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In situ immunopathological changes in cutaneous leishmaniasis due to *Leishmania donovani*

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Abstract

Introduction: Cutaneous leishmaniasis in Sri Lanka is a newly established parasitic disease caused by the usually visceralizing *Leishmania donovani*. Skin lesions manifest as non-itchy, non-tender papules, nodules or ulcers. In situ cytokine expression provides clues for immunopathogenesis of this localized form of disease.

Methods: Skin biopsies from 58 patients were analyzed for histological appearance and in situ cytokine expression of T- helper 1 (Th1) and T- helper 2 (Th2) cytokines, namely interferon (IFN)- γ , interleukin (IL)-12A, tumor necrosis factor (TNF)- α , IL-4 and IL-10 by real-time RT- PCR.

Results: Significant up regulation of the Th1 cytokine IFN- γ and down regulation of the Th2 cytokine IL-4 was seen in patients compared to healthy controls. Significantly elevated tissue expression of IFN- γ and TNF- α was seen in lesions that presented later than 6 months from the time of onset, while IL-4 expression was more prominent in lesions that responded poorly to antimony therapy.

Conclusion: A prominent Th1 response appears to support resolving of lesions, whereas a Th2 biased milieu tends to favor poor responsiveness to antimony and delayed lesion healing in *L. donovani* infections in Sri Lanka.

Key words: Cell mediated immunity, IFN- γ , IL-4, TNF- α , histopathology, Sri Lanka

Introduction

Leishmaniasis is a vector-borne parasitic disease, caused by the genus *Leishmania*, which are obligate intracellular parasites of the macrophage – dendritic cell lineage¹. It presents in three major forms as cutaneous, mucocutaneous or visceral leishmaniasis (VL). Leishmaniasis is an important global health problem; currently endemic in 98 countries, with an estimated 0.2 to 0.4 million VL and 0.7 to 1.2 million cutaneous leishmaniasis (CL) cases occurring each year². It was considered as an exotic disease in Sri Lanka until the first locally-acquired case was described in 1992³. At present, it is an established parasitic disease with CL as the predominant form of presentation⁴. Cutaneous Leishmaniasis in Sri Lanka is caused by *Leishmania donovani* MON-37⁵, which is closely related to *Leishmania donovani* MON-2 that causes VL in the Indian subcontinent⁶. This zymodeme has also been recently isolated from cutaneous lesions in a tribal population from Kerala, India⁷, but is better known for its ability to cause VL in many countries including India, Kenya, Israel and Cyprus⁸.

In addition to CL, several cases of mucosal leishmaniasis^{9,10} and VL^{11,12} have been recently reported in Sri Lanka. The causative agent of autochthonous VL also has been confirmed as *Leishmania donovani* MON-37¹². From the current picture, it is clear that Sri Lanka is dealing with a *Leishmania donovani* strain that manifests mainly as CL, however with visceralizing potential. Hence, it could be assumed that the host genetics and immunological response may undoubtedly have a role in apparently diverse clinical outcome.

Histopathology of CL is influenced by the geographical origin through diverse host or parasite related factors. It shows a two way spectrum: from anergic macrophage to tuberculoid granuloma in one and from digestion of amastigotes within macrophages to necrosis of infected macrophages in the other. Such necrosis may be either diffuse or focalized¹³. Histopathological spectrum of CL in Sri Lanka has been described as similar to

leprosy and grouped into four using a modified Ridley classification. It ranged from diffuse infiltrate of parasitized macrophages in group I to well-formed epithelioid granulomata in group IV ¹⁴.

Experimental mouse models have shown that outcome of *Leishmania major* infection depend on preferential activation of Th1 or Th2 subsets of CD4+ T cells, with Th1 type response leading to host resistance and Th2 type response causing progressive disease ¹⁵. Different mouse strains are recognized as genetically resistant or susceptible to *L. major*, depending on their ability to produce Th1 or Th2 cytokine profiles respectively. However, this clear dichotomous response is apparently not obvious in human leishmaniasis ¹⁶.

Varying cytokine profiles have been associated with different clinical manifestations and infecting species in leishmaniasis. *Leishmania braziliensis* infection which may manifests as localized cutaneous leishmaniasis (LCL), mucocutaneous leishmaniasis (MCL) or disseminated cutaneous leishmaniasis (DCL), has demonstrated a predominant Th1 response in LCL, a mixed Th1/Th2 response in MCL and a prominent Th2 response in DCL ^{17,18}.

Similarly, a predominant Th1 type cytokine profile is seen in localized *L. mexicana* and *L. tropica* infections ^{19,20}. In contrast a prominent Th2 response was observed in lesions caused by *L. guyanensis* ²¹. Unfavorable clinical evolution in *L. donovani*-induced post kala-azar dermal leishmaniasis (PKDL) has been attributed to a mixed Th1/Th2 cytokine response ²².

Hence, the in situ cytokine profile in leishmaniasis is determined by the causative *Leishmania* spp. ^{18,19,21}, the parasite strain ^{23,24}, and the host immune response ²⁵. Cytokine expression in dermatropic *L. donovani* infections however, has not been previously evaluated. The present study is the first to characterize the in situ cytokine gene expression in CL due to *Leishmania donovani*.

Materials and Methods

Ethical issues. This study received ethical approval from the Ethics Review Committee of the Faculty of Medicine, University of Kelaniya, Sri Lanka and was conducted adhering to the approved protocol and in agreement with the Helsinki Declaration. Patients and controls were recruited on a voluntary basis and informed written consent was obtained prior to sample collection.

Study population. A total of 108 patients with suspected skin lesions attending dermatology clinics in the Sri Lanka Army and District General Hospitals of Polonnaruwa and Hambantota were included. Patients with major co-morbidities such as diabetes mellitus and those already on treatment for CL were excluded from the study.

Sample collection. Two punch biopsies each measuring 2-3 mm were obtained from the lesion under local anesthesia. One was immediately submerged in RNAlater (Qiagen, Hilden, Germany), to stabilize RNA, and stored at -20°C until further analysis. The second biopsy was gently rolled over a microscope slide to prepare an impression smear. The biopsy specimen thereafter was fixed in 10% neutral buffered formalin (NBF) for routine histopathological processing, where tissue sections separately stained with haematoxylin and eosin (H & E) and Giemsa were prepared. Impression smears made on glass slides were air dried, fixed in methanol, stained with Giemsa and examined under a light microscope for the presence of parasites. Fifty eight patient samples with confirmed diagnosis of leishmaniasis, following the demonstration of parasites in impression smears or histological sections were further analyzed for cytokine gene expression. Control skin specimens were obtained from incision sites of 25 patients with no signs or symptoms of leishmaniasis, who underwent minor surgical procedures due to unrelated surgical causes. Patients were treated by the

dermatologists in charge of the clinics with intralesional sodium stibogluconate weekly or fortnightly until the lesions healed.

Histopathological analysis. Forty six H and E stained sections were examined under a conference microscope (Olympus BX50, Tokyo, Japan) and categorized according to modified Ridley's criteria for leishmaniasis as previously described¹⁴. These categories are defined and illustrated in figure 2 and 3. In the group IV pattern where amastigotes were not seen, diagnosis was based on positive impression smear result. Histology was not performed in 12 patients due to inadequacy of specimen or its poor quality. Each specimen was examined by two histopathologists for confirmation of findings.

Altogether, cytokine gene expression was quantified in 58 patient and 25 control samples and it was correlated with histopathology in 46 patient samples. Forty four patients were followed up to assess the duration of treatment.

Total RNA extraction. Tissue specimens in RNAlater were disrupted and homogenized with RNA lysis buffer containing guanidine isothiocyanate using 1 g of 2.00 mm diameter Zirconia beads in a Mini-Beadbeater (Biospec, Bartlesville, OK, USA). Total RNA was extracted using the RNeasy Plus Universal Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. RNA purity and concentration measured using the NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Integrity of RNA was established by the presence of 28s and 18s bands on a 1% agarose gel electrophoresis according to established methods²⁶.

Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) for cytokine gene expression. Gene expression was analyzed for 5 cytokine genes (IL-12A,

IFN- γ , TNF- α , IL-4 and IL-10) that represent both the Th1 and Th2 responses in the lesion.

Cytokine primers validated for quantitative real-time PCR were selected from the

PrimerBank²⁷ (Table 1). Optimization of RT-qPCR was carried out following the MIQE

guidelines²⁸. Complementary DNA (cDNA) was synthesized using a unique blend of oligo

(dT) and random hexamer primers provided by the iScript[®] cDNA synthesis kit (Bio-Rad,

Hercules, CA, USA), according to manufacturer's instructions. Briefly, 100 ng of RNA was

used per 20 μ l total reaction volume, which included 4 μ l of 5x iScript[®] reaction mix and 1 μ l

of iScript[®] reverse transcriptase. Reactions were incubated for 5 minutes at 25°C, 30 minutes

at 42°C and 5 minutes at 85°C. A water control was included for each batch of cDNA

synthesized. As recommended by the manufacturer, one-tenth of the cDNA reaction volume

(2 μ l) was used for qPCR. Real-time PCR reactions were set up with 2 μ l of cDNA, 2.5 μ l of

primer mix with a concentration of 2.5 μ M of each forward and reverse primers, 12.5 μ l of

iQ[™]SYBR[®] Green Supermix (Bio-Rad, Hercules, CA, USA) and 8 μ l of nuclease free water

in a total reaction volume of 25 μ l in each well on Microseal[®]96-well skirted PCR plates (Bio-

Rad, Hercules, CA, USA). For each set of primers, all samples were run in duplicates. Beta-

actin was used as the reference gene and qPCR was performed for 58 patient samples and 25

control samples in a CFX96[™] Real-time system (Bio-Rad, Hercules, CA, USA). Thermal

cycling protocol consisted of an initial denaturation step at 95°C for 10 min followed by 45

cycles each consisting of 95°C for 15s, 60°C for 30s, 72°C for 30s and 78°C for 5s, and held

at 72°C for 10 min. Post-amplification melting curve analysis was done to ensure reaction

specificity, starting at 50°C and increasing up to 95°C in increments of 0.5°C for 5s. A

negative control was included for each batch of cDNA samples.

Relative quantification of gene expression was done using the equation $2^{-\Delta\Delta Ct} = 2^{-(Ct_{\text{target gene}} - Ct_{\beta\text{-actin}})_{\text{sample}} - (Ct_{\text{target gene}} - Ct_{\beta\text{-actin}})_{\text{control}}}$ as previously described²⁹.

Statistical analysis. Data were analyzed by IBM SPSS statistics for Windows, version 21.0 (Armonk, NY, USA). Non parametric tests were used for comparison of cytokine gene expression between patient and control groups as cytokine levels did not show a normal distribution. Mann – Whitney U test was used for comparison between two groups and Kruskal-Wallis test was used for comparison between more than two groups. Spearman’s correlation test was used for analyzing correlation between continuous variables. Significance was estimated at 0.05 level for all above methods.

Results

Patient and control group characteristics. The two groups were comparable with no significant difference in their age ($p = 0.19$) and sex ($p = 0.72$) distribution (Table S1).

Patients presented with 1- 4 lesions and majority (75.9%, 44/58) had only a single lesion.

Lesions were mainly on upper limb (51.7%, 30/58) and lower leg (34.5%, 20/58), and lesion type varied from discrete papules and nodules to ulcerated plaques (Figure 1). Majority had nodular lesions (44.8%, 26/58). Lesions had a mean duration of 6.75 ± 9.1 months (range: 1-48 months) and a mean size of 176.59 ± 185.76 mm² (range: 12.6 – 908.3 mm²).

Histopathological characteristics. Epidermal changes included hyperkeratosis (91.3%, 42/46), irregular acanthosis (54.3%, 25/46), parakeratosis (34.8%, 16/46), follicular plugging (21.7%, 10/46) and hyperplasia (10.9%, 5/46) (Figure S1). Dermal changes were characterized by marked inflammatory infiltrate composed of macrophages, lymphocytes and plasma cells with or without granuloma formation (Figure 2 and 3). Distribution of sample

numbers, lesion types and mean lesion duration among histopathological groups are given in table 2. Necrosis was not seen in any of these specimens. Histological grouping failed to show any obvious association with the lesion type, size or duration.

In situ cytokine gene expression and association with patient and lesion characteristics.

All cytokines tested were expressed at detectable levels in patients' tissue samples. In the control group all had detectable levels of IL-10, IFN- γ and TNF- α . However, IL-12A and IL-4 were detectable only in 17/25 and 21/25 controls respectively. Significant up regulation of IFN- γ ($p < 0.001$) and down regulation of IL-4 ($p < 0.001$) was seen in the patients compared to controls (Figure 4).

Expression of TNF- α increased significantly with lesion duration (Spearman $r = 0.294$, $p = 0.025$). When lesions were categorized as recent (those with a duration of < 6 months at presentation) and late (duration of 6 months or more), there was significant increase in both IFN- γ ($p = 0.018$) and TNF- α ($p < 0.001$) in late lesions (Figure 5). Females showed higher mean level of Th1 type cytokine IL-12A ($p = 0.03$). On the other hand, males had higher mean levels of Th2 type cytokines IL-4 and IL-10 as well as Th1 type IFN- γ , but none were statistically significant. Cytokine expression was not significantly associated with the lesion size, lesion type, ulceration or histopathological grouping. Expression of all cytokine genes correlated significantly with each other, with the strongest correlation in the expression of IL-12A with IFN- γ ($r = 0.786$) (Table 3).

All lesions followed up achieved complete healing and the treatment duration ranged from 1.5 to 8 months with a mean of 3.0 ± 1.75 months. Time taken to heal showed a significant positive correlation with in situ expression level of IL-4 (Spearman's $r = 0.321$, $p = 0.034$).

Discussion

Localized immune response at the site of infection plays an important role in pathogenesis and outcome of CL^{18-20,30,31}. To better understand the role of cytokines in the pathogenesis of CL due to dermatropic *L. donovani*, we investigated and quantified for the first time, the in situ expression of both Th1 and Th2 type cytokine profiles together with local histopathological changes. Different types of cutaneous lesions, as previously described⁴ were studied and all appeared to heal completely following treatment with intralesional sodium stibogluconate, although the rate of healing varied between patients. There was a mixed Th1/Th2 cytokine profile at the site of infection, similar to observations made on human CL due to other *Leishmania* species¹⁷⁻¹⁹. However, it was interesting to note a markedly up-regulated IFN- γ and down-regulated IL-4 expression with varying histopathological characteristics.

The most striking feature in histopathology was the presence of a marked inflammatory cell infiltrate in the dermis, composed of histiocytes, plasma cells and lymphocytes. Its organization ranged from diffuse inflammatory infiltrate with parasitized macrophages to varying degrees of granuloma formation that extended from ill-formed histiocytic to epithelioid granulomata. Similar histological changes have been observed in previous studies done on CL due to *L. major*³² and *L. donovani* in the local setting¹⁴. Varying degrees of necrosis have been described in American tegumentary leishmaniasis^{33,34} as well as in CL in the old world³². However, necrosis was not a prominent feature in this study, and therefore parasite elimination in CL due to *L. donovani* appears to depend on activation of macrophages to form epithelioid granulomas, rather than through a necrotizing process, as previously suggested¹⁴. Interestingly, up-regulation of IFN- γ seen in this study provide further support for this assumption, as IFN- γ is a potent activator of macrophages³⁵. Therefore, it can be concluded that macrophage activation plays a key role in parasite

elimination in CL due to *L. donovani* in Sri Lanka. No significant association between histological grouping and cytokine expression was seen however, which may have been due to the inadequate numbers in some of the groups that hindered a proper comparison.

Furthermore, failure to measure the cytokines produced also may have been an added limitation.

Localized CL is associated with increased in situ expression of IFN- γ as demonstrated in *L. tropica*²⁰ and *L. major*³¹ infections, which are clinically comparable to *L. donovani*-induced CL in Sri Lanka. Interferon- γ is a potent activator of macrophages³⁵ and acts synergistically with TNF- α to kill intracellular pathogens by induction of Nitric Oxide Synthase (iNOS) to produce nitric oxide³⁶. Significant up-regulation of IFN- γ and significant positive correlation between IFN- γ and TNF- α gene expression seen in this study further suggest that, macrophage activation is responsible for parasite elimination. Interleukin-12 plays a major role in the development of a Th1 response at the site of infection³⁵. *Leishmania* spp. are known to down-regulate production of IL-12, which enable their establishment within macrophages without activation, and hence facilitate 'silent entry'³⁷. A similar tendency to down-regulate IL-12 was seen in the patient population compared to controls, but the strong positive correlation seen between the expressions of IL-12A and IFN- γ indicates a well regulated expression of these two cytokines in the local milieu.

Although essential for parasite clearance, an exaggerated Th1 response with excessive IFN- γ levels causes damage to host tissues. Subclinical infections have been associated with low levels of IFN- γ production in *L. mexicana* and *L. braziliensis* infections^{30,38}. Furthermore, elevated in situ expression of IFN- γ has been demonstrated in late lesions (> 6 months) in *L. major* infections³⁹. In this study, significantly increased expression of IFN- γ was seen in lesions diagnosed late (\geq 6 months) and a tendency to have higher levels of IFN- γ was observed in lesions that healed more slowly. Further enhancement of the pro-inflammatory

milieu at the site of lesions as they evolved with time was evident by the significant positive correlation between the expression of TNF- α and lesion duration. This adds to the existing theory of the role of TNF- α in MCL and chronic manifestations in human leishmaniasis ¹⁷⁻¹⁹.

Development of an anti-inflammatory milieu at the site of infection favors persistence of parasites and disease progression in mouse models ¹⁵. Interleukin - 4 secreted by Th2 subset of lymphocytes plays a major role in down regulating the Th1 response and inhibiting nitric oxide production ¹. In general, in situ expression of IL-4 is low in human CL ^{19,31}, but a greater expression has been evident in MCL and DCL ¹⁷⁻¹⁹. In human infections with *L. major*, IL-4 production was associated with severe disease, while patients with mild disease had no IL-4 production ⁴⁰. A very low and significantly down regulated IL-4 response was observed in patients compared to healthy controls, in the current study. This might provide a clue as to the non-visceralizing nature of the local parasite with relatively more favorable outcome of leishmaniasis in Sri Lanka, despite the virulence and visceralizing potential of the species *L. donovani*. Furthermore, a significant positive correlation was observed between IL-4 expression and time taken for lesions to heal, which points toward a role of IL-4 in interfering with the healing process. Interleukin-10, which is another cytokine with anti-inflammatory properties, was co-expressed with IL-4 demonstrating a significant positive correlation. Modulatory actions of IL-10 is important to prevent excessive tissue damage in inflammatory reactions by inhibiting pro-inflammatory cytokines ^{41,42} and hence higher expression has been recorded in MCL than LCL ¹⁸. High IL-10 has also been associated with poor response to treatment in infections with *L. guyanensis* ⁴³, more slowly healing lesions in *L. major* ³¹, persistence of disease with *L. mexicana* ⁴⁴ and Indian PKDL ⁴⁵, which has led to the suggestion that an optimum balance between macrophage activating IFN- γ and deactivating IL-10 is required for favorable outcome in leishmaniasis ⁴⁶. Interestingly, we observed a significant positive correlation between expression of IFN- γ and IL-10 in lesions,

with suggestive favorable balance between the pro-inflammatory and immune-regulatory responses among CL patients in Sri Lanka, which possibly explains its uncomplicated course in almost all patients.

Male and female sex hormones have a role in modulating the immune responses as evidenced by the pro-inflammatory properties of estrogen or 17 β -estradiol and anti-inflammatory properties of testosterone⁴⁷. Furthermore, studies have shown that 17 β -estradiol can increase nitric oxide production from macrophages in a pro-inflammatory cytokine independent pathway⁴⁸. However, experimental models have shown different outcomes in male and female mice depending on the *Leishmania* species⁴⁷. Influence of sex was not very marked in the present study though there was an apparent tendency for increased expression of pro-inflammatory cytokine TNF- α and IL-12A in females and IL-4 and IL-10 with anti-inflammatory bias in males. However, the use of large sample numbers may increase the statistical power of such analysis.

Evidence from these cytokine profiles points out a significant correlation between all the genes studied, with strong correlations apparent for IFN- γ with IL-12A and TNF- α . Similar significant correlation for IFN- γ with IL-12 and TNF- α has been found in other studies that involve *Leishmania*^{20,31}. Interestingly we also observed a significant positive correlation between the expression of IFN- γ and IL-4 and IL-4 and IL-10, indicating the fine balance between the pro- and anti-inflammatory modulators in local tissues of CL patients.

In conclusion, these observations point towards an existence of a mixed Th1/ Th2 cytokine expression profile at the site of lesion with an obvious bias towards Th1 response, that has positively influenced the outcome of the resultant localized form of leishmaniasis caused by a variant strain of *Leishmania donovani* among Sri Lankan patients. However, in order to directly implicate cytokines in disease pathogenesis and/or its outcome, more detailed

investigations with adequate number of samples in each category of histopathology and proteomic studies would be required, which may be combined with a comprehensive analysis of chemokines and other anti-inflammatory cytokines such as IL-17, TGF- β .

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Author Contributions

NHM designed, performed the experiments, analyzed the data and wrote the manuscript. SO contributed to experimental work by designing cytokine mRNA quantification. Clinical management was done by NP. VCdeS performed the histopathological analysis of specimens. WA contributed to the design and provided supervision. ARS provided essential reagents and

laboratory facilities. NDK contributed to design, undertook overall supervision, managed collaborations and edited the manuscript.

Disclosures

The authors have no potential conflict of interest to disclose.

References

1. Alexander J, Satoskar AR, Russell DG. *Leishmania* species: models of intracellular parasitism. *J Cell Sci.* 1999;112 Pt 18:2993-3002.
2. Alvar J, Vélez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One.* 2012;7(5). doi:10.1371/journal.pone.0035671.
3. Athukorale DN, Seneviratne JK, Ihalamulla RL, Premaratne UN. Locally acquired cutaneous leishmaniasis in Sri Lanka. *J Trop Med Hyg.* 1992;95(6):432-433.
4. Karunaweera ND, Rajapaksa US. Is Leishmaniasis in Sri Lanka benign and be ignored? *J Vector Borne Dis.* 2009;46(1):13-17.
5. Karunaweera ND, Pralong F, Siriwardane YD, Ihalamulla RL, Dedet JP. Sri Lankan cutaneous leishmaniasis is caused by *Leishmania donovani* zymodeme MON-37. *Trans R Soc Trop Med Hyg.* 2003;97(4):380-381. doi:10.1016/S0035-9203(03)90061-7.
6. Alam MZ, Haralambous C, Kuhls K, et al. The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. *Microbes Infect.* 2009;11(6-7):707-715. doi:10.1016/j.micinf.2009.04.009.

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7. Kumar NP, Srinivasan R, Anish TS, Nandakumar G, Jambulingam P. Cutaneous leishmaniasis caused by *Leishmania donovani* in the tribal population of the Agasthyamala Biosphere Reserve forest, Western Ghats, Kerala, India. *J Med Microbiol.* 2015;64(Pt_2):157-163. doi:10.1099/jmm.0.076695-0.
 8. Karunaweera ND. *Leishmania donovani* causing cutaneous leishmaniasis in Sri Lanka: a wolf in sheep's clothing? *Trends Parasitol.* 2009;25(10):458-463. doi:10.1016/j.pt.2009.07.002.
 9. Rajapaksa US, Ihalamulla RL, Karunaweera ND. First report of mucosal tissue localisation of leishmaniasis in Sri Lanka. *Ceylon Med J.* 2005;50(2):90-91.
 10. Rathnayake D, Ranawake RR, Sirimanna G, Siriwardhane Y, Karunaweera N, De Silva R. Co-infection of mucosal leishmaniasis and extra pulmonary tuberculosis in a patient with inherent immune deficiency. *Int J Dermatol.* 2010;49(5):549-551. doi:10.1111/j.1365-4632.2010.04376.x.
 11. Abeygunasekara P, Costa Y, Seneviratne N, Ratnatunga N, Wijesundera MDS. Locally acquired visceral leishmaniasis in Sri Lanka. *Ceylon Med J.* 2007;52(1):30-31. doi:10.4038/cmj.v52i1.1047.
 12. Ranasinghe S, Zhang W-W, Wickremasinghe R, et al. *Leishmania donovani* zymodeme MON-37 isolated from an autochthonous visceral leishmaniasis patient in Sri Lanka. *Pathog Glob Health.* 2012;106(7):421-424. doi:10.1179/2047773212Y.0000000054.
 13. Ridley DS, Ridley MJ. The evolution of the lesion in cutaneous leishmaniasis. *J Pathol.* 1983;141:83-96.
 14. Herath CH, Ratnatunga NV, Waduge R, Ratnayake P, Ratnatunga CN, Ramadasa S. A

histopathological study of cutaneous leishmaniasis in Sri Lanka. *Ceylon Med J.* 2010;55:106-111.

15. Heinzl FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J Exp Med.* 1989;169(1):59-72. doi:10.1084/jem.169.1.59.
16. Alexander J, Brombacher F. T helper1/T helper2 cells and resistance/susceptibility to *Leishmania* infection: Is this paradigm still relevant? *Front Immunol.* 2012;3:80. doi:10.3389/fimmu.2012.00080.
17. Cáceres-Dittmar G, Tapia FJ, Sánchez MA, et al. Determination of the cytokine profile in American cutaneous leishmaniasis using the polymerase chain reaction. *Clin Exp Immunol.* 1993;91(3):500-505.
18. Pirmez C, Yamamura M, Uyemura K, Paes-Oliveira M, Conceicao-Silva F, Modlin RL. Cytokine patterns in the pathogenesis of human Leishmaniasis. *J Clin Invest.* 1993;91(4):1390-1395. doi:10.1172/JCI116341.
19. Melby PC, Andrade-Narvaez FJ, Darnell BJ, Valencia-Pacheco G, Tryon V V, Palomo-Cetina A. Increased expression of proinflammatory cytokines in chronic lesions of human cutaneous leishmaniasis. *Infect Immun.* 1994;62(3):837-842.
20. Kumar R, Bumb RA, Salotra P. Evaluation of localized and systemic immune responses in cutaneous leishmaniasis caused by *Leishmania tropica*: interleukin-8, monocyte chemotactic protein-1 and nitric oxide are major regulatory factors. *Immunology.* 2010;130(2):193-201. doi:10.1111/j.1365-2567.2009.03223.x.
21. Bourreau E, Prévot G, Pradinaud R, Launois P. Interleukin (IL)-13 is the predominant

Th2 cytokine in localized cutaneous leishmaniasis lesions and renders specific CD4+ T cells unresponsive to IL-12. *J Infect Dis.* 2001;183(6):953-959. doi:10.1086/319249.

22. Ansari NA, Ramesh V, Salotra P. Interferon (IFN)-gamma , tumor necrosis factor-alpha , interleukin-6, and IFN-gamma receptor 1 are the major immunological determinants associated with post-kala azar dermal leishmaniasis. *J Infect Dis.* 2006;194(7):958-965. doi:10.1086/506624.
23. Asadpour A, Riazi-Rad F, Khaze V, Ajdary S, Alimohammadian MH. Distinct strains of *Leishmania major* induce different cytokine mRNA expression in draining lymph node of BALB / c mice. *Parasite Immunol.* 2013;35(1):42-50. doi:10.1111/pim.12018.
24. Teixeira MJ, Fernandes JD, Teixeira CR, et al. Distinct *Leishmania braziliensis* isolates induce different paces of chemokine expression patterns. *Infect Immun.* 2005;73(2):1191-1195. doi:10.1128/IAI.73.2.1191-1195.2005.
25. Gollob KJ, Viana AG, Dutra WO. Immunoregulation in human American leishmaniasis: balancing pathology and protection. *Parasite Immunol.* 2014;36(8):367-376. doi:10.1111/pim.12100.
26. Forlenza M, Kaiser T, Savelkoul HF, Wiegertjes GF. The use of real-time quantitative PCR for the analysis of cytokine mRNA levels. *Methods Mol Biol.* 2012;820:7-23. doi:10.1007/978-1-61779-439-1_2.
27. PCR primers for gene expression detection and quantification. PrimerBank web site. <https://pga.mgh.harvard.edu/primerbank/>. Accessed August 20, 2015.
28. Bustin SA, Benes V, Garson JA, et al. The MIQE Guidelines : Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin Chem.* 2009;55(4):1-12. doi:10.1373/clinchem.2008.112797.

29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta CT)}$ method. *Methods*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262.
30. Valencia-Pacheco G, Loría-Cervera EN, Sosa-Bibiano EI, et al. In situ cytokines (IL-4, IL-10, IL-12, IFN- γ) and chemokines (MCP-1, MIP-1 α) gene expression in human *Leishmania (Leishmania) mexicana* infection. *Cytokine*. 2014;69(1):56-61. doi:10.1016/j.cyto.2014.05.016.
31. Louzir H, Melby PC, Ben Salah A, et al. Immunologic determinants of disease evolution in localized cutaneous leishmaniasis due to *Leishmania major*. *J Infect Dis*. 1998;177:1687-1695.
32. Gaafar A, el Kadar AY, Theander TG, et al. The pathology of cutaneous leishmaniasis due to *Leishmania major* in Sudan. *Am J Trop Med Hyg*. 1995;52(5):438-442.
33. Bittencourt AL, Barral A. Evaluation of the histopathological classification of American cutaneous and mucocutaneous leishmaniasis. *Mem Inst Oswaldo Cruz*. 1991;86(1):51-56.
34. Martins ALGP, Barreto JA, Lauris JRP, Martins ACGP. American tegumentary leishmaniasis: Correlations among immunological, histopathological and clinical parameters. *An Bras Dermatol*. 2014;89(1):52-58. doi:10.1590/abd1806-4841.20142226.
35. Alexander J, Bryson K. T helper (h)1/Th2 and Leishmania: Paradox rather than paradigm. *Immunol Lett*. 2005;99(1):17-23. doi:10.1016/j.imlet.2005.01.009.
36. Liew FY, Li Y, Millott S. Tumor necrosis factor-alpha synergizes with IFN-gamma in

mediating killing of *Leishmania major* through the induction of nitric oxide. *J Immunol.* 1990;145(12):4306-4310.

37. Gupta G, Oghumu S, Satoskar AR. Mechanisms of immune evasion in leishmaniasis. *Adv Appl Microbiol.* 2013;82:155-184. doi:10.1016/B978-0-12-407679-2.00005-3.
38. Bittar RC, Nogueira RS, Vieira-Gonçalves R, et al. T-cell responses associated with resistance to *Leishmania* infection in individuals from endemic areas for *Leishmania (Viannia) braziliensis*. *Mem Inst Oswaldo Cruz.* 2007;102(5):625-630.
39. Hoseini SG, Javanmard SH, Hejazi SH, et al. Comparison of immune regulatory factors in acute and chronic lesions of cutaneous leishmaniasis due to *Leishmania major*. *J Res Med Sci.* 2014;19(Suppl 1):S36-40.
40. Gaafar A, Kharazmi A, Ismail A, et al. Dichotomy of the T cell response to *Leishmania* antigens in patients suffering from cutaneous leishmaniasis; absence or scarcity of Th1 activity is associated with severe infections. *Clin Exp Immunol.* 1995;100(2):239-245.
41. Bacchetta R, Gambineri E, Roncarolo M-G. Role of regulatory T cells and FOXP3 in human diseases. *J Allergy Clin Immunol.* 2007;120(2):227-235. doi:10.1016/j.jaci.2007.06.023.
42. Cummings HE, Tuladhar R, Satoskar AR. Cytokines and their STATs in cutaneous and visceral leishmaniasis. *J Biomed Biotechnol.* 2010;2010. doi:10.1155/2010/294389.
43. Bourreau E, Prévot G, Gardon J, Pradinaud R, Launois P. High intralésional interleukin-10 messenger RNA expression in localized cutaneous leishmaniasis is associated with unresponsiveness to treatment. *J Infect Dis.* 2001;184:1628-1630.

doi:10.1086/324665.

44. Melby PC, Andrade-Narvaez F, Darnell BJ, Valencia-Pacheco G. In situ expression of interleukin- 10 and interleukin- 12 in active human cutaneous leishmaniasis. *FEMS Immunol Med Microbiol*. 1996;15(2-3):101-107.
45. Ganguly S, Das NK, Panja M, et al. Increased levels of interleukin-10 and IgG3 are hallmarks of Indian post-kala-azar dermal leishmaniasis. *J Infect Dis*. 2008;197(12):1762-1771. doi:10.1086/588387.
46. Gomes-Silva A, De Cássia Bittar R, Dos Santos Nogueira R, et al. Can interferon- γ and interleukin-10 balance be associated with severity of human *Leishmania (Viannia) braziliensis* infection? *Clin Exp Immunol*. 2007;149(3):440-444. doi:10.1111/j.1365-2249.2007.03436.x.
47. Snider H, Lezama-Dávila CM, Alexander J, Satoskar AR. Sex hormones and modulation of immunity against leishmaniasis. *Neuroimmunomodulation*. 2009;16(2):106-113. doi:10.1159/000180265.
48. Lezama-Dávila CM, Isaac-Márquez AP, Barbi J, Oghumu S, Satoskar AR. 17β -estradiol increases *Leishmania mexicana* killing in macrophages from DBA/2 mice by enhancing production of nitric oxide but not pro-inflammatory cytokines. *Am J Trop Med Hyg*. 2007;76(6):1125-1127.

Abbreviations: **CL**-cutaneous leishmaniasis, **DCL**- disseminated cutaneous leishmaniasis, **LCL**- localized cutaneous leishmaniasis, **MCL**-mucocutaneous leishmaniasis, **VL**- visceral leishmaniasis, **IFN** – Interferon , **IL** – Interleukin, **TGF**- Transforming growth factor , **TNF**- Tumor necrosis factor, **Th**-T helper

Legends to Tables and Figures

Table 1. Cytokine primer sequences used in this study for mRNA quantification by RT-qPCR. Source: ²⁹

Abbreviations: bp, base pairs.

Table 2. Distribution of sample numbers and lesion characteristics among histopathological groups

Table 3. Spearman's rank correlation coefficients for intralesional cytokine gene expressions.

Values indicate correlation coefficients with level of significance in parentheses, determined by the non-parametric Spearman's rank correlation test to calculate the degree of linear correlation among the relative copy numbers for cytokine mRNAs expressed at the site of lesion. A correlation coefficient close to 1 indicate a linear relationship in the expression of the two cytokines.

Abbreviations: IL,interleukin; IFN- γ ,interferon-gamma; TNF- α , tumor necrosis factor-alpha.

Figure 1. Clinical presentations of cutaneous leishmaniasis due to *L. donovani* in Sri Lanka.

Pictures A, B, C and D demonstrate a papule, nodule, ulcer and an indurated plaque lesion respectively.

Figure 2. Histopathological groups I and II.

Group I (pictures A and B): Parasitized macrophages with variable lymphocytes and plasma cells in a diffuse infiltrate (amastigotes are shown by arrows in B).

Group II (pictures C and D): Parasitized macrophages (shown by arrows in D) with lymphocytes, plasma cells and ill formed histiocytic granulomata. (A and C: haematoxylin and eosin 10 x 20, B and D: haematoxylin and eosin 10x40).

Figure 3. Histopathological groups III and IV.

Group III (pictures A and B): A mixture of macrophages with or without parasites (amastigotes focally present are indicated by arrows in B), lymphocytes, plasma cells and epithelioid granulomata (shown by arrows in A).

Group IV (pictures C and D): Epithelioid granulomatous response with or without Langhans type multinucleated giant cells (shown with arrow in D), few lymphocytes, plasma cells but no amastigotes (A and C: haematoxylin and eosin 10 x 20, B and D: haematoxylin and eosin 10x40).

Figure 4. Gene expression for IL-12A, IFN- γ , TNF- α , IL-4 and IL-10 in patient and control groups. Cytokine mRNA in dermal lesions and control skin specimens were quantified by RT-qPCR and expressed as relative mRNA copy numbers by $2^{-\Delta\Delta C_t}$ method. Beta actin was used as the reference gene and controls as the calibrators. Graphs represent the median relative mRNA copy numbers and interquartile range. Difference between patient and control groups were significant by Mann-Whitney U test for IFN- γ and IL-4 (**p <0.001).

Figure 5. Expression of IFN-gamma and TNF-alpha in recent (< 6 months) and late (≥ 6 months) lesions. Both genes were expressed significantly more in late lesions compared to recent lesions. Graphs represent median relative mRNA copy numbers and interquartile range. (p values as calculated by Mann Whitney U test: * p <0.05 and ***p <0.001).

Table 1. Cytokine primer sequences used in this study for mRNA quantification by RT-qPCR

Gene	Genbank accession	PrimerBank ID	Primer Sequence (5'-3')	Amplicon size (bp)	Melting Temperature (°C)
β-actin	NM_001101	4501885a1	F: CATGTACGTTGCTATCCAGGC	250	60.8
			R: CTCCTTAATGTCACGCACGAT		60.2
IFN-γ	NM_000619	56786137c1	F: TCGGTAAGTACTGACTTGAATGTCCA	93	61.2
			R: TCGCTTCCCTGTTTTAGCTGC		62.9
IL-12A	NM_000882	325974478c1	F: CCTTGCACTTCTGAAGAGATTGA	181	60.2
			R: ACAGGGCCATCATAAAAAGAGGT		61.1
TNF-α	NM_000594	25952110c1	F: CCTCTCTCTAATCAGCCCTCTG	220	60.8
			R: GAGGACCTGGGAGTAGATGAG		60.2
IL-4	NM_000589	4504669a1	F: CCAACTGCTTCCCCCTCTG	150	62
			R: TCTGTTACGGTCAACTCGGTG		61.7
IL-10	NM_000572	24430216c1	F: GACTTTAAGGGTTACCTGGGTTG	112	60.5
			R: TCACATGCGCCTTGATGTCTG		63

Source: ²⁹

Abbreviations: bp, base pairs.

Table 2. Distribution of sample numbers and lesion characteristics among histopathological groups

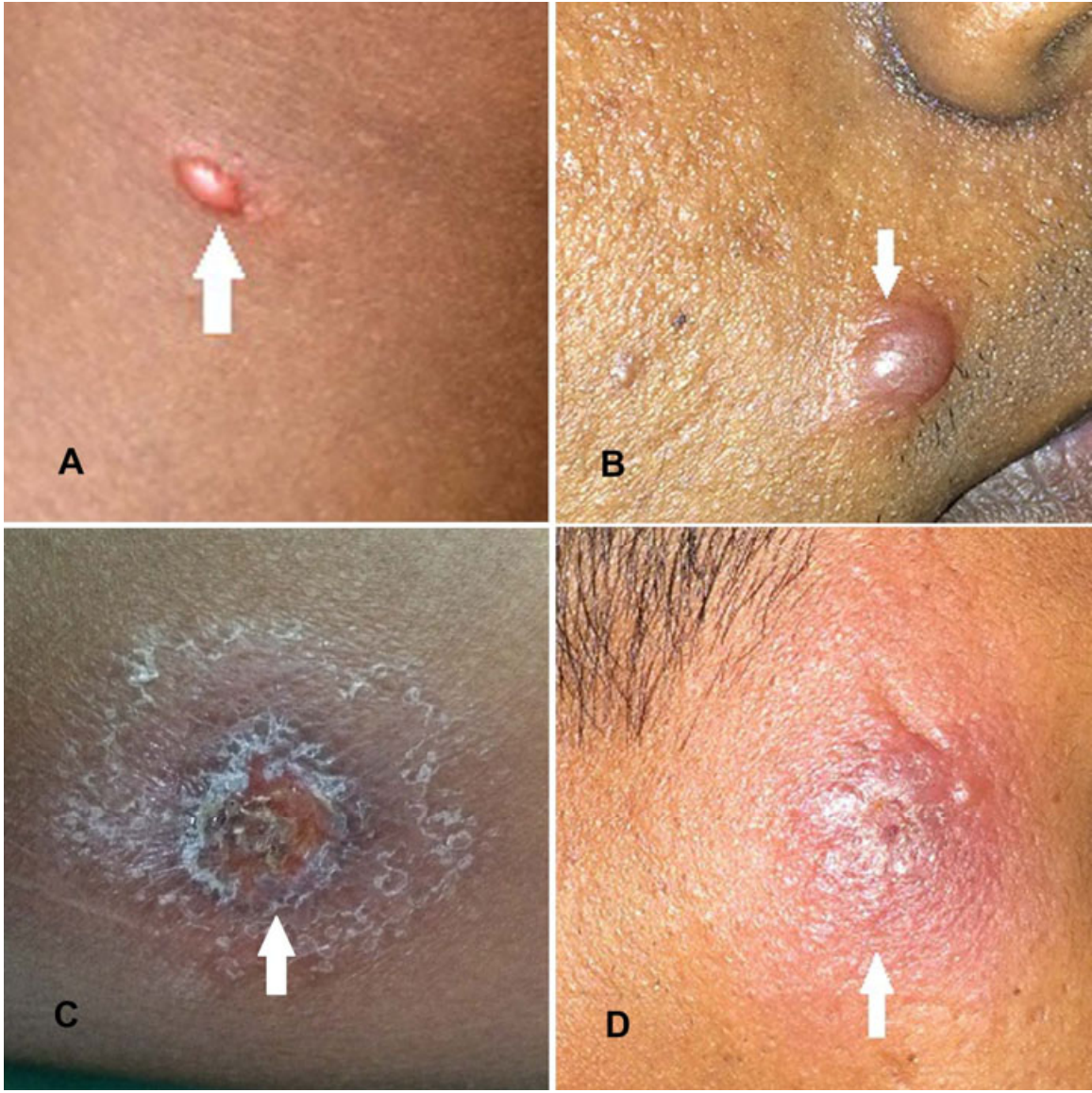
	Group I	Group II	Group III	Group IV
Number of samples	13	17	10	6
Papules	0	0	1	0
Nodules	3	10	3	5
Plaques	2	2	1	0
Ulcers	8	5	5	1
Mean lesion duration in months (SD)	4.6 (2.5)	8.1 (11.6)	11.8 (14.4)	5.7 (4.3)

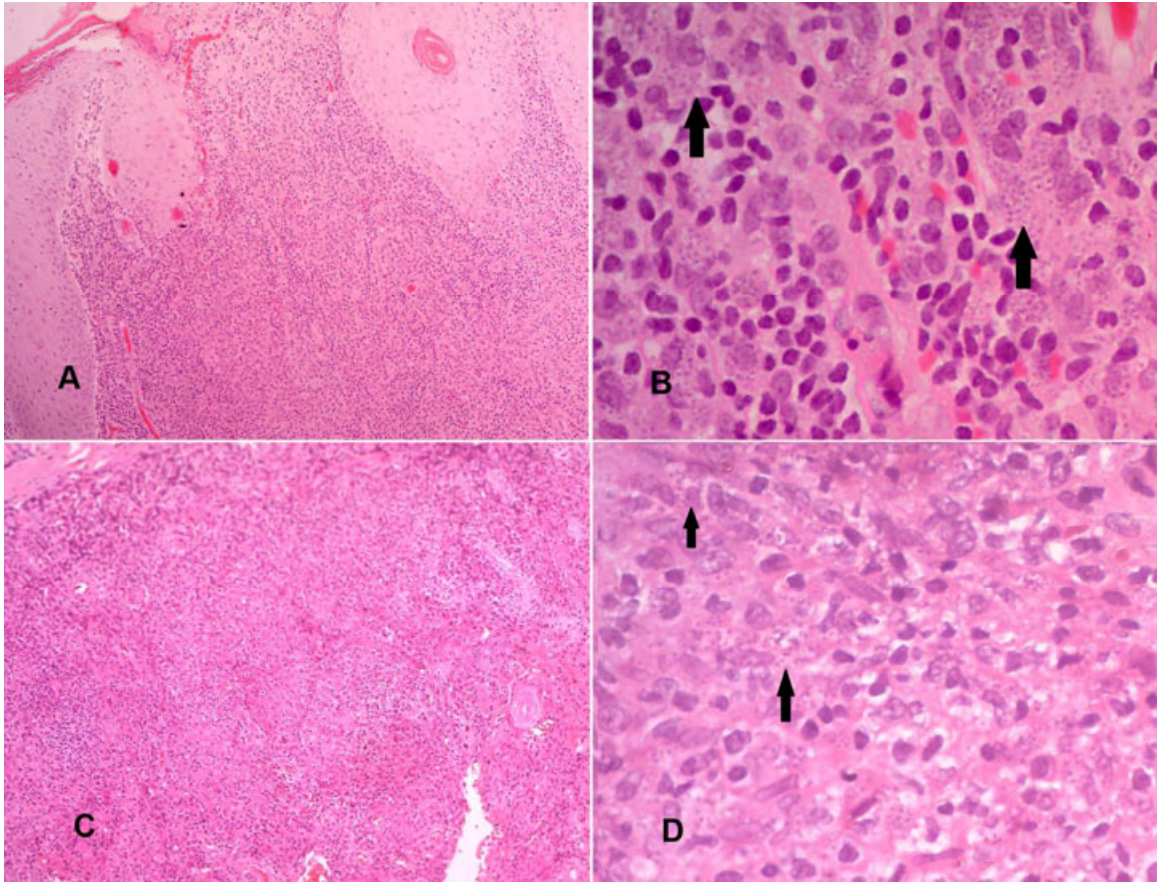
Table 3. Spearman's rank correlation coefficients for intralesional cytokine gene expressions

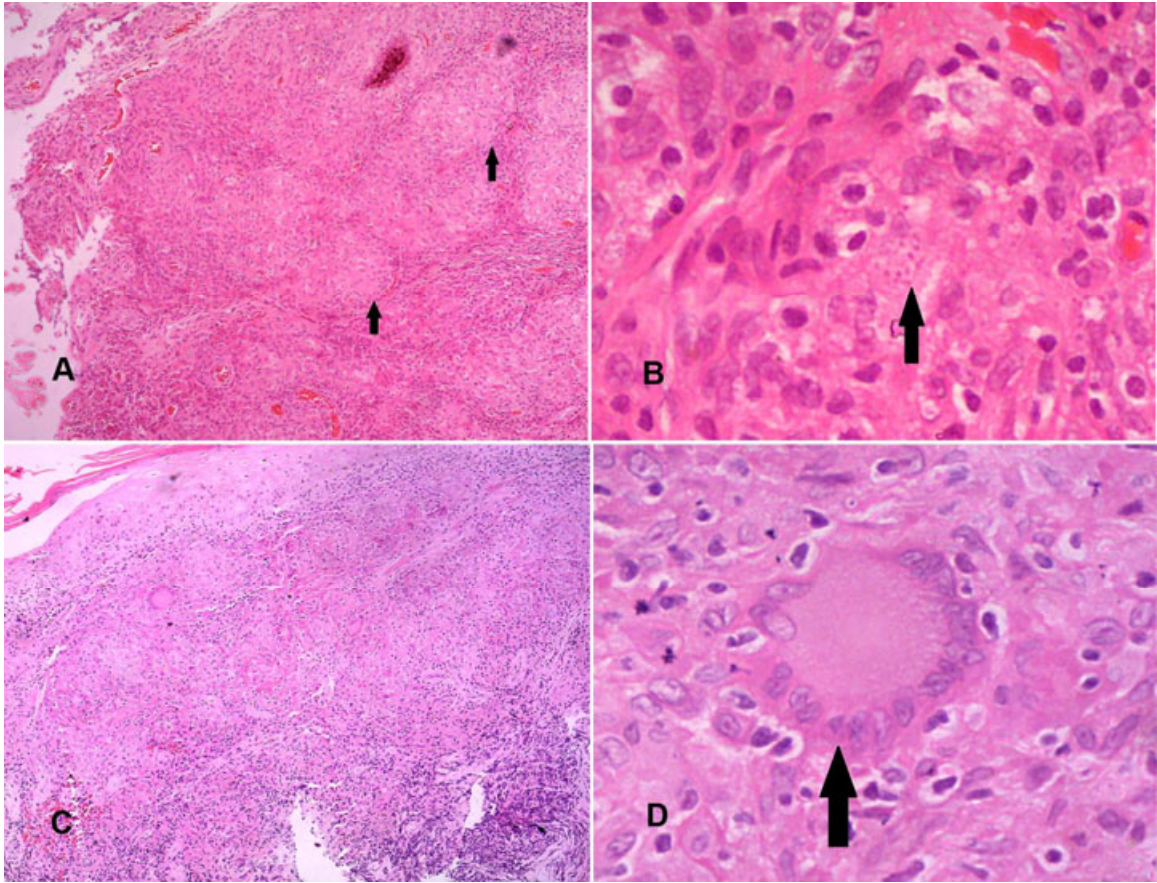
	IL-12A	IFN- γ	TNF- α	IL-4
IFN- γ	0.786 (p <0.001)			
TNF- α	0.594 (p <0.001)	0.679 (p <0.001)		
IL-4	0.565 (p <0.001)	0.615 (p <0.001)	0.395 (p =0.002)	
IL-10	0.507 (p <0.001)	0.597 (p <0.001)	0.485 (p <0.001)	0.659 (p <0.001)

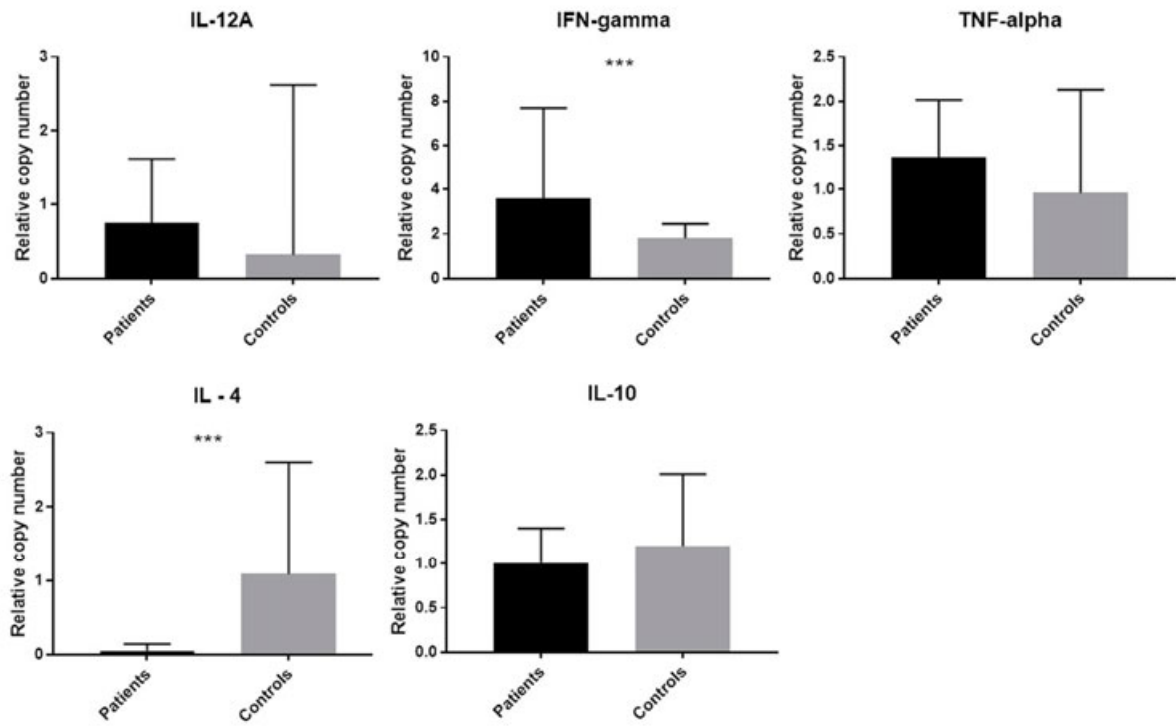
Values indicate correlation coefficients with level of significance in parentheses, determined by the non-parametric Spearman's rank correlation test to calculate the degree of linear correlation among the relative copy numbers for cytokine mRNAs expressed at the site of lesion. A correlation coefficient close to 1 indicate a linear relationship in the expression of the two cytokines.

Abbreviations: IL,interleukin; IFN- γ ,interferon-gamma; TNF- α , tumor necrosis factor-alpha.









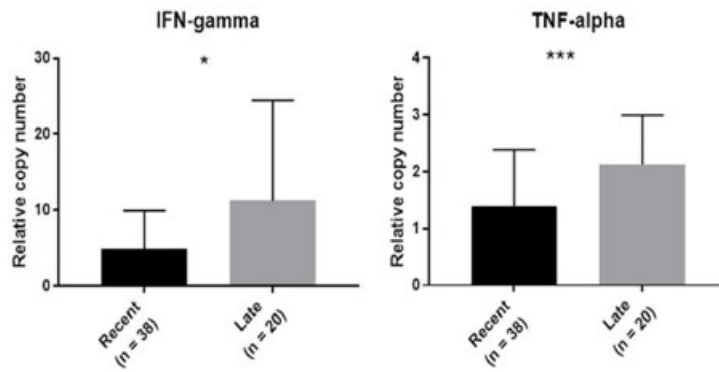


Figure 5. Expression of IFN-gamma and TNF-alpha in recent (< 6 months) and late (≥ 6 months) lesions. Both genes were expressed significantly more in late lesions compared to recent lesions. Graphs represent median relative mRNA copy numbers and interquartile range. (p values as calculated by Mann Whitney U test: * p < 0.05 and ***p < 0.001).