Evaluation of IL-12RB1, IL-12B, CXCR-3 and IL-17a expression in cases affected by a non-healing form of cutaneous leishmaniasis: an observational study design


ABSTRACT

Introduction: Seldom cutaneous leishmaniasis (CL) may present as a lasting and active lesion(s), known as a non-healing form of CL (NHCL). Non-functional type 1 T helper (Th1) cells are assumed the most important factor in the outcome of the disease. The present study aims to assess some molecular defects that potentially contribute to Th1 impairment in NHCL.

Methods and analysis: This prospective observational study will be implemented among five groups. The first and second groups comprise patients afflicted with non-healing and healing forms of CL, respectively. The third group consists of those recovered participants who have scars as a result of CL. Those participants who have never lived or travelled to endemic areas of leishmaniasis will comprise the fourth group. The fifth group comprises participants living in hyperendemic areas for leishmaniasis, although none of them have been afflicted by CL. The aim is to recruit 10 NHCL cases and 30 participants in each of the other groups. A leishmanin skin test (LST) will be performed to assess in vivo immunity against the Leishmania infection. The cytokine profile (interleukin (IL)-12p70, interferon (IFN)-γ, C-X-C motif chemokine ligand (CXCL)-11 and IL-17a) of the isolated peripheral blood mononuclear cells (PBMCs) will be evaluated through ELISA. Real-time PCR will determine the C-X-C motif chemokine receptor (CXCR)-3 and IL-17a gene expression and expression of IL-12RB1 will be assessed by flow cytometry. Furthermore, IL-12B and IL-12RB1 mutation analysis will be performed.

Discussion: It is anticipated that the outcome of the current study will identify IL-12B and IL-12RB1 mutations, which lead to persistent lesions of CL. Furthermore, our expected results will reveal an association between NHCL and pro-inflammatory cytokines (IL-12p70, IFN-γ IL-17a and CXCL-11), as well as CXCR-3 expression.

Strengths and limitations of this study

- This is a prospective study that has not yet been carried out; it aims to assess the role of interleukin (IL)-12RB1 and IL-12B mutation in a non-healing form of cutaneous leishmaniasis (NHCL).
- For the first time, this study design aims to assess C-X-C motif chemokine ligand (CXCL)-11 and IL-17a cytokines in patients afflicted by NHCL and the C-X-C motif chemokine receptor (CXCR)-3 gene expression.
- NHCL cases are rare; hence, we faced limitations in the number of cases affected by non-healing lesions of cutaneous leishmaniasis (CL).

INTRODUCTION

Leishmaniasis is considered to be a neglected tropical as well as an emerging and uncontrolled disease, which demonstrates highly variable clinical signs and symptoms. This disease is causing significant morbidity and mortality among 380 million susceptible individuals that are at risk in 98 countries. According to the WHO, the global incidence of visceral and cutaneous leishmaniasis (CL) varies from 200 000 to 400 000 and 700 000 to 1 200 000 patients, respectively. CL mostly appears as singular or multiple lesions, which spontaneously heal within 2–10 months. However, CL seldom manifests as long-lasting and active Leishmania lesions for more than a few years.

Much data has demonstrated that innate and acquired immunity is clearly involved in the outcome of the disease. According to the previously published articles regarding animal modelling, the clinical manifestation of the Leishmania infection is based on type 1
T helper (Th1) and type 2 T helper (Th2) dichotomies. In fact, the induction and maintenance of a Th1 immune response are indispensable for Leishmania clearance and lesion healing. Hence, primary immunodeficiency deficiencies (PIDs), which impede interferon (IFN)-γ production, probably predispose patients to intracellular pathogens such as Leishmania infection. Mutations in the interleukin (IL)-12Rβ1 and IL-12B genes lead to IL-12 receptor β1 (IL-12β1) and IL-12p40 deficiencies, respectively. All of these deficiencies impair IFN-γ production, which potentially increases one’s susceptibility to refractory Leishmaniasis. For example, Sanal et al. reported a 5-year-old patient, whose natural killer and T cells did not express IL-12Rβ1. This patient suffered from a refractory form of Kala Azar disease, which lasted about 6 months.

IL-12p40 and IL-12β1 (CD-212) deficiencies may also lead to fewer Th17 cells. The Th17 lineage of T helper cells potentially provides protection against pathogenic intracellular organisms such as the Leishmania species. This explains why IL-17R knockout mice show an increased susceptibility to systemic Leishmania infections. However, IL-17A knockout mice demonstrated attenuated Leishmania lesions. Hence, the role of IL-17 during a Leishmania infection is poorly understood.

The Th1 subset of CD4 cells, as well as CD8 T cells, expresses C-X-C motif chemokine receptor 3 (CXCR-3) molecules, which recruit these cells in inflammatory lesions. Expression of the CXCR-3 gene is clearly regulated through T-bet, which is the major transcription factor of Th1 subset. Furthermore, it has been shown that CXCR-3 expression gives protection from intracellular parasites such as different Leishmania species. For example, BALB/c mice, which are genetically susceptible to Leishmania infection, cannot upregulate the CXCR-3 gene expression in response to Leishmania infections. Nevertheless, CXCL-11 chemokine, which is assumed as a major ligand of the CXCR-3 gene, can induce Th2-driven responses. Therefore, the precise roles of CXCR-3 and its major ligand in the Leishmania infection remain ambiguous.

Based on the previous studies, consanguinity can be assumed as a risk factor, contributing to the high prevalence of primary immunodeficiency diseases (PIDs). Furthermore, high levels of consanguinity, as well as PIDs, are documented in Iran. In addition, some reports document the existence of chronic or non-healing leishmaniasis in Middle East countries. Hence, assessment of IL-12Rβ1 and IL-12p40 deficiencies as well as CXCL-11 and IL-17A could describe new features of PIDs, which present as non-healing form of CL (NHCL). To the best of our knowledge, none of the researches implemented throughout the world have aimed to evaluate the possible physiopathological role of PIDs, which constitute NHCL.

### Methods and analysis

This is a prospective observational study, which will evaluate in vitro and in vivo immune responses against the Leishmania infection. The study will be undertaken from July 2016 to September 2017 in the Acquired Immunodeficiency Research Center, Isfahan University of Medical Sciences (IUMS), Isfahan, Iran.

#### Study groups

This study comprises five different groups (table 1). The first group includes patients affected by healing lesions of CL. The second group includes patients affected by NHCL. Leishmania major causes NHCL when its cutaneous lesion lasts for >1 year. CL induced by L. tropica is considered as a non-healing form when its active lesion lasts for >2 years. NHCL generally does not respond to three courses of chemotherapy based on antimonials. In comparison, healing lesions of CL caused by L. major will heal with or without medical treatment in <1 year. In the case of L. tropica, healing lesions will be maximally cured in 2 years (with or without medical intervention). The third group includes individuals who have recovered from CL and have scars of leishmaniasis. CL in

<table>
<thead>
<tr>
<th>Group number</th>
<th>Active lesion*</th>
<th>Duration of lesion†</th>
<th>Scar of lesion‡</th>
<th>History of living or travel§</th>
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</thead>
<tbody>
<tr>
<td>1</td>
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<td>&lt;2 years in case of L tropica</td>
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<td>2</td>
<td>+</td>
<td>&gt;1 year in case of L major</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;2 years in case of L tropica</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>≤1 year in case of L major</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt; years in case of L tropica</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>–</td>
<td>NA</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

*Active lesion induced by cutaneous leishmaniasis.†Duration of active lesion induced by cutaneous leishmaniasis.‡Scar of cutaneous leishmaniasis.§History of living or travel to endemic areas of leishmaniasis. NA, not applicable.
these patients was previously confirmed through parasitological methods. The fourth group is comprised of healthy individuals who have not lived or travelled to endemic areas for leishmaniasis. The fifth group comprises individuals who have lived a minimum of 5 years in hyperendemic areas for CL. None of the participants in the last group have a clinical history of CL (table 1). Inclusion criteria are males and females who are: (1) 5–60 years of age, (2) without HIV infection and (3) without addiction. Exclusion criteria are: (1) pregnancy, (2) steroid intake, (3) diabetes, (4) hypersensitivity, (5) hypertension and (6) lactation.

Sample size
All available patients affected by NHCL (ie, n=10) will be recruited; however, for statistical power analysis’s purposes, three controls in each of the four remaining groups will be considered, resulting in a 1:3 prospective follow-up study. Accordingly, 10 patients afflicted with NHCL and 30 participants in each of the four control groups will be investigated.

Leishmania diagnosis
Direct smear and parasite culture will be applied to verify suspected leishmaniasis in patients. For this, the active lesions of the suspected patients will be cleaned and disinfected by the use of povidone-iodine and ethanol 70%. Afterwards, cutaneous scraping will be made from the lesion base by a sterile surgical blade (no. 10). Serosa and skin tissue obtained through scraping will be applicable for direct smear and Leishmania culture.40

Direct smear
For a microscopic diagnosis of Leishmania infection, two thin smears will be prepared for every patient. Smear fixation through absolute methanol (for 30 s) will be preceded by drying in air. Afterwards, the smear will be stained with Giemsa (10%) for 30 min. The smears will be assumed to be positive if amastigote forms of Leishmania species can be detected in macrophages, or out of the cells through a light microscope (magnification 1000×).40

Leishmania culture
All of the specimens, which include negative and positive smears, will be evaluated by the culture method. For this aim, the extracted tissue and serosa will be transferred to tubes containing biphasic Nowy-Nicol-Mac Neal (NNN) medium (6 g/L NaCl, 14 g/L plain non-nutrient agar, 150cc/L of rabbit defibrinated blood and up to 1200 mL H2O) in sterile conditions.40 The inoculated medium will be incubated at 26±1°C and its contents will be checked for promastigote forms of Leishmania species every 3 days. To enhance parasite proliferation, the cultivated parasites will be isolated and transferred to Roswell Park Memorial Institute 1640 medium (RPMI 1640) plus 20 U penicillin (per mL), 20 μg of streptomycin (per mL) and 10% fetal calf serum (FCS). The parasites will be incubated at 26±1°C for 4 weeks, while the liquid medium will be replaced every 3 days. The parasite proliferation will be assessed through an inverted light microscope (magnification 40×).41

Leishmania species identification
We will spin 10 mL of medium, which contains proliferated promastigotes, at 400 g for 5 min. Afterwards, we will resuspend and wash the pellets in 1 mL sterile phosphate buffer solution (PBS (Cytomatingen Co, Isfahan, Iran)) three times.42

Nucleic acid extraction
Leishmania pellets will be resuspended in 10 mL lysis buffer: 0.15 M sodium chloride, 0.1 EDTA, 100 μg/mL proteinase K and 0.5% sodium dodecyl sulfate (SDS). This suspension will be incubated at 50±1°C for half an hour. The extraction will proceed further by pellet washing and resuspension: twice in phenol (300 μL every time) at 60°C, once in phenol-chloroform-isoamyl alcohol (25:24:1) and once in chloroform-isoamyl alcohol (24:1). Extracted nucleic acid will be washed and collected by diethyle ether and ethanol, respectively. In all of the steps, the pellets will be formed through centrifugation at 400 g for 15 min.42

Identification of Leishmania species through internal transcribed spacer-ribosomal DNA (ITS-rDNA)
ITS-1, ITS-2 and 5.8s tracts, which form some parts of the ribosomal DNA (rDNA), could be efficiently exploited for the identification of the Leishmania species. For DNA amplification, forward (F:5’-CAACA CGCCGCCTCTCTCTCT-3’) and reverse (R:5’-CCTCTCTCT TTTTTCNCTGTGC-3’) primers will be applied.42 Standard PCR will be implemented through a Qiagen PCR kit, in 50 μL total volume for every sample, including 1× Q solution, 2.5 mM MgCl2, 1× standard PCR buffer, dNTPs (200 μM), 0.5 μM of each primer 1 U HotStarTag Plus and 20–40 ng of genomic DNA. This concentration will be evaluated at 260 nm through spectrophotometry (Bio Photometer, Eppendorf, Germany). PCR amplification conditions will be: denaturation at 95°C for 5 min proceeded by 31 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 45 s, extension at 72°C for 60 s and a final extension step at 72°C for 5 min. Positive controls will include nucleic acid extracted from standard strains of L major (MRHO/IR/75/ER) and L tropica (MHOM/IR/2002/Mash2). Distilled DNA-free water will be applied instead of extracted nucleic acid as a negative control. The amplicon sizes will be analysed on 1.5% agarose gel and visualised through ethidium bromide. Leishmania species will be discriminated, since the amplicon sizes differ between L major and L tropica (626 base pair [bp] and 800 bp, respectively).42

Preparation of soluble Leishmania antigen

A standard strain of \textit{L major} (MRHO/IR/75/ER) will be applied for soluble \textit{Leishmania} antigen (SLA) preparation. Promastigot forms of this strain will be harvested up to stationary phase in RPMI 1640 (Gibco RL, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS, Gibco, UK), 2 mM glutamine (Invitrogen), 20 U of penicillin (Gibco, UK) per mL and 20 mg of streptomycin (Gibco, UK) per mL. For this aim, the parasite culture will be implemented in 26±1°C for five days. The harvested parasites will be washed four times through centrifugation (300 g for 30 min) and pellet resuspension in sterile PBS at pH 7.2. The parasite concentration will be adjusted to 10^6 parasites per mL by 100 mM Tris-HCl, 1 mM EDTA (pH 8), supplemented with 50 μg of Leupeptin per mL and 1.6 mM phenyl methyl sulfonyl fluoride (all from Sigma, St. Louis, MO, USA). To prevent denaturation of protein, a protease inhibitor cocktail (Sigma) will be added to the above-mentioned parasite suspension (50 μL in 1 mL). Afterwards, the parasite bodies will be subjected to seven cycles of freezing and thawing at −70°C and 35°C, respectively. The procedure will be followed by sonication (10 times, each time 20 s in power 60% at 4°C). Parasite suspension will be centrifuged at 30000×g for 20 min at 4°C and the supernatant will be extracted. Then, the collected supernatant will be centrifuged again at 100000×g for 4 hours at 4°C. Importantly, the supernatant collection should exclude parasite lipid, which appears as a milky-colored layer on the top of the centrifuged supernatant. A dialysis pack (3500 dalton cut-off) will be included against the above-mentioned protease inhibitor cocktail. The prepared antigen will be sterilised through a 0.22 μm membrane filter.43

Leishmanin skin test

The leishmanin skin test (LST), or Montenegro skin test, is an in vivo assay, which demonstrates partial immunity against the \textit{Leishmania} infection. All of the study participants will receive a suspension of killed promastigotes. This suspension is prepared in the Pasteur Institute, Tehran, Iran. Every phial of the suspension includes PBS (1 cc), thiomersal (0.1%), and killed \textit{L major} promastigotes (6×10^6 bodies of standard strain (MRHO/IR/75/ER)). This suspension is kept in a refrigerator at 6°C up to the time of the application. For the administration, 0.1 mL of leishmanin suspension will be intradermally injected through a 27-gauge needle, in the palmar surface of the left forearm in every individual. Additionally, a placebo suspension, including PBS (1 cc) and thiomersal (0.1%), will be injected into the dorsal surface of the left arm. This placebo will also be produced by the same institute. Induration of delayed type hypersensitisation (DTH) resulting from the LST will be measured after 48 hours through a ballpoint pen. However, the induration measurement shall be repeated after 72 hours if there is no reaction. The outcome of the LST is negative unless the induration diameter is >5 mm. The positive results may also be classified as either normal positive or strongly positive, according to the diameter of induration (5–14 mm and ≥15 mm, respectively).44 45

Isolation of peripheral blood mononuclear cells

In order to isolate peripheral blood mononuclear cells (PBMCs), heparinised human peripheral blood (10 mL) will be collected from every individual in one of the above-mentioned study groups. The collected blood will be diluted with an equal volume of sterile PBS (Cytomatin Gene Co, Isfahan, Iran) adjusted to pH 7.4 and 260 m Osmo. This diluted blood will be gently layered on top of Ficoll-Hypaque (Lymphodex, Inno-Train, Germany) within sterile and Dnase/Rnase-free conical tubes in an equal volume. These tubes will be centrifuged at 800×g for 15 min at room temperature. After the centrifugation, the isolated PBMCs located in the second layer (out of the three formed layers) will be retrieved through a sterile Pasteur pipette. The collected layer will be washed twice in a sterile PBS (10 mL) through centrifugation (at 450×g for 10 min) and pellet resuspension. Washed PBMCs will be resuspended in RPMI 1640 (Gibco). To assess cell viability, the PBMCs suspension (10 μL) will be dyed by trypan blue (10 μL) hemocytometer-based technique.38 46

Cell culture

Isolated PBMCs will be cultured at 1×10^6/mL/well in a 12-well plate. The culture medium will include RPMI 1640 supplemented with 10% FBS (Gibco, UK), 2 mM glutamine (Invitrogen), 20 U of penicillin (Gibco, UK) per mL and 20 mg of streptomycin (Gibco, UK). The cells will be stimulated with purified protein derivative (PPD), SLA, phytohemagglutinin (PHA) and recombinant IL-12 (rIL-12) in four treatments, as follows: (1) the first treatment will consist of SLA antigen (5 μg/mL); (2) the second treatment will consist of SLA (5 μg/mL) + rIL-12(10 μg/mL); (3) the third treatment will be stimulated through PHA (10 mg/mL); (4) the final treatment (control negative) will contain 12 μg of PPD (Razi Institute, Karaj, Iran), as an irrelevant antigen (table 2).5 All of the plates will be incubated for 72 hours at 37°C under 5% CO_2.38 46

Cytokine measurement

After the plate incubation, the cell culture supernatant will be collected by centrifugation at 400 g for

Table 2 Stimulators of peripheral blood mononuclear cells (PBMCs) cultured in four different wells

<table>
<thead>
<tr>
<th>Well number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulator(s)</td>
<td>SLA</td>
<td>SLA+rIL-12</td>
<td>PHA</td>
<td>PPD</td>
</tr>
<tr>
<td>PPD, purified protein derivative; PHA, phytohemagglutinin; rIL, recombinant interleukin; SLA, soluble Leishmania antigen.</td>
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<td></td>
</tr>
</tbody>
</table>
Flow cytometry of IL-12Rβ1 (CD212)

To assess whether there is an association between IL-12Rβ1 expression and pro-inflammatory cytokines (IL-12, IFN-γ, IL-17a and CXCL-11), a flow cytometry analysis of IL-12Rβ1 will be performed. IL-12Rβ1 has a low expression on unstimulated T cells and is highly expressed on activated T cells. Hence, we aim to restimulate PHA-treated cells (as described in the Cell culture section) with recombinant IL-2 (r-IL2).

Restimulation of PHA-treated cells

PHA-treated cells will be pelleted and resuspended in a density of 2 x 10^5 cells/mL. PHA, PPD and PHA (10 mg/mL) will be added to the cells to stimulate the cells. After 72 hours, the cells will be harvested and washed with PBS. Afterwards, these cells will be incubated with an anti-human IgG1 monoclonal antibody (BD Biosciences, USA, cat. no # 556065) or its appropriate isotype antibody (BD Biosciences, USA, cat. no # 555749) on ice for 20 min (all of the antibodies are fluorolabeled with phycoerythrin (PE)). The antibody-incubated cells will be washed twice with a blocking solution (2% FBS in PBS). Finally, cell analysis will be performed through a FACScan machine, using CellQuest software (Becton Dickinson San Jose, CA).

Cell preparation for flow cytometry and flow cytometry analysis

PHA and rIL-2-treated cells will be pelleted and washed with PBS. Afterwards, these cells will be incubated with either an IL-12Rβ1 specific mouse antihuman IgG1 monoclonal antibody (BD Biosciences, USA, cat. no # 556065) or its appropriate isotype antibody (BD Biosciences, USA, cat. no # 555749) on ice for 20 min (all of the antibodies are fluorolabeled with phycoerythrin (PE)). The antibody-incubated cells will be washed twice with a blocking solution (2% FBS in PBS). Finally, cell analysis will be performed through a FACScan machine, using CellQuest software (Becton Dickinson San Jose, CA).

Genetic analysis

RNA extraction

As described, RNA will be extracted from cell cultures using the standard protocol. The supernatant will be analysed for cytokine measurements by an indirect form of ELISA using four commercial kits: (1) IFN-γ ELISA kit eBioscience (Ready-SET-Go! , USA catalog number # 88-731622); (2) IL-12 p70 ELISA kit eBioscience (Ready-SET-Go!, USA, catalog number # 88-7126-22); (3) CXCL-11 (San Diego, CA, CXCL-11 catalog number # 443307) and (4) IL-17a ELISA kits Biolegend (San Diego, CA, catalog number # 439197). Briefly, for each of the cytokines, a standard curve will be established using a twofold serial dilution of recombinant corresponding proteins (r IFN-γ, rIL-12, rCXCL-11 and rIL-17a). Additionally, an appropriate dilution factor of every sample will be determined if the optical density falls outside of the corresponding standard curve. All of the data will be calculated through computer-based curve-fitting software when the mean absorbance for each set of duplicate standards, controls and samples is determined.
efficiencies of all genes are roughly identical at or >90%.\textsuperscript{51, 52}

**IL-12B and IL-12RB1 exon sequencing**

Six coding exons of the IL-12B gene of non-healing patients will be sequenced in the following conditions: (1) lack of IFN-\(\gamma\) production in the SLA-stimulated well (first well); (2) retrieval of IFN-\(\gamma\) production in the presence of SLA and rIL-12 (second well). On the other hand, 16 coding exons of IL-12RB1 will be sequenced, providing IFN-\(\gamma\) will not be appropriately produced in SLA-stimulated and IL-12-added wells (first and second well). For this reason, PCR amplification will be implemented in two different conditions, as follows:

**IL-12B1 PCR amplification**

PCR amplification will be performed in a reaction volume (25 \(\mu\)L), which consists of 1.25 U of Taq DNA polymerase (Fermentase), 0.4 mm dNTPs, 2.5 \(\mu\)L of 10X reaction buffer, 2.5 mM MgCl\(_2\), 20 ng of cDNA and 10 \(\mu\)L of appropriate primers (table 4).\textsuperscript{63} This amplification protocol consists of an initial denaturation at 95°C for 5 min; 36 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 45 s and the final extension at 72°C for 7 min.\textsuperscript{53}

**Exon amplification of IL-12RB1 gene**

20 ng of cDNA will be amplified in a 25 \(\mu\)L of reaction mixture: 2.5 \(\mu\)L of 10X reaction buffer, 1 \(\mu\)L of 2 mM MgCl\(_2\), 0.5 \(\mu\)L of 25 mM dNTP, 0.5 \(\mu\)L of 10 mM appropriate primer (table 5), 1.25 U of Taq DNA polymerase (Fermentase) and 2.5 \(\mu\)L of 50% dimethyl sulfoxide (DMSO) solution.\textsuperscript{64} The PCR condition includes an initial denaturation at 95°C for 5 min; 38 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 45 s and the final extension at 72°C for 7 min. The amplicon quality will be checked by way of electrophoresis on 1% agarose gel. Afterwards, bidirectional sequencing will be implemented on an ABI 3130 automated sequencer (XL genetic analyser) through the BigDye terminator V3.1.\textsuperscript{46}

**Statistical analysis**

All data will be analysed using SPSS V20 (SPSS, Chicago, USA). Categorical variables will be reported as frequency and percentage, while continuous variables will be described as mean and SD or median (IQR), as appropriate. Repeated measures analysis of variance (ANOVA), with five between group factors and three within group measures, will be applied to compare different cytokine levels between and within groups, respectively. Participant classification in low IFN-\(\gamma\) or high IFN-\(\gamma\) categories will be based on a receiver operating characteristics curve (ROC) analysis. Additionally, sensitivity and specificity of various levels of IFN-\(\gamma\) for determining the best cut-off point will be evaluated. The IL-12R\(\beta\)1 expression will be compared through \(\chi^2\) or Fisher exact test.

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### Table 3 Sequences of oligonucleotide primers for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence(forward and reverse (5'-3'))</th>
<th>Length Primer</th>
<th>Product</th>
<th>Tm</th>
<th>CG%</th>
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<td>TCAGCTCTTTTGGGTTGT</td>
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<tr>
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<td></td>
<td>GCCCTCCGAGTACAGA</td>
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<td>TCTTAGAGCAGGTCAAGGT</td>
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<td>34</td>
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</table>

CXCR, C-X-C motif chemokine receptor; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; IL, interleukin; Tm, melting temperature.

### Table 4 Sequences of oligonucleotide primers for PCR amplification of IL-12B coding exons

<table>
<thead>
<tr>
<th>Covering exons</th>
<th>Primer sequence(forward and reverse (5'-3'))</th>
<th>Length Primer</th>
<th>Product</th>
<th>Tm</th>
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<td>22</td>
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IL, interleukin; Tm, melting temperature.
will analyse IL-12Rβ1 and analysed by IL-12B sequencing. Moreover, there will be a probability of IL-12Rβ1 mutations which lead to persistent lesions of CL. Indeed, NHCL cases may be afflicted by IL-12B mutation(s) if the PBMCs of these patients can retrieve IFN-γ production in SLA+rIL-12 wells (table 2). However, IL-12B mutation(s) will be further assessed and analysed by IL-12B sequencing. Moreover, there will be a probability of IL-12Rβ1 deficiency unless IFN-γ will be sufficiently produced in the presence of rIL-12. We will analyse IL-12Rβ1 deficiency by flow cytometry, and we will also assess IL-12RB1 mutation(s) through sequencing. It is anticipated that the outcome of the current study could identify IL-12B and IL-12RB1 mutations, which lead to persistent lesions of CL.

In addition, our expected results can pave the way for further immunotherapy of Leishmania lesions. Indeed, scientists can provide NHCL cases with appropriate immunotherapy when the pathophysiology of the persistent lesions is clearly described. For example, identification of NHCL cases afflicted by IL-12B or IL-12RB1 mutations suggest animal modelling, and even future clinical trials, aiming to apply rIL-12 or rIFN-γ for the treatment of the chronic lesions. Furthermore, our expected results will reveal an association between NHCL and pro-inflammatory cytokines (IFN-γ, IL-12, IL-17a, CXCL-11), as well as CXCR-3 expression.

DISCUSSION

Low amounts of IFN-γ production have been previously shown in some patients afflicted by non-healing leishmaniasis. On the other hand, some studies have determined genetic defects, which impede IFN-γ production, predisposing some patients to intracellular microorganisms. For example, IL-12B and IL-12RB1 mutations are considered as two major components, which induce increased susceptibility to non-tuberculous mycobacteria (NTM) species. Neither investigation, however, has evaluated genetic defects, which potentially lead to NHCL.

The current study could discriminate between IL-12B and IL-12RB1 mutations through dichotonic responses induced by rIL-12. Indeed, NHCL cases may be afflicted by IL-12B mutation(s) if the PBMCs of these patients can retrieve IFN-γ production in SLA+rIL-12 wells (table 2). However, IL-12B mutation(s) will be further assessed and analysed by IL-12B sequencing. Moreover, there will be a probability of IL-12Rβ1 deficiency unless IFN-γ will be sufficiently produced in the presence of rIL-12. We will analyse IL-12Rβ1 deficiency by flow cytometry, and we will also assess IL-12RB1 mutation(s) through sequencing. It is anticipated that the outcome of the current study could identify IL-12B and IL-12RB1 mutations, which lead to persistent lesions of CL.

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**DISSEMINATION**

Information regarding the nature of the research project will be clearly described for all of the participants in written and verbal format. Afterwards, informed consent will be obtained from every participant, or their guardians, by trained medical staff. The informed consent will assure the patients that they can leave the study at their convenience.

Biological samples will be identified through a code number and the medical data of participants will be analysed anonymously. Paper-based data of the participants will be kept in a locked cabinet at the Acquired Immunodeficiency Research Center, and electronic information will be stored on a password-protected computer.

The final results will be disseminated through peer-reviewed journals and scientific conferences. All of the reports will be submitted to the project funder.

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**Data sharing statement** Final results will be disseminated through peer-reviewed journals and scientific conferences.

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