Research article

The first documentation of the cytokine response to cutaneous leishmaniasis caused by *Leishmania donovani* in Sri Lanka

D Atapattu[1], D Iddawela[1], S Adikari[2], S Wickramasinghe[1],
L Bandara[1], S Samaraweera[3]

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Abstract

Introduction and Objectives: The predominant form of leishmaniasis seen in Sri Lanka is cutaneous leishmaniasis (CL) caused by *Leishmania donovani*, a species commonly causing visceral disease. The objective of this study was to explore the human host immune response to CL in Sri Lanka.

Methods: A descriptive comparative study was carried out on nine CL patients referred to the Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka, during 2011-2013. mRNA was extracted from the skin biopsy samples and reverse transcribed to cDNA and tested for Th1 and Th2 cytokines by using specific primers for IFN-γ, IL-2 (Th1 cytokines) and IL-4, IL-10 (Th2 cytokines). The results were compared with different characteristics of the lesion (number, duration, size and type of lesion).

Results: This study revealed that the majority of CL patients (5/9) elicited a Th1 response whereas a Th2 response was not detected in any of the patients. Of the Th1 cytokine positive patients, majority had single lesions, with a shorter duration and smaller sized lesions which were of the plaque type. Of those which exhibited a negative Th1 response, the majority were larger lesions with a longer duration and were of the papular-nodular type.

Conclusions: The study suggests that the predominant immune response to CL caused by *L. donovani* in Sri Lanka, is a Th1 type of response which may explain the localization of *L. donovani* which is usually a viscerotropic species, to skin tissue.

Limitations of study: This study was done only in nine patients due to resource limitations. A continuation of this study is indicated to validate these results.

Keywords: Cutaneous leishmaniasis, cytokines, immune response, leishmaniasis, Leishmania donovani, tropical diseases

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[1]*Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka.*
[2]*Department of Anatomy, Faculty of Medicine, University of Peradeniya, Sri Lanka.*
[3]*Teaching Hospital, Kurunegala, Sri Lanka.*

Address for correspondence: Dr Dhilma Atapattu, Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka. +094 715313760. Email: dhilmaa@yahoo.com *https://orcid.org/*0000-0002-7596-9698

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Introduction

Leishmaniasis is caused by a protozoan flagellate of the genus *Leishmania,* which includes around 30 different species. The clinical manifestations of leishmaniasis have a wide spectrum that includes cutaneous leishmaniasis, muco-cutaneous leishmaniasis and visceral leishmaniasis.[1] In leishmaniasis, multiple and complex interactions resulting from the invasiveness of the parasite, tropism and pathogenicity and the genetically determined immune status of the host contribute to the ultimate clinical manifestations of the disease.[2,3]

Even among patients with cutaneous leishmaniasis (CL), the clinical presentation may show a spectrum, from single small lesions to large, multiple, disfiguring lesions that may be resistant to treatment. In Sri Lanka, the predominant form of the disease is cutaneous leishmaniasis caused by the parasite *Leishmania donovani* of the zymodeme MON-37.[4] The same parasite, *L. donovani,* also causes visceral leishmaniasis (VL) in East Africa, Himachal-Pradesh (State) in India and in Israel[5]. DNA sequencing and microsatellite analyses have shown that these parasites are closely related to those causing VL in the Indian subcontinent (*L. donovani,* MON-2).[6,7] The term "leishmaniasis" encompasses a spectrum of acute or chronic cutaneous and visceral diseases. Evidence has shown that resistance to *Leishmania* infection is mainly due to the cellular immune response of the host, which results in leishmaniacidal macrophage activation.[3]

Studies have found that a Th1 cytokine response enhances cell mediated immunity that is effective against intra-cellular pathogens, whereas a Th2 cytokine response enhances humoral immunity that is effective against extra-cellular pathogens.[8] In murine leishmaniasis, resistance to the disease has been associated with a predominant Th1 response (by secreting IFN-γ and IL-2) and susceptibility with a prominent Th2 response (by secreting IL-4 and IL-10).[9] These findings have been strengthened by subsequent studies showing that the ability to control infection is a result of production of Th1 cytokines, especially IFN-γ and inhibition of the production of Th2 cytokines, especially IL-4.[10,11] However, the clinical responses seen in murine models and humans are not identical, although according to a different study, murine infection with *L. major* mimics human infection due to *L. donovani.*[12,13] . In human CL, healing or asymptomatic disease is correlated with IFN-γ/Th1 cytokine production, whereas VL and diffuse cutaneous leishmaniasis (DCL) appear to result from an inability to produce sufficient Th1 cytokines to control infection.[12] Reasons for this difference in the clinical presentation of the disease may well be revealed by studying associated cellular response patterns.

Research has shown that this polarized response was related to the outcome of the disease which depends on the balance of Th1 and Th2 responses.[8] In Sri Lanka, there is a scarcity of information regarding immunological response to *Leishmania spp.*

This is the first documented study of the cytokine response to *L. donovani* causing CL in Sri Lanka in which the Th1 versus Th2 immune response was studied.

Methods

This study was conducted as a descriptive comparative study.

Nine patients with CL, referred to the Department of Parasitology, Faculty of Medicine, University of Peradeniya, Sri Lanka, from November 2011 to January 2013 were selected for the study. Their diagnosis was based on microscopy of slit skin smears (SSS) obtained from skin lesions and/or clinical criteria. A detailed description of each lesion, including the number, type (papulo-nodular, ulcerative, plaque types), size (expressed in mm^2, great axis x small axis), duration and presence of inflammation, were documented. The cytokine patterns in the 9 biopsies were compared with 4 skin biopsy samples obtained from apparently healthy volunteers.

Collection of slit skin smear (SSS). Skin scrapings and serous dermal fluid was collected from the border of the lesions and used to prepare Giemsa stained smears which were visualized under oil immersion for *Leishmania* amastigotes.

Clinical Diagnosis. A clinical diagnosis of CL was made based on the criteria described by Ranawaka *et al.*[15] This study demonstrated that a clinical diagnosis by a dermatologist reaches an accuracy of 96% and is reliable enough in diagnosing typical CL lesions in endemic areas in Sri Lanka.

Collection of biopsy specimens. Nine biopsies were collected from patients with a suspected clinical diagnosis of CL with or without a positive microscopy result. Biopsies were obtained after local anaesthesia with lignocaine and epinephrine injected at the border of the ulcer. A 3mm dermal thickness circular piece of skin was taken from the border of the ulcer using Visipunch™ biopsy needle. The biopsy was immediately labeled and frozen in liquid nitrogen and later transferred to a -80 °C freezer. Four punch biopsies were also obtained from consenting healthy individuals (from the upper limb).

RNA extraction. Total RNA was extracted from the frozen tissues using the Promega Total RNA isolation kit, in accordance with the manufacturer's instructions. RNA in the biopsy specimen was lysed using 175µl of RNA lysis buffer. Thereafter, 350µl of RNA dilution buffer was added to the lysate and mixed and placed in a heating block at 70 °C for 3 minutes. This was then centrifuged for 10 minutes at 12,000–14,000 × g and the cleared lysate transferred to a spin column purified by centrifugation using the spin columns. The RNA was eluted into nuclease free water and stored at -80 °C overnight.

Reverse transcription and cDNA synthesis. Using theGoScript™ Reverse Transcription System, the extracted RNA was reverse transcribed to cDNA. The extracted RNA (3µl) was mixed and incubated with 2 µl of Primer OligodT and nuclease free water. Thereafter it was combined with the reverse transcription mix and annealed at 25 °C for 5 minutes and extended at 42 °C for up to one hour. It was then stored at -20 °C till the polymerase chain reaction (PCR) was performed to detect the Th1 and Th2 cytokines.

Polymerase Chain Reaction. PCR was done according to a standard protocol.[16] The PCR reaction mixture contained PCR buffer, supplemented with 50mM $MgCl_2$, 2.5mM dNTP, 10pM 5' and 3' oligonucleotide primers and 2U Taq Polymerase. Samples were then amplified in a DNA Thermocycler for 40 cycles. Each cycle consisted of denaturation at 94 °C for 1 minute and annealing/extension at 55 °C (IFN-γ and IL-2) or 65 °C for 2 minutes (IL-4 and IL-10). An

aliquot of PCR product was then electrophoresed on 2% agarose gels and visualized by ethidium bromide staining, The sequence of cytokine specific primer pairs , 5' and 3', are as follows:

IFN-γ: AGTTATATCTTGGCTTTTCA and ACCGAATAATTAGTCAGCTT
IL-2: ACTCACCAGGATGCTCACAT and AGGTAATCCATCTGTTCAGA
IL-4: CCTCTGTTCTTCCTGCTAGCATGTGCC and CCA CGT ACT CTG GTT GGC TTC CTT CAC
IL-10: ATGCCCCAAGCTGAGAACCAAGACCCA and TCTCAAGGGGCTGGGTCAGCTATCCCA

Results

All 9 skin lesions were clinically diagnosed as CL, according to the diagnostic criteria mentioned above.[15] Of the 9, only 3 were microscopy positive for CL.

Cytokine patterns of the biopsy samples from CL patients.

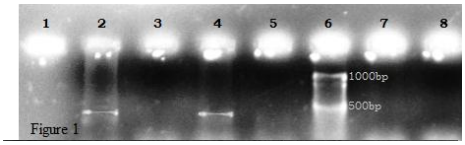

Figure 1: IFN- γ response in 6 CL samples

Ethidium bromide stained 1.5% agarose-gel showing PCR amplified products of cytokine mRNA of IFN-γ from 6 CL biopsies.

Lane 1 - negative control for IFN-γ (no template);
Lanes 2, 4 - IFN-γ bands
Lane 6 - 100bp marker

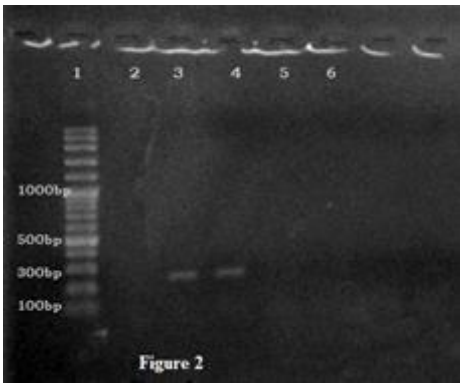

Figure 2: IL-2 response in 4 CL samples

Ethidium bromide stained 1.5% agarose-gel showing PCR amplified products of cytokine mRNA of IL-2 from 4 CL biopsies.
Lane 1 - 100bp marker,
Lane-2 - negative control for IL-2 (no template);
Lanes 3, 4 - IL-2 bands,
Lanes-5, 6 - CL biopsies negative for IL-2.

A Figure 2 for both IFN- γ and IL-2. Figure 1 shows the IFN- γ response in 6 of the 9 patients tested. IFN-γ was positive in 4 samples. (Figure 1 shows 2 of those 4 samples). Figure 2 shows the IL-2 response in 4 of the 9 patients tested. IL-2 was positive in 2 samples as shown in Figure 2.

Both these cytokines were positive in one patient.

All the CL samples tested negative for IL-4 and IL-10. The PCR on these samples were repeated with different temperatures and concentrations of template but with similar results.

Cytokine patterns of the biopsy samples from healthy volunteers

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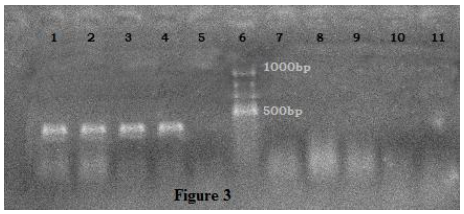

Ethidium bromide stained 1.5% agarose-gel showing PCR amplified products of cytokine mRNA of IFN-γ in biopsies of control samples.

Lanes 1-4 - positive for IFN-γ
Lane-5 - negative control for IFN-γ (no template)
Lane-6 - 100bp marker
Lane-7 - negative control for IL-2 (no template)
Lanes-8-11 - normal biopsies negative for IL-2.

Figure 3: Th1 cytokine response in healthy biopsy samples

Figure 3 shows the Th1 response (IFN- γ and IL-2) in healthy control subjects. Of these skin biopsy samples, all 4 tested positive for IFN-γ (Figure 3) but IL-2, IL-4 and IL-10 were not detected.

Characteristics of CL biopsy samples which tested positive for Th1 cytokines.

Five of the 9 CL biopsies showed the presence of a Th1 response and their lesion duration ranged from 2 to 6 months. Majority of these lesions which showed a positive Th1 response were single lesions (n=4/5), plaque type (n=3/5) with a lesion size of 100-500mm^2 (n=3/5) (Table 1).

Table 1. Characteristics of CL biopsy samples which tested positive for Th1 cytokines.

Patient	Microscopy	Th1 cytokines		No. of lesions	Duration (months)	Presence of inflammation	Type of lesion	Size
		IFN-γ	**IL-2**					
1	positive	negative	positive	1	3	yes	plaque	>1000mm^2
2	negative	positive	positive	1	6	yes	plaque	100-500
3	positive	positive	negative	2	2	yes	ulcerative	>1000
4	negative	positive	negative	1	4	no	ulcerative	100-500
5	negative	positive	negative	1	3	no	plaque	100-500

All 4 biopsies that gave a positive band for IFN-γ, were lesions on the upper limb, with duration from 2 to 6 months and except for one lesion all others had a surface area of 100-500mm.^{2} Two of the 4 lesions had inflammatory signs and 2 out of 4 were plaque like lesions. Two lesions out of the 9 CL lesions were ulcerative type, and they both tested positive for IFN-γ.

Both lesions which tested positive for IL-2 were single, plaque type lesions with inflammatory signs of 3-6 months duration.

Characteristics of lesions tested negative for Th1 cytokines

All lesions tested negative for Th1 cytokine response were of the papulo-nodular type with durations ranging from 3 to 11 months (Table 2). None of the 9 CL lesions tested positive for IL-4 or IL-10.

Table 2. Characteristics of lesions which tested negative for Th1 cytokines

		Th1 cytokines				Presence of Inflammation		
Patient	Microscopy	IFN-γ	IL-2	No. of lesions	Duration (months)		Type	Size mm^2
6	negative	negative	negative	3	11	no	Papulo-nodular	<100
7	negative	negative	negative	1	5	yes	Papulo-nodular	500-1000
8	negative	negative	negative	2	6	no	Papulo-nodular	100-500
9	positive	negative	negative	1	3	no	Papulo-nodular	100-500

Discussion

This is the first study done in Sri Lanka to identify the cytokine expression at the site of the lesion in CL patients using molecular biological means. A predominant Th1 response in CL patients was detected.

Some studies on immune response to CL caused by *L. major* have been performed on cells of the peripheral blood, which may not give a true picture.[17] There is a dearth of information regarding immunological response to CL caused by *L. donovani.*

According to the findings in this study, the majority (5/9) of CL biopsies were positive for Th1 cytokines (IFN-γ and/or IL-2). Conversely, a Th2 response (IL-4 and IL-10) was not detectable in any of the nine CL samples. These results suggest that the Th1 response is more prominent compared to the Th2 response in skin lesions of CL patients. A similar study done on localized cutaneous leishmaniasis (LCL) patients caused by *L. braziliensis* in Venezuela revealed the presence of a predominant Th1 response (IFN-γ and IL-2).[18]

Of the CL biopsies that showed a positive Th1 response, IFN-γ was seen as the predominant response. In the above Venezuelan study performed on 10 LCL, 9 muco-cutaneous leishmaniasis (MCL) and 10 DCL patients, the quantification studies have detected a prominent IFN-γ expression and a weak expression of IL-2 in LCL patients by qPCR[18]. They also detected low

expression of Th2 cytokines (IL-4 and IL-10) in those lesions. Furthermore, they have detected a preponderance of IFN-γ in the localized form of cutaneous leishmaniasis (LCL) and a predominantly Th2 response in the diffuse form of cutaneous leishmaniasis (DCL). These findings lead to the belief that a prominent Th1 response causes resistance to disease or a milder form of localized disease.

The majority of the lesions in the present study that gave a positive Th1 response were single lesions, of the plaque type with a lesion size of < 500 mm^2 and duration of < 6 months. Those which gave a negative Th1 response were papular-nodular type, with a lesion size between 100-1000 mm^2 (except 1) with a duration of 3-11 months. According to a study done in India, a Th1 response is considered to influence a mild or self-curing form of the disease, whereas a Th2 response, which results in the production of IL-4 and IL-10, will influence the dissemination of the infection.[19] All the CL biopsy samples in this study were from localized lesions and the majority showing a Th1 cytokine response might have contributed to limit the dissemination of the disease. This study failed to detect a Th2 cytokine response which might have been due to low concentration of Th2 cytokines in the tissues and/or low sensitivity of the PCR. A predominant Th2 response is seen in lesions with dissemination whereas all the samples in this study were from localized lesions that could partly explain the absence of a Th2 response in the present study.

In a study done on American Cutaneous Leishmaniasis (ACL) patients, it was shown that in humans, the localization of the disease is due to a predominant Th1 response with a preponderance of IFN-γ over IL-4, whereas in the invasive forms (DCL and MCL), the response seen is a mixture of Th1 and Th2 with a preponderance of IL-4 and IL-10.[18]

Cytokine studies on leprosy patients show a similar picture in leprosy.[16] The milder form of leprosy, the tuberculoid form, is characterized by a Th1 response whereas the more severe lepromatous leprosy, is characterized by a Th2 response.

The present study also revealed that the lesions in which it was not possible to detect a cytokine response were all of the nodular type (as opposed to plaque and ulcerative types). Cytokine studies on Buruli disease caused by *Mycobacteria* also showed that there was a difference in the cytokine response seen in ulcerative lesions when compared to nodular lesions.[19]

All 4 normal skin biopsy samples displayed bands for IFN-γ but did not display bands for the other 3 cytokine mRNAs. A similar study carried out in Rajasthan detected low levels of mRNA for both IFN-γ (Th1) and IL-10 (Th2) by qPCR in control samples compared to CL lesions caused by *L. tropica*.[17] However, since mRNA was not quantified in the present study, we are not in a position to comment on their levels in normal skin.

Conclusion

The present study suggests that there may be a distinct Th1 cytokine expression pattern in CL caused by *L. donovani* in Sri Lanka, which could be one reason for the preponderance of LCL seen in this country. The strong cell mediated immunological response mounted by the patients against this vicerotropic *Leishmania* species may be the reason for limiting the VL patients

reported from Sri Lanka to a minimum, but further studies with more samples and quantification of cytokine mRNA are necessary to draw conclusions about the cytokine pattern of CL in this setting.

Competing interests: the authors declare there are no competing interests.

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Ethical Clearance was obtained from the Ethics Review Committee, Faculty of Medicine, University of Peradeniya (2009/EC/40).

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