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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

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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-12Rβ1 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.

Ethics and dissemination: This study has been approved by a local ethical committee. The final results will be disseminated through peer-reviewed journals and scientific conferences.

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.[1–3] 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.[4–9]

Much data has demonstrated that innate and acquired immunity is clearly involved in the outcome of the disease.[10] 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.[11] 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.[12 13] Mutations in the interleukin (IL)-12RB1 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.[14 15] For example, Sanal *et al*[16] 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.[16]

IL-12p40 and IL-12Rβ1 (CD-212) deficiencies may also lead to fewer Th17 cells.[17] 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.[18 19] However, IL-17a knockout mice demonstrated attenuated *Leishmania* lesions.[20 21] 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.[22] 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.[23] Nevertheless, CXCL-11 chemokine, which is assumed as a major ligand of the CXCR-3 gene, can induce Th2-driven responses.[24] 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).[25] Furthermore, high levels of consanguinity, as well as PIDs, are documented in Iran.[26–29] In addition, some reports document the existence of chronic or non-healing leishmaniasis in Middle East countries.[30–38] 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).[37 38] The third group includes individuals who have recovered from CL and have scars of leishmaniasis. CL in

Table 1 Clinical characteristics of participants in different groups

Group number	Active lesion*	Duration of lesion†	Scar of lesion‡	History of living or travel§
1	+	≤1 year in case of *Leishmania major* ≤2 years in case of *L tropica*	–	±
2	+	>1 year in case of *L major* >2 years in case of *L tropica*	–	±
3	–	≤1 year in case of *L major* ≤ years in case of *L tropica*	+	±
4	–	NA	–	–
5	–	NA	–	+

*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.[39] 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 Novy-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 H_2O) 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 lysing 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 recentrifugation 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 CGCCGCCTCCTCTCT-3′) and reverse (R:5′-CCTCTCT 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 $MgCl_2$, 1× standard PCR buffer, dNTPs (200 mM), 0.5 µM of each primer 1 U HotStarTaq 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 *L major* (MRHO/IR/75/ER) will be applied for soluble *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 10^9 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 37°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 30 000×g for 20 min at 4°C and the supernatant will be extracted. Then, the collected supernatant will be centrifuged again at 100 000×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) filled with 10 mM 4–(2-hydroxyethyl)-1-piperazineëthanesulfonic acid (HEPES) and sucrose 10% (all from Sigma) will be applied to exclude the 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 *Leishmania* infection. All of the study participants will receive a suspension of killed promastigotes. This suspension is prepared in the Pastor Institute, Tehran, Iran. Every phial of the suspension includes PBS (1 cc), thiomersal (0.1%), and killed *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 U/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

Well number	1	2	3	4
Stimulator(s)	SLA	SLA+rIL-12	PHA	PPD

PPD, purified protein derivative; PHA, phytohemagglutinin; rIL, recombinant interleukin; SLA, soluble *Leishmania* antigen.

7 min.[38 46] 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# 88-7316-22); (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# 433917). 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.

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 was described in the Cell culture section) with recombinant IL-2(r-IL2).[46]

Restimulation of PHA-treated cells

PHA-treated cells will be pelleted and recultured in a density of 2×10^5 cells/mL for 48 hours at 37°C under an atmosphere containing 5% CO_2. These cells will be restimulated by human rIL-2 (50 IU/mL; R&D system) in Panserin 401 medium (Pan Biotech) with 10% FBS and 2 mM L-glutamine (Gibco).[46]

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).[46]

Genetic analysis

RNA extraction

RNA will be extracted from cells cultured in the presence of one of the above-mentioned antigens (SLA (5 µg/mL), PHA (10 mg/mL) or PPD (12 µg/mL)) using a total RNA extraction kit (Jena Bioscience, GmbH, Jena, Germany) according to the manufacturer's instructions. To avoid genomic contamination, the extracted RNA will be treated with RNA-free DNAse I (10 U/L; cat no. EN0525, Fermentas, St. Leon-Rot, Germany) at 22°C for 15 min. This enzyme will be inactivated through EDTA (2 µL of 7.5 mM) at 65°C for 10 min.[47]

cDNA synthesis

In the first step, the purity and concentration of the extracted RNA will be assessed through the absorbance at 260 nm (260A), 280 nm(280A) and 230 nm (230A) using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).[48] Highly purified 5 µg of mRNA (optical density (OD) ratios: 260/280=1.92.0 and 260/230=2.02.2) will be reverse transcribed using a Reverta-L kit (Central Institute of Epidemiology, Moscow, Russia) through oligo-dT.

Real-time PCR for CXCR-3 and IL-17a expression

Primer designing for real-time PCR All of the primers aiming for either target (CXCR-3 and IL-17a) or reference (glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)) gene amplification were designed according to GenBank sequence information (table 3).[49 50]

Real-time PCR protocol Quantification of gene expression will be performed through a comparative C_T method in a commercial real-time thermocycler (ABI Prism 7000) in accordance with the manufacturer's instructions. For this aim, 1 µL of our cDNA solution will be mixed with 19 µL of a master mix: 20 pMol of every primer, optimised concentration of $MgCl_2$ (ranging from 1–8 mM) and 2 µL of SYBR Green I master mix (Roche Burgess Hill, UK). Real-time PCR conditions will be set at: 95°C for 3 min (as an initial denaturation step), followed by 40 cycles of 95°C for 10 s. After the implementation of optimal primer annealing temperature ranging from 50 to 60°C for 30 s and primer extension at 72°C for 45 s, the florescent amplitude will be measured for a period of 1 s. Final extension step will be performed at 72°C for 5 min. Besides this, a melting curve, which potentially ranges from 60 to 99°C, will be acquired (heating rate: 0.1°C/s) to ensure uniformity of PCR products.[51]
Moreover, to detect potential genomic contamination, negative control, which will contain non-reverse transcribed RNA and GAPDH primers, will be included in each run (table 3).

Relative quantification of gene expression through real-time PCR To quantify the gene expressions, the amount of CXCR-3, IL-17a expressions will be normalised to GAPDH cDNA and normalised in accordance with unstimulated cells (calibrator). This quantification will be calculated by $2^{-\Delta\Delta CT}$ where $\Delta\Delta Ct=(CT_{target}-CT_{reference})_{calibrator}-(CT_{target}-CT_{reference})_{sample}$. This calculation is valid when the amplification

Table 3 Sequences of oligonucleotide primers for real-time PCR

Gene	Primer sequence(forward and reverse (5′-3′))	Length		Tm	CG%	Accession number
		Primer	Product			
CXCR-3	GGTGCCCTCTTCAACATCAAC	21	90	44.3	52.4	NC_000023
	GGTGGCATGAACTATGTTCAGGTA	24		46.5	45.8	
IL-17a1	CCCCTAGACTCAGGCTTCCT	20	135	42.7	60	NC_000006
	TCAGCTCCTTTCTGGGTTGT	20		41.9	50	
IL-17a2	GAAGGCAGGAATCACAATC	19	1461	36.8	47.4	NC_000006
	GCCTCCCAGATCACAGA	17		34		
GAPDH	ACCCAGAAGACTGTGGATGG	20	200	36.8	58.8	NC_000012
	TTCTAGACGGCAGGTCAGGT	20		34	47.4	

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

efficiencies of all genes are roughly identical at or >90%.[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-γ production in the SLA-simulated well (first well); (2) retrieval of IFN-γ 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-γ 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 µL), which consists of 1.25 U of Taq DNA polymerase (Fermentase), 0.4 mm dNTPs, 2.5 µL of 10X reaction buffer, 2.5 mM $MgCl_2$, 20 ng of cDNA and 10 µL of appropriate primers (table 4).[53] This amplification protocol consists of an initial denaturation at 95°C for 5 min; 36 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 150 s and the final extension at 72°C for 7 min.[53]

Exon amplification of IL-12RB1 gene 20 ng of cDNA will be amplified in a 25 µL of reaction mixture: 2.5 µL of 10X reaction buffer, 1 µL of 2 mM $MgCl_2$, 0.5 µL of 25 mM dNTP, 0.5 µL of 10 mM appropriate primer (table 5), 1.25 U of Taq DNA polymerase (Fermentase) and 2.5 µL of 50% dimethyl sulfoxide (DMSO) solution.[46] 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 V.3.1.[46]

Statistical analysis

All data will be analysed using SPSS V.20 (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-γ or high IFN-γ categories will be based on a receiver operating characteristics curve (ROC) analysis. Additionally, sensitivity and specificity of various levels of IFN-γ for determining the best cut-off point will be evaluated. The IL-12Rβ1 expression will be compared through χ^2 or Fisher exact test.

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

Covering exons	Primer sequence(forward and reverse (5′-3′))	Length		Tm	CG%	Accession number
		Primer	Product			
Exon 2,3,4	GACTCTCCGTCCTGCCCA	18	493	60.68	66.7	NC_000005
	GACACTGAATGTCAAATCAG	20		52.22	40	
Exon 5,6,7	TCTGGACGTTTCACCTGCTG	20	656	60.25	55	NC_000006
	GTCTATTCCGTTGTGTCTTT	20		53.28	40	
Exon 8	CATCTGTGCCCTGCAGTTAG	20	1328	58.62	55	NC_000006
	AAGAGTTTTTATTAGTTC	18		41.41	22	

IL, interleukin; Tm, melting temperature.

Table 5 Sequences of oligonucleotide primers for PCR amplification of IL-12RB1 coding exons

Covering exons	Primer sequence(forward and reverse (5′-3′))	Length		Tm	CG%	Accession number
		Primer	Product			
Exon:1,2,3,4,5,6,7,8	TCGCAGGTGGCAGAGAGG	18	845	61.39	66.67	XM_011527977
	GCTGGGTTGGCTGCTCTTT	19		60.91	57.89	
Exon:6,7,8,9,10,11	CGGACACCCAGCAGCCCA	18	801	64.71	72.22	XM_011527976
	CAGGACCGTAGACCACAAG	19		57.19	57.89	
Exon:10,11,12,13,14,15,16,17	CATTGAATGGCAGCCTGTG	19	903	57.27	52.63	XM_011527975
	GAGTCACTCACCCTCTCTG	19		56.18	57.89	

IL, interleukin; Tm, melting temperature.

DISCUSSION

Low amounts of IFN-γ production have been previously shown in some patients afflicted by non-healing leishmaniasis.[37 38 54] 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.[55] 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 dichotic 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.

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.

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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