMS) approved the study, and the author group collected written, informed consent from all patients (Project Number: 9211166007).

Microscopic Examination

After careful sterilization of the biopsy sites and local anesthesia with 2% lidocaine, skin biopsies from the cutaneous lesions of the patients were done. The tissue samples were fixed in 10% neutral-buffered formalin (Sigma, USA), dehydrated in increasing concentrations of ethanol, cleared with xylene and impregnated with paraffin wax. The paraffin embedded specimens were sectioned at 3µm thickness and were subsequently stained using hematoxylin and eosin (H&E), and IHC stain.

Immunohistochemistry staining

Three micrometer thick sections were used for the IHC analysis, as previously described by Shirian *et al.* (Shirian *et al.*, 2014). Briefly, the slides were deparaffinized in xylol, rehydrated, and to quench the endogenous peroxides treated with 3% hydrogen peroxide solution for 10 minutes at room temperature. The antigen retrieval was done by pretreatment in a microwave oven (power 100 for 10minutes; then, power 20 for 20 minutes) using 10-mmol/L concentration of citrate buffer (pH: 6.0). The primary antibodies including CD161(Dako, Denmark) was applied for 1 hour (diluted 1:300). For IHC, Envision+ (DakoCytomation) system was used and developed by using diaminobenzidine (DakoCytomation) (Shahbazi *et al.*, 2015; Soleimanpour *et al.*, 2011. To calculate the percentage of positive cells, images were acquired from ten microscopic fields of each IHC stained slide using the Labomed light microscopy (LX-300-USA) and analyzed using Image J software (version 1.50). The average of positive cells of each slide (number of positive cells/mm2) was recorded as density.

Nested-PCR Assay

The primers for the first-round PCR assay set for CSB1XR (ATTTTTCGCGAT TTT CGCAGAACG) and CSB2XF (CGAGTAGCA GAAACTCCCGTTCA) sequences.

Standard PCR amplification was carried out in a reaction mixture of 25µl containing 200 µM of deoxynucleoside triphosphate (Roche, Penzberg, Germany), 1.0 unit Taq polymerase (CinnaGene, Tehran, Iran), 50 mMTris-HCl (pH 7.6), 1.5mM MgCl2, 5µl template DNA, 10 µM CSB1XR and 10 µM CSB2XF. The thermocycler (Eppendorf AG, Hamburg, Germany) was set to 5 min at 94°C for first denatured, followed by 30cycles of denaturation, each consisting of 30 s at 94°C,

1 min at 55°C for annealing, and extension at 72°C for 1 min, and then a final extension for 5 min at 72°C. The PCR products of the first step of PCR were diluted 1:9 in ultrapure water, and 2µlof this dilution was then used as the template for the second round of PCR, which was carried out under the same conditions and reaction mixture as the first round, except that LiR (TCGCAGAACG CCCCT) and 13Z (ACTGGGGGTTGGTG TAAAATAG) were used as the primers. The PCR products of second round were electrophoresed on 1.5% agarose gel. The DNA extracted from the promastigote cultures of the reference strains of *L. tropica* (MHOM/IR/89/ARA2), *L. infantum* (MCAN/IR/97/LON490) and *L. major* (MCAN/IR/97/LON490) were also run on each gel as the positive controls. The tissue sections from 10 patients who referred to plastic surgery were used as the negative controls (Shirian *et al.*, 2014).

Statistical analysis

Descriptive statistics such as mean and standard deviation and frequency with percentage were reported for continuous and categorical variables, respectively. In order to compare the percentage of Th17 cell population between acute, chronic and control groups, Kruskal Wallis test with Dunn's post hoc test for pairwise comparison was used. In order to compare two independent groups, mean percentage of CD161 in acute *vs.* chronic group with *L. major* infected patients and *L. major* vs. *L. tropica* infected patients with acute disease, Mann Whitney nonparametric test was used. *P*-value less than 0.05 were consider statistically significant. All statistical analysis were performed by SPSS version 22.

RESULTS

Clinical findings

The clinical manifestations of the lesions of 33 patients with acute CL (25 males and 8 females, mean age 45.24±16.43) were nodule (16), pluque (8), tumor (7), sporotrichoid (1) and erysipeloid (1). While, 16 patients with non-healing CL (14 males and 2 females, 33.56±15.87) were infected with plague (6), tumor (3), nodule (2), sporotrichoid (2), erysipeloid (1), papule (1) and erythematous annular form (Fig. 1 A to D).

Molecular findings

The kDNA fragments of *L. tropica* (750-bp long) and *L. major* (560–590-bp long), were amplified from the 8 (24.24%) and 24 (72.72%) of 33 acute CL samples. Out of 16 non-healing CL samples, *L. tropica* and *L. major* were detected from 2 and 13 patients by second-round PCR assay, respectively. PCR assay of one patient with acute CL was negative as well as one patient with non-healing CL. These two patients with negative PCR had positive direct smear for CL. PCR findings of study population have shown in Table 1.

Microscopic findings

With H&E staining in acute lesions, parasitized macrophage lymphocytes and plasma cell were seen in dermis. Sometimes hyperkeratosis of epidermis was seen, in slides of 54.5% of acute patients with CL / Leishman body was observed. Twenty four of acute CL patients were infected with *L. major* and eight CL patients were infected with *L. tropica*. In one patient with positive smear, the species of *leishmania* was not determined.

Table 1. Direct smear and PCR findings in acute and chronic CL patients

	Direct smear	PCR (Species)		
	+	*L. major*	*L. tropica*	Negative (Undetermined)
Acute	33	24(72.7%)	8(24.2%)	1(3%)
Chronic	16	13(81.3%)	2(12.5%)	1(6.3%)

Table 2. Mean and median of Th17 surface markers (CD161) expression in patients with acute and chronic CL and control groups

Group	N	Mean ± SD	Median ± IQR	*P*-value*
Acute	33	2.92±2.21	2.56±2.9	<0.0001
Chronic	16	2.1±1.99	1.54±2.81	
Control	30	0.72±0.41	0.61±0.58	

* Kruskal Wallis Nonparametric test.

In H&E staining of histopathological slides of patients with chronic CL, granuloma and mostly tuberculoid granuloma (81.25%) while Leishman bodies were sparse. Lymphocytes and plasma cells were seen in vicinity of granulomas. In slides of 6.25% of chronic patients with CL / Leishman body were observed. Out of 16 with chronic (non-healing) CL, 13 cases were due to *L. major* and 2 due to *L.tropica* and in one case the species was not determined.

Immunohistochemical findings

The mean percentage (±SD) and median (±IQR) of CD161expression in patients with acute and chronic CLand control groups are shown in Table 2.

Significant differences was observed between median of acute, chronic and control groups (*P*-value<0.001) Table 2. Based on pair wise comparison there were significant differences between median of acute group with control group (*P*-value<0.001) and chronic group with control group (*P*-value=0.043) but there was no significant difference between acute group with chronic group (*P*-value=0.435) Table 3 and Figures 2 and 3. Similarly the level of CD161 indexes are significantly higher in acute and chronic groups in compare with control group.

Table 3. Pairwise comparison of acute, chronic and control groups

Pairwise comparison	*P*-value*
Acute vs Chronic	0.435
Acute vs Control	<0.0001
Chronic vs Control	0.043

* Dunn's post hoc test for pairwise comparison.

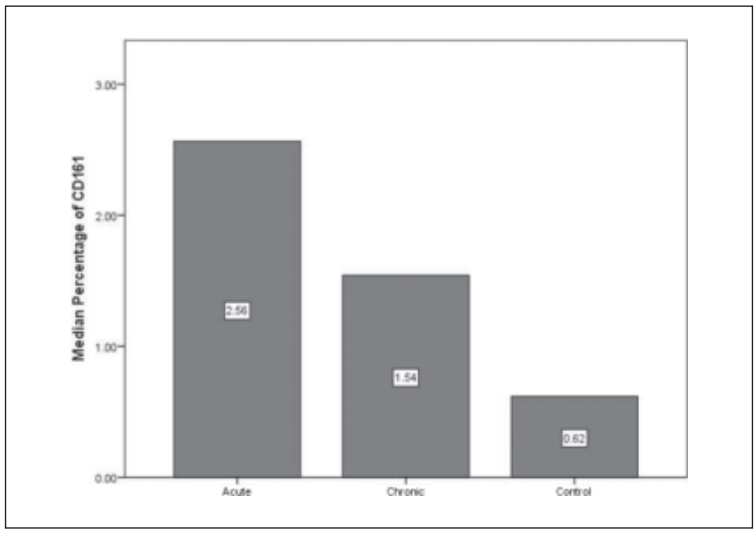

Figure 2. Bar plot of median percentage of CD161 in Acute, Chronic vs. Control groups.

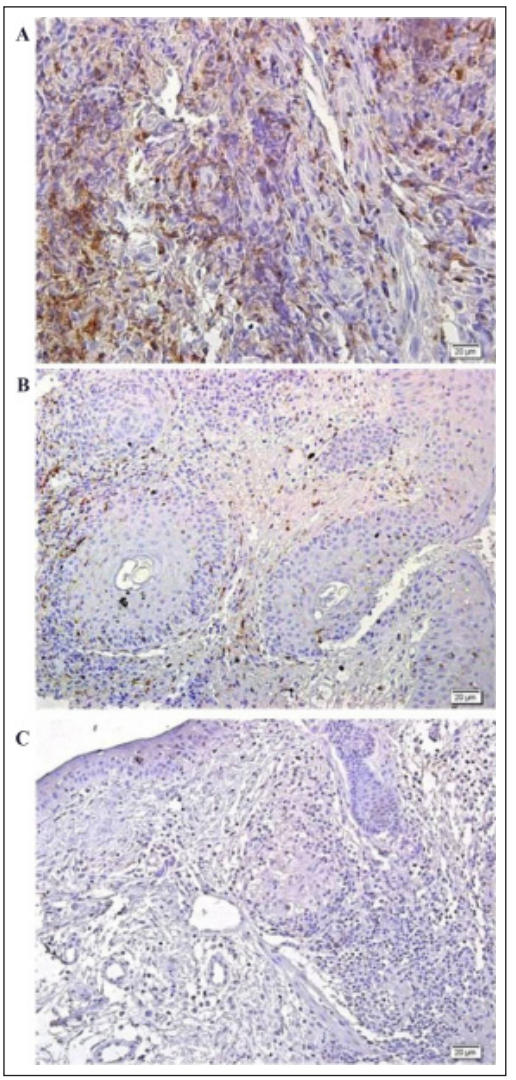

Figure 3. The mean positive cell percentage for Th17 surface marker in various groups, A: acute cutaneous leishmaniasis (CL); B: chronic CL, C: control group, immunohistochemical staining, Scale bar= 20µm.

Comparison of CL patients with *L. major* infection versus patients infected with *L. tropica*

Due to the low number of *L. tropica* observations (just two observations) in chronic group the statistical comparison between *L. major* and *L. tropica* are not reasonable. When in acute CL group, we compared mean CD161 marker of *L. major* with *L. tropica*, we observed no significant difference (*P*-value=0.192) Table 4.

In comparing mean percentage of CD161 of acute CL patients with chronic patients infected with *L. major*, there was not significance difference between two group (*P*-value=0.131) Table 5.

Regarding comparison of patients infected with *L. tropica* in acute *vs.* chronic group, due to low number of observations infected with *L. tropica* statistical comparing between these two groups are not reasonable.

DISCUSSION

The primary aim of this study was to investigate the role of Th17 cells in acute form of CL lesions defined as lesion with duration less than one year versus chronic CL lesion defined as more than 3 years using IHC. Cutaneous leishmaniasis with duration more than 2 years is considered as non-healing or chronic CL (Mortazavi *et al.*, 2016). CL infection lasting over two years should be considered as chronic cutaneous leishmaniasis (CCL), which is more common in infections due to *L. tropica* than to *L. major* (Dowlati 1996). CCL develops in only 5–10% of patients with CL. The CCL form also occurs

Table 4. Comparison of mean percentage of acute CL patients with regard to infectious agents (*L. major* or *L. tropica*)

	Infection	N	Mean	SD	Median	Min-Max	P-value*
CD161	*L. major*	24	3.2	2.3	3.07	0.36-8.46	0.192
	L. tropica	8	2.1	1.83	1.44	0.09-5.65	

* Mann Whitney Non-parametric test.

Table 5. Comparison of mean percentage of acute versus chronic CL patients with regard to infectious agents

	Group	N	Mean	Std. Deviation	Median	Min-Max	P-value*
CD161	Acute	24	3.21	2.3	3.07	0.36-8.46	0.131
	chronic	13	2.21	2.14	1.3	0.04-6.21	

* Mann Whitney Non-parametric test.

as a result of a failure of the host immune response (Akilov *et al.*, 2007). The IHC preparations is the best method for the antibody-antigen reaction, diagnosis and immunophenotyping of T cell surface markers (Rahman & Bari 2006; Campanelli *et al.*, 2006; Boaventura *et al.*, 2010). The chromogenic substrate produces a precipitate layer at the site of the antibody-antigen reaction and results in an overlying brown layer which is readily detectable at any lower or higher power views (Shirian *et al.*, 2014). Therefore, in this study CD161 as Th17 surface markers was used (Pianta *et al.*, 2014). To the best of our knowledge, this is the first study to compare the T helper 17 cell population in patients with acute and chronic CL in Old World. In Old World CL, there was only one report in which the immune response of acute CL lesions was compared with healed lesions (Rahman & ul Bari 2006).

The *in situ* regulatory immune response has been evaluated by IHC using anti-human CD4, FoxP3, IL-10, and TGF-β antibodies. It has been suggested that T regulatory FoxP3+ cells play an important role in the immunopathogenesis of non-ulcerative CL (Flores *et al.*, 2018). Katara *et al.* (2012) have utilized the IHC to authenticate translation of Th17 type responses by analyzing mRNA and protein expression of Th17 markers in post-kala-azar dermal leishmaniasis (PKDL) lesions. They have shown the evidence for the involvement of Th17 type in the disease pathogenesis (Katara *et al.*, 2012).

Data obtained from both PCR assay and microscopic examination showed that all 49 presented patients were infected with leishmaniasis. Cutaneous Leishmaniasis is a protozoan disease that causes a wide spectrum of clinical manifestations, from self-healing to non-healing and diffuse cutaneous lesions in the endemic areas such as Iran (Shirian *et al.*, 2014; Ostad *et al.*, 2016). The severity of the disease is characterized by both genetic of parasite and the immune response of the host (Oryan *et al.*, 2013).

Regarding high number of CL patients with *L. major* infection in this study the following reasons could be stated. Razi hospital of Tehran is the largest referral dermatologic center of Iran, since there are many endemic zones of CL due to *L. major* in central part of Iran (Isfahan and Kashan) near Tehran, therefore, the CL patients in this endemic zone are referred (especially with chronic CL) to Razi Hospital of Tehran. Therefore most of CL patients entered this study were referred from *L. major* endemic zones near Tehran to Razi Hospital, Iran.

In patients with acute and chronic CL, the mean percentage and median expression of CD161 were higher than control group. The difference between acute and control groups was significant ($P<0.001$). The difference between chronic and control groups was also significant ($P=0.043$). Th17 cells, particularly by IL-17 production represent another arm of T cell-mediated immune responses which plays a significant role in the development of protective cellular immunity against *Leishmania* parasite (Bacellar *et al.*, 2009). Experimental studies have been shown the crucial role of IL-17 in the recruitment and activation of defense cells such as neutrophils (Wu *et al.*, 2010). Based on the potential role of neutrophils, their important effectors and regulatory functions in *Leishmania* infection, it would be expected that Th17 cell is a major player during *Leishmania* disease (Charmoy *et al.*, 2010). Th17 cells produce cytokines such as IL-17 and IL-22 which have been demonstrated to enhance protection

against certain pathogens in human kala-azar infection (Pitta *et al.*, 2009). It has been suggested that IL-17 and IL-22 play complementary roles in human protection against visceral leishmaniasis (VL) along with the Th1 cytokines. Therefore, a defect in Th17 induction may increase the risk of VL. However, human studies have not reliably demonstrated a crucial role of Th17 cells in protection against CL infections.

According to Souza *et al.* in active phase of American CL elevated level of IL-17 have been observed (in cultured PBMC), as we know IL-17 is a product of Th17 cell, therefore, in current study, the higher number of Th17 in active (acute) phase of CL *vs.* chronic phase of CL could be explained by the above experiment. However, to the best of our knowledge, there is no data about IL-17 in *L. major* infection (Souza *et al.*, 2012; Banerjee *et al.*, 2016).

Regarding expression of CD161 cells (Th17) when we compared CL lesions due to *L. major* and *L. tropica* there was no significant difference between CL lesion due to these pathogenic agent (*L. major* versus *L. tropica*) Table 4 and 5. With regard to these findings, we did not find any publication to compare with our findings. Therefore, for elucidation of the role of Th17 in *L. major* and *L. tropica* human infection more studies are needed.

In this study we showed that *L. major* and *L. tropica* which can cause acute CL and chronic CL, stimulates the differentiation of Th17 cells. Th1/Th2 paradigm is not the only pathogenetic mechanisms that determine the fate and outcome of *Leishmania* infection, many pathomechanisms including neutrophils and Th17 cells may contribute in pathogenesis and outcome of *Leishmania* infection (Mortazavi *et al.*, 2016; Gonçalves-de-Albuquerque *et al.*, 2017).

CONCLUSION

In conclusion, there was increased Th17 cellular response in both acute and chronic CL patients. However, Th17 was significantly higher in patients with acute and chronic CL lesions in comparison with healthy control group. There was no significant difference between acute and chronic infection concerning to Th17 cells. Therefore, this cellular population was increased in CL caused by *L. tropica* and *L. major* similarly in acute and chronic phases of infection. More studies with larger sample sizes are suggested to elucidate the role of Th17 in the pathogenesis of CL.

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