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Gene expression profile of cytokines produced in biopsies from patients with American cutaneous leishmaniasis

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ABSTRACT

American cutaneous leishmaniasis (ACL) causes a local inflammatory process, inducing expression of several cytokine genes. Particularly, IFN-γ can predict to disease susceptibility. Based in these data, this study was aimed to investigate the gene expression profile of IFN-γ, IL-10, IL-27, TNF-γ, TGF-β and IL-6 produced in biopsies from ACL patients; and whether the gene expression profile of IFN-γ could determine the disease evolution. Gene expression of 6 cytokines was investigated in 40 formalin-fixed paraffin embedded (FFPE) biopsies from patients with cutaneous leishmaniosis (CL); and 10 FFPE biopsies from patients with mucosal leishmaniasis (ML) (control). All 50 patients were infected with *Leishmania (Viannia) braziliensis*. Gene expression was determined by qPCR; and a normal control group was used for calculations (5 normal biopsies). Values were expressed as Relative Quantification (RQ). The 40 CL patients were classified into 2 groups. CLlowIFN-γ, 35 patients with RQ for IFN-γ below 100; and CLhighIFN-γ, 5 (12.5%) patients with RQ above 100. Significant increase of mRNA levels of IFN-γ, IL-10 and IL-27 was shown in CLhighIFN-γ group when compared with CLlowIFN-γ and ML groups. TNF-α levels in CLlowIFN-γ group were higher than CLhighIFN-γ and ML groups. TGF-β and IL-6 were similar in 3 groups. Comparison of cytokine expression/group showed that CLlowIFN-γ group had an equilibrium between the cytokines analyzed. In ML group, IFN-γ was over-expressed; but in CLhighIFN-γ group, besides IFN-γ, IL-27 was also over-expressed. The immune response to *Leishmania* induces to identification of some markers, which can be determined by analysis by gene expression of cytokines produced in biopsies.

1. Introduction

Leishmaniases are neglected tropical diseases, emergent and uncontrolled, which causes several clinical manifestations. These infections cause significant morbidity and mortality among 380 million susceptible individuals, who are at risk in 98 countries. According to WHO, the prevalence of cutaneous forms caused by *Leishmania* is estimated to be between 0.7 million to 1.0 million new cases worldwide annually (WHO, 2018).

Cutaneous and mucosal leishmaniasis occur in 20 countries in the Americas and is endemic in 18 of them. From 2001–2015, 843,931 new cutaneous and mucosal cases were reported to PAHO/WHO with an annual average of 56,262 cases (PAHO, 2017). In Sao Paulo State (Brazil), during 1998–2017 occurred around 10,000 confirmed cases of American cutaneous leishmaniasis (ACL) (SES, 2018).

More than 20 *Leishmania* species can infect humans causing different clinical presentations, varying since cutaneous lesions that heal spontaneously within 2–10 months (Barry et al., 2014; Eiras et al., 2015), severe chronic mucocutaneous infections or fatal visceral lesions if untreated (Desjeux, 2004; Murray et al., 2005). The different clinical

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forms of disease are attributed to several parasite species and/or host immune response to infection (Carvalho et al., 2012).

The role of immune response and immunological mediators involved in the clinical presentation of cutaneous leishmaniasis has been discussed and has become the subject of several studies on the immunopathogenesis of ACL (Tripathi et al., 2007; Hoseini et al., 2012). Cytokines are the keys in the induction and development of the immune response (Carvalho et al., 2012). The immune response against intracellular parasites depends on the balance between Th1 and Th2 cells (Choi and Kropf, 2009). In the murine model, the immune response has been clearly described as a Th2 profile during disease progression and Th1 in infection control. In humans, the exact role of Th1 and Th2 dichotomy in persistence of cutaneous lesions has not been totally elucidated. In addition, an association of both immune responses appear to be present in acute, as well as, in chronic forms of the infection (Jafari-Shakib et al., 2009; Frade et al., 2011).

Interferon gamma (IFN-γ) plays a role in controlling infection through macrophage activation (Alexander and Bryson, 2005; Mansueto et al., 2007; Castellano et al., 2009). IFN-γ is synergistically linked to tumor necrosis factor alpha (TNF-α) to activate macrophages and induce inducible nitric oxide synthase (Faria et al., 2005). Interleukin 10 (IL-10) plays an important role in the pathogenesis of the disease, since regulates the proinflammatory effects of IFN-γ and TNF-α. Additionally, IL-10 can, also, facilitate parasite growth and replication by decreasing the ability of macrophages to kill *Leishmania* (Vieth et al., 1994; Scott, 2003; Salhi et al., 2008; Assis Souza et al., 2013).

The severity and prevalence of ACL vary greatly. Such variations are correlated with parasite species, well as, genetic and status of host immune system (Desjeux, 2004; Gomes et al., 2008). Studies have been described that infected individuals respond differently to *Leishmania* stimulation. According IFN-γ, they had high or low production and represented a potential tool to predict to disease susceptibility (Pompeu et al., 2001; Carneiro et al., 2016).

In a previous study, our group examined biopsies collected from ACL patients. The analysis of immunostaining cells established that patients with recent lesions expressed higher levels IL-10, TNF-α and IFN-γ than patients with late lesions (Gomes et al., 2017). The choice for biopsies was related by the microenvironment of lesions, which seem to be an important immunopathogenic event occurring *in situ*. In addition, a local inflammatory process, induce the expression of several cytokine genes (Costa-Silva et al., 2014).

Studies on T cell-mediated immunity in ACL are important due to the need of new approaches concerning a rapid treatment and prophylaxis. Gene expression analysis by quantitative polymerase chain reaction (qPCR) to determine immunological mediators in ACL is a recent approach that must be considered by the good sensitivity and accuracy of this methodology.

Based in these data, this study was aimed, firstly, to evaluate the gene expression profile (mRNA) for IFN-γ, IL-10, IL-27, TNF-γ, TGF-β and IL-6 produced in biopsies from patients with ACL. In parallel, we investigated whether the gene expression profile of IFN-γ could determine the clinical evolution of patients.

2. Materials and methods

2.1. Ethical considerations

The Ethics Committees of all involved Institutions approved the entire study, which was performed according to recommendations of these Committees (CONEP-IAL number: 424.827). The procedures were performed after patients provided written informed consent from the institutional review boards of Ethics Committee.

2.2. Patients and clinical samples

This study evaluated 55 biopsies collected during for 28 months (January 2014 to April 2016). Out of then, 40 skin biopsies (CL) were collected from patients living and attending public dermatology clinics of Sorocaba Region, Sao Paulo, Brazil, a high endemic region for ACL. For control of infection evolution, 10 skin/mucosal biopsies were collected from patients with clinical diagnosis for mucosal leishmaniasis (ML). These biopsies were collected from patients attending in Instituto de Infectologia Emilio Ribas, São Paulo, Brazil. All patients with typical lesions of leishmaniasis were given a complete dermatological examination performed before skin/mucosal biopsy collection. The medical procedures and biopsy collection were performed according previously described (Gomes et al., 2008). Briefly, lesions were cleaned with an antiseptic and a local anesthetic was administered. The borders of the lesions were scraped and samples were obtained by punch biopsy (0.2 mm) and immediately added to two tubes. One, contained 1–2 mL of sterile 0.85% NaCl and 200 μg/mL gentamicin solution and were processed for molecular diagnosis. The second tube contained 1–2 mL of sterile 0.85% NaCl and 10% formalin and was processed for histopathological diagnosis, immunohistochemistry (IHC) analysis and gene expression. The samples were sent to the laboratory within 48 h, where they were immediately processed. None of them had acquired the infection before or had been treated with drugs for leishmaniasis. A normal control (NC) group was included and was composed of 5 biopsies of normal skin tissues collected from females who had undergone to breast surgery. The skin tissue samples were collected and samples were obtained and immediately added in a tube contained 1–2 mL of sterile 0.85% NaCl and 10% formalin for histopathological, IHC and molecular analysis.

2.3. Laboratorial diagnosis

For molecular diagnosis, DNA extraction from biopsy samples as well as, conventional PCR were performed exactly as described before (Gomes et al., 2007, 2008). L. *(V.) braziliensis* complex was determined by amplification of a 146 to 149 bp fragment, using the primer pair LU-5 A/LB-3C, which amplifies a variable non-transcribed spacer. This region is specific for L. *(V.) braziliensis complex* (Harris et al., 1998). The quality of the extracted DNA and presence of PCR inhibitors were verified in DNA samples using a PCR that amplifies a housekeeping gene that amplified a 140-bp fragment of the human β-globin gene using primers β1/β2 following the same conditions as previously described (Gomes et al., 2007).

For histopathological analyses, samples were fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin, after microtome sectioning. Histologic sections were examined under light microscope to determine tissue characteristics (Gomes et al., 2017).

For IHC, 4 mm histological sections obtained from formalin-fixed paraffin embedded (FFPE) tissue samples were deparaffinized and submitted to antigen retrieval before incubating with mouse polyclonal anti-*Leishmania* spp. The entire protocol was performed exactly as described before (Gomes et al., 2017). Positive and negative controls consisted of human lymph-node tissue sample and the primary-antibody omission, respectively. Immunostaining parasites were shown under light microscopy.

2.4. RNA isolation and cDNA synthesis

All materials and working surfaces were cleaned using RNaseZap® RNase Decontamination Solution (Ambion™) prior to handling the samples, to minimize the RNA degradation by RNases during the experimental process. RNA molecules were extracted from FFPE biopsies using RNeasy FFPE isolation Kit (Qiagen, GmbH, Hiden, Germany), according to the manufacturer's instructions. Extractions were made using 6–8 fragments of 5–8 μm. Initially, fragments were dissolved in xylene (1 mL) and, centrifuged at 13,000 *g* for 1 min. This step was carried out twice. Supernatants were removed and ethanol 96–100% (500 μL) was added to pellets, centrifuged at 13,000 *g* for 2 min and

pellets were concentrated in speed-vac (RC 10.09 – Jouan) for 5 min. Next, PKD buffer (240 µL) and proteinase K (10 µL) were added to the samples incubated at 56 °C to a complete lysis and followed the manufacturer's instructions.

Extraction yield and concentrations were assessed by Quantus™ Fluorometer (Promega). RNA integrity and levels of degradation were assessed by on-chip electrophoresis using RNA 6000 Pico kit and Bioanalyzer 2100 (Agilent Technologies). Based on the electropherogram, RNA Integrity Number (RIN) was calculated, considering 18S/28S rRNA peaks, as well as, the background and possible degradation products (Schroeder et al., 2006).

Next, 100 ng/µL of total RNA was reverse-transcripted (RT) using a High-Capacity cDNA RT kit (Applied Biosystems). RT was performed using a Veriti® 96-Well Thermal Cycler (Applied Biosystems) according to the manufacturer's instructions under the following thermal conditions: 10 min at 25 °C, 120 min at 37 °C followed by 5 min at 85 °C. All cDNA samples were stored at −70 °C until use in real time qPCR.

2.5. Gene expression of immunological mediators

The amplification mixture contained 10 µL of 2X TaqMan Universal PCR Master Mix and 1 µL TaqMan® Gene Expression Assays (both Applied Biosystems) for the following genes: IFN-γ, IL-10, IL-27, TNF-α, TGF-β, IL-6, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Assay IDs are given in Table 1. Template cDNA (4 µL) and 5 µL of RNAse free water were added to a total volume of 20 µL. Reactions were performed in triplicate for each sample and all assays. Samples were amplified and detected using a StepOne™ Real-Time PCR System (Applied Biosystems) using the following thermal profile: 2 min, 50 °C, and 95 °C for 10 min, followed by 40 cycles performed at 95 °C for 15 s and 60 °C for 1 min. The results with no amplification in qPCR were repeated at least three times.

2.6. Data analysis

Values of mRNA expression were showed as "Relative Quantification" (RQ) and were calculated by the comparative C_T method ($2^{-\Delta\Delta CT}$) as described before (Livak and Schmittgen, 2001). NC group consisted in the calibrators, which were used for calculations in mRNA expression experiments. Expression values of this group by the comparative C_T method assume the value of 1.0. GAPDH gene was chosen as reference gene by its uniform expression throughout FFPE samples (Meira-Strejevitch et al., 2017). Statistical analyses were performed using Graph Pad Prism software version 6.0 (San Diego, CA, USA). Comparison of expression between groups of patients with leishmaniasis was determined by unequal-variance *t*-test based on a critical value of $p \leqslant 0.05$ and F test to compare variances, by the analysis of one-way variance. In all cases, differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Patient groups and laboratorial diagnosis

Studies have shown that in ML patients present an exacerbated inflammatory nature of the cellular immune response as compared to CL patients. Normally, ML patients has higher IFN-γ production than CL patients (Pompeu et al., 2001; Gaze et al., 2006; Carvalho et al., 2007; Carneiro et al., 2016). In this study, out of the 40 patients with CL, 5 (12.5%) of them, had high mRNA expression levels for IFN-γ (statistically significant $p < 0.0001$). Based in these previous studies, we considerate to classify the 40 FFPE biopsies from CL patients into 2 groups according the gene expression of IFN-γ. CLlowIFN-γ group was constituted of 35 patients with RQ results for IFN-γ below 100. CLhighIFN-γ group had 5 patients with RQ results for IFN-γ above 100.

The laboratorial diagnosis for ACL included PCR, IHC, and histopathology. Out of them, 50 biopsies (CL and ML) had positive diagnosis for L. *(V.) braziliensis* complex. These DNA samples also had positive results for human β-globin (control for PCR inhibitors) confirming the good quality of the DNA extracted from biopsies.

Mild intracellular and free amastigotes were visible on histopathological and IHC procedures in 87% (35/40) of CL samples. Although, the biopsy preparations were used to identify amastigotes, histopathological observations showed different epidermal changes such as pseudoepitheliomatous, hyperplasia, acanthosis hyperkeratosis. Dermis presented inflammatory infiltrate with lymphocytes, plasma cells, macrophages containing amastigotes, and rare eosinophils (Gomes et al., 2017).

As the majority of cases of ACL in Brazil is caused by L. *(V.) braziliensis* (Grimaldi and Tesh, 1993; Ramos-E-Silva et al., 2002) and parasites were determined in a typical population target for ACL, as previously well described (Grimaldi and Tesh, 1993; Gomes et al., 2008), patients had laboratorial, clinical and epidemiological diagnosis for ACL and ML.

The 55 patients were separated as following. The 40 patients as having CL were divided into 18 males and 17 females (CLlowIFN-γ group), and 4 males and 1 female (CLhighIFN-γ group). The ages ranged from 11 to 79 years old in both Groups. The 10 patients defined as having ML were 4 males and 6 females. The ages ranged from 17 to 75 years old. Individuals from NC group were 5 females ranging from 34 to 54 years old. All samples were negative for leishmaniasis in all laboratorial procedures.

3.2. Modulation on expression of IFN-γ, IL-10 and IL-27

Total RNA molecules were successfully extracted from all 55 FFPE biopsies. The mean for RNA yield was 64.19 ng/µL, and for RIN value was 4.91.

Fig. 1 shows in detail the results of mRNA relative levels, expressed as RQ, after normalization with the endogenous reference gene (GAPDH). The values refer to IFN-γ, IL-10, IL-27, TNF-α, TGF-β and IL-6 of CL patients (CLlowIFN-γ and CLhighIFN-γ groups); ML patients

Table 1
Description of the genes analyzed in this study.

Gene Symbol	*Gene Name*	*Accession number*	*Biological function*	*Assay ID*[a]	*Amplicon Length*	*Location Chromosome*
IFN-γ	Interferon gamma	[RefSeq: NM_000619.2]	Protein coding	Hs00989291_m1	73	12
IL-10	Interleukin 10	[RefSeq: NM_000572.2]	Protein coding	Hs00961622_m1	74	1
IL-27	Interleukin 27	[RefSeq: NM_145659.3]	Protein coding	Hs00377366_m1	75	16
TNF-α	Tumor necrosis factor alpha	[RefSeq: NM_000594.3]	Protein coding	Hs01113624_g1	143	6
TGF-β	Transforming growth factor beta 1	[RefSeq: NM_000660.5]	Protein coding	Hs00998133_m1	57	19
IL-6	Interleukin 6	[RefSeq: NM_000600.4]	Protein coding	Hs00985639_m1	66	7
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	[RefSeq: NM002046.5]	Involved in glycolysis	Hs02758991_g1	93	12

TaqMan real-time PCR assays were chosen to span at least one exon-exon boundary and can be purchased with the given assay IDs (Applied Biosystems, Darmstadt, Germany). [a] Purchased from Applied Biosystems.

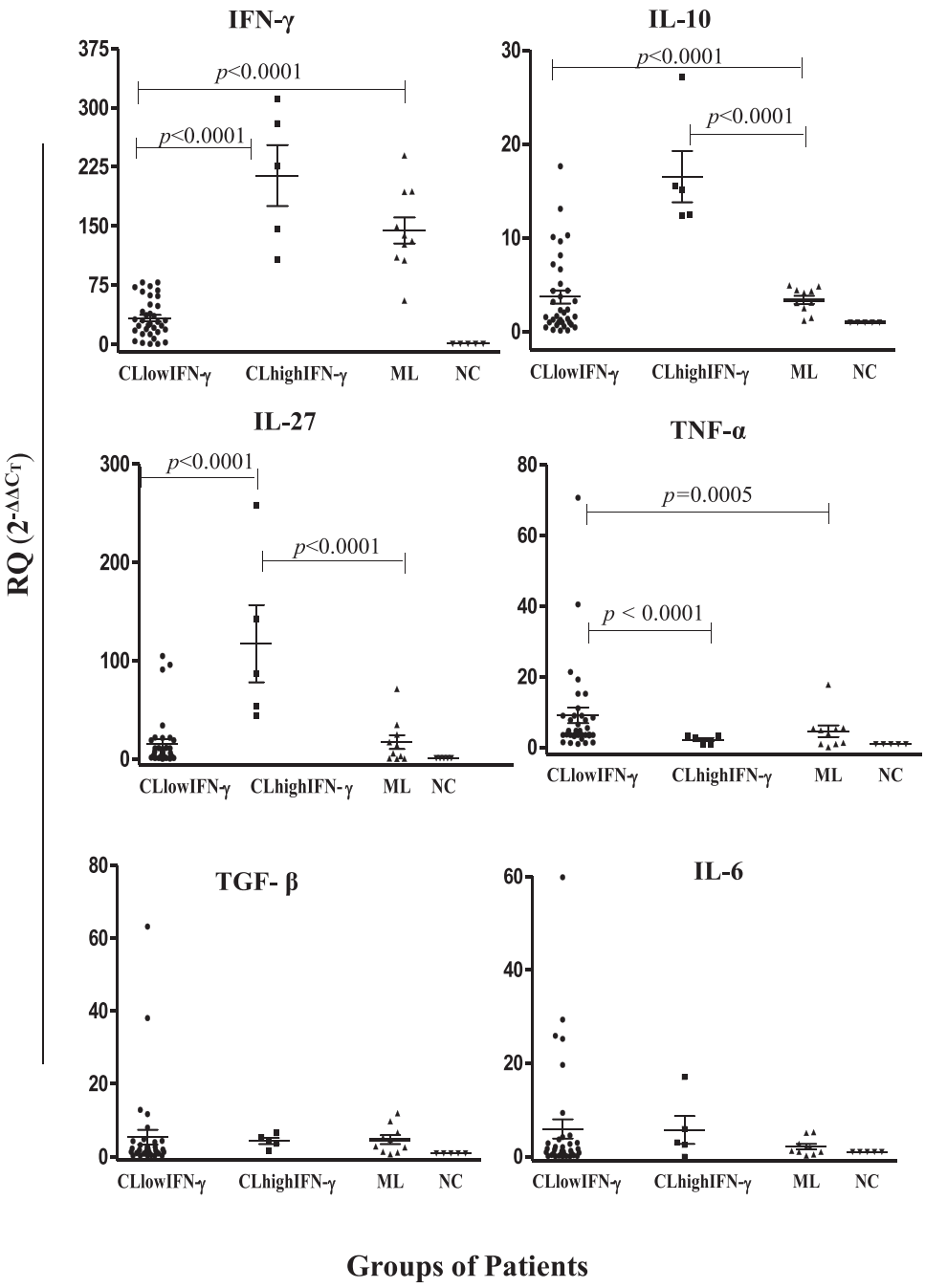

Fig. 1. mRNA relative expression of cytokines listed in each graphic of FFPE biopsies from 35 CL patients expressing low IFN-γ (CLlowIFN-γ – circles), 5 CL patients expressing high IFN-γ (CLhighIFN-γ - squares); 10 patients with clinical diagnosis of mucosal leishmaniasis (ML - triangles); and 14 negative controls (NC – inverted triangles). After RNA isolation and cDNA synthesis, qPCR determined gene expression of each cytokine. mRNA expression levels are shown as Relative Quantification (RQ), whose calculation was determined by "comparative C_T", ($2^{-\Delta\Delta CT}$), as described in Material and Methods section. The vertical lines indicate the means ± SEM. Comparison of expression between groups of patients with leishmaniasis was determined by unequal-variance *t*-test based on a critical value of $p \leqslant 0.05$ and F test to compare variances by the analysis of one-way variance. Significant differences between Groups are shown in horizontal lines.

Table 2
mRNA relative expression (in RQ) of IFN-γ, IL-10, IL-27, TNF-α, TGF-β of the 50 patients with leishmaniasis.

RQ	Patients with leishmaniasis CLlowIFN-γ (n = 35)	CLhighIFN-γ (n = 5)	ML (n = 10)
IFN-γ	33.34 ± 4.08	214.10 ± 38.65	144.50 ± 16.65
IL-10	3.71 ± 0.71	16.54 ± 2.72	3.41 ± 0.43
IL-27	15.81 ± 4.49	117.30 ± 39.24	17.29 ± 7.05
TNF-α	9.07 ± 2.22	2.21 ± 0.50	4.59 ± 1.61
TGF-β	5.38 ± 2.04	4.33 ± 0.83	4.72 ± 1.20
IL-6	5.92 ± 2.07	5.69 ± 2.98	2.11 ± 0.56

RQ (Relative Quantification) were calculated by the comparative C_T method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001).

(ML); and normal control group (NC). Table 2 shows RQ values of patient groups.

Considering IFN-γ, ML group had mRNA gene expression 144.50 times higher than NC group, followed by CLhighIFN-γ group, with 214.10 and CLlowIFN-γ group, with 33.34. The differences between CLlowIFN-γ group and the other groups (CLhighIFN-γ and ML) were statistically significant at $p < 0.0001$.

mRNA expression levels for IL-10 were 3.71, 16.54 and 3.41 times higher than NC group in CLlowIFN-γ, CLhighIFN-γ, and ML groups, respectively. Differences between results of CLlowIFN-γ and CLhighIFN-γ groups from ML group were statistically significant at $p < 0.0001$.

mRNA expression levels for IL-27 were 15.81, 117.30 and 17.29 times higher than NC group in CLlowIFN-γ, CLhighIFN-γ, and ML groups respectively. Differences between result of CLhighIFN-γ group and the other groups (CLlowIFN-γ and ML) were statistically significant at $p < 0.0001$.

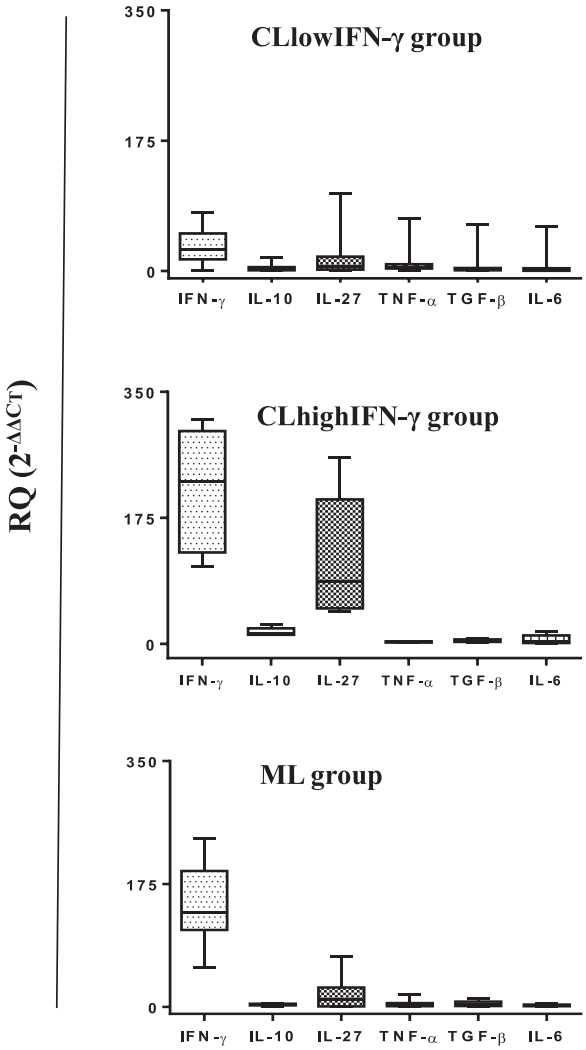

Fig. 2. Comparison of mRNA relative expression of IFN-γ, IL-10, IL-27, TNF-α, TGF-β, and IL-6 in FFPE biopsies from 35 CL patients (CLlowIFN-γ group); 5 CL patients expressing high IFN-γ (CLhighIFN-γ group); and 10 patients with clinical diagnosis of mucosal leishmaniasis (ML group). After RNA isolation and cDNA synthesis, qPCR determined gene expression of each cytokine. mRNA expression levels are shown as Relative Quantification (RQ), whose calculation was determined by "comparative CT", ($2^{-\Delta\Delta CT}$), as described in Material and Methods section. The vertical lines indicate the means ± SEM.

For TNF-α, mRNA expression levels were 9.07, 2.21, and 4.59 times higher than NC group in CLlowIFN-γ, CLhighIFN-γ, and ML groups, respectively. Differences between results of CLlowIFN-γ group from CLhighIFN-γ and ML groups were statistically significant at $p < 0.0001$ and $p = 0.0005$ respectively.

For TGF-β, mRNA expression levels were 5.38, 4.33 and 4.72 times higher than NC group in CLlowIFN-γ, CLhighIFN-γ, and ML groups respectively. For IL-6, mRNA expression levels were 5.92, 5.69 and 2.11 times higher than NC in CLlowIFN-γ, CLhighIFN-γ, and ML groups, respectively. No significant statistical difference was observed between groups, when mRNA expression levels for TGF-β and IL-6 were analyzed.

In the next step, the mRNA relative expressions of IFN-γ, IL-10, IL-27, TNF-α, TGF-β and IL-6 in FFPE biopsies were compared in each group of patients (Fig. 2). CLlowIFN-γ group had an equilibrium between the cytokines analyzed. On the other hand, in CLhighIFN-γ group, IL-27 was over-expressed. Finally, in ML group only IFN-γ was over-expressed.

4. Discussion

Leishmania survival depends on the escape of host immune system, that is highly adapted to these effects, either by humoral or cellular systems (Kemp, 1997; Janeway, 2001; Sacks and Sher, 2002; Zambrano-Villa et al., 2002). In humans, these interactions cause a large spectrum that may range from subclinical infection to manifestations such as localized, disseminated, diffuse skin lesions, or nasopharyngeal mucosa. In all these forms, the expansion of helper T cells exerts a fundamental influence and the difference between resistance and susceptibility to infection is related to the level of Th1/Th2 cell expansion (Bacellar et al., 2002; Gollob et al., 2008). For these reasons, the evaluation the immunological mechanisms and ACL development were investigated studding the genic expression in FFPE biopsies collected from patients with clinical and laboratorial diagnosis of ACL. In order to determine whether some these patients could develop severe forms of infection, a group of patients with ML were included in study for comparison.

Although the ability to trigger Th1 response with production of IFN-γ and TNF-α is associated with the control and cure of infection (Mougneau et al., 2011; França-Costa et al., 2015), it is knowing, that during L. *(V.) braziliensis* infection, parasites in lesions can cause an exacerbated Th1 response, related with the disease pathology, principally, in ML patients (Lessa et al., 2001; Pompeu et al., 2001; Gaze et al., 2006; Carvalho et al., 2007; Novais et al., 2013; Carneiro et al., 2016; Gomes et al., 2017). In addition, previous studies have been documented that there is a direct correction between the frequency of T CD4 + cells expressing IFN-γ and the size of the lesion (Antonelli et al., 2005; Novais et al., 2013; Vieira et al., 2013; Carneiro et al., 2016). These studies lead us to divide the 40 CL patients into two groups. The 35 patients (87.5%) of CLlowIFN-γ group had low IFN-γ with RQ below 100. On the other hand, the 5 patients (12.5%) of CLhighIFN-γ group had high IFN-γ, with RQ up to 100. These data are in agreement with other regions that occur per year around 500 cases of ACL and 10–20 cases of ML (Miranda et al., 2007). Interestingly, CLhighIFN-γ group had similar IFN-γ results of patients with mucosal leishmaniasis (ML group). These data can suggest that CLhighIFN-γ patients, if not quickly treated could develop more dangerous forms of cutaneous leishmaniasis (Pompeu et al., 2001; Oliveira et al., 2014; Cardoso et al., 2015; Carneiro et al., 2016).

The prevention of the immunopathology can also to be correlated with an immunosuppressive response with production of anti-inflammatory cytokines as IL-10 and TGF-β (Bacellar et al., 2002; Oliveira et al., 2014). IL-10 plays a key role in the pathogenesis of human leishmaniasis. In initial phase of ACL caused by L. *(V.) braziliensis*, IL-10 contributes for parasite persistence and may limit the tissue damage caused by the inflammatory process (Salhi et al., 2008; Assis Souza et al., 2013; Rodrigues et al., 2014; Gomes et al., 2017). Patients from CLhighIFN-γ group developed high mRNA expression for IL-10 when compared with those from the other groups (CLlowIFN-γ and ML). While L. *(V.) braziliensis* infection progresses, IFN-γ levels increase and predominate over IL-10 levels (Gomes et al., 2017), which corroborates our findings. Although such levels of IL-10 mRNA may help limit immune-mediated changes, the immunosuppressive activities of interleukin may promote low macrophage activation, collaborating with the replication of the parasite, and thus culminating in the progression of the disease (Kane and Mosser, 2001). After the progression of the disease, mRNA expression for IL-10 can be controlled as showed in ML group. In ML normally occurs an exaggerated Th1 immune response and an enhanced Th2 immune response likely due to the low production of IL-10 and the decreased ability of IL-10 in down modulate the Th1 and Th2 immune response. IL-10 values from ML group are in agreement with a previous study (Gonzalez-Lombana et al., 2013).

Patients from CLhighIFN-γ group, over-expressed IL-27 when compared to other patient groups. Although the same was not observed in ML group, these data suggest that IL-27 could contribute for

propagation of parasites and possible evolution for the mucosal form since the anti-inflammatory activity of this cytokine exerts a local and systemic effect on the growth and propagation of parasites. IL-27 has a vast inhibitory action on Th1 cells (Pflanz et al., 2002; Takeda et al., 2003; Yoshimura et al., 2006; Findlay et al., 2010).

Patients from CLlowIFN-γ group expressed more TNF-α than the CLhighIFN-γ and ML groups. The results of CLlowIFN-γ group suggested a balance between IFN-γ, TNF-α and IL-10 and appear to be important for controlling the infection and inflammatory process, since the presence of TNF-α and IFN-γ in equilibrium can be correlated with the control of parasites as previously described in other studies (Follador et al., 2002; Díaz et al., 2006; Lanier, 2008; Bogdan, 2012; Carneiro et al., 2016; Gomes et al., 2017). However, the same equilibrium between these cytokines were not shown in CLhighIFN-γ and ML groups that produced high IFN-γ expression and low TNF-α expression (Ulloa and Tracey, 2005). These findings are expressed in Fig. 2, which shows the comparison of cytokine expression per group. In ML group, IFN-γ was over-expressed; but in CLhighIFN-γ group, besides IFN-γ, IL-27 was also over-expressed.

All these data together suggested that patients from CLlowIFN-γ group developed an immunological balance between inflammatory-anti-inflammatory agents. Patients from CLhighIFN-γ group developed a strong immune response with production of high levels of IFN-γ and IL-27. This scenario could promote inflammation with tissue destruction and formation of severe lesions, common in severe cutaneous or mucosal forms. However, all patients were treated in order to prevent the evolution of severe forms.

The immune response to *Leishmania* induces some markers that are associated with the ACL evolution. Such markers can be determined by the analysis of gene expression of cytokines produced in biopsies. Although the results of mRNA expression, sometimes are not totally associated with protein synthesis ones, can contribute to understanding the pathology and the evolution of ACL.

Author contributions

VL Pereira-Chioccola, DDC Hippólito and CS Meira-Strejevitch designed the study and experiments; performed the data analysis, interpreted data, and wrote the manuscript. MM Maia, DDC Hipólito and CT Kanamura performed the laboratorial experiments (RNA purification, qPCR, IHC, histopathology and data analysis). AHS Gomes and JAL Lindoso performed the inclusion of patients, sample collection; developed the clinical evaluation.

All authors revised the manuscript, approved the final version submitted, published and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Conflict of interest statement

The authors have no other relevant affiliations or financial involvement with any organization or entire with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript or financial relationships that could be construed as a potential conflict of interest.

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