

# Inducible nitric oxide synthase and cytokine pattern in lesions of patients with American cutaneous leishmaniasis

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## Summary

American tegumentary leishmaniasis has three forms: localized (LCL), found in resistant individuals; diffuse (DCL), found in susceptible individuals; and intermediate cutaneous leishmaniasis (ICL), found in individuals with exacerbated immunity. We evaluated cytokines and inducible nitric oxide synthase (iNOS) in lesions of LCL, ICL and DCL using immunohistochemistry. LCL granulomas showed a preponderance of interferon (IFN)- $\gamma$  and interleukin (IL)-12 expression, whereas ICL granulomas had more IL-4-, IL-10- and mainly transforming growth factor (TGF)- $\beta$ 1-expressing cells. Higher densities of iNOS+ cells were observed in ICL and LCL than in DCL. iNOS was also expressed in keratinocytes of LCL and ICL lesions, and in epidermal dendritic cells of ICL lesions. In LCL and ICL, most keratinocytes expressed IL-12 and a portion expressed IFN- $\gamma$ . IL-12+ and IFN- $\gamma$ + dendritic cells were absent or sparse in LCL and ICL epidermis. Our results show the importance of iNOS, IL-12 and IFN- $\gamma$  in LCL and ICL lesions, emphasizing the existence of a mixed cytokine pattern in ICL different from the Th1 and Th2 responses established in LCL and DCL lesions.

American tegumentary leishmaniasis (ATL) has three active forms: localized cutaneous leishmaniasis (LCL), characterized by a Th1 response; diffuse cutaneous leishmaniasis (DCL) characterized by a Th2 response; and mucocutaneous or intermediate cutaneous leishmaniasis (ICL), characterized by a mixed pattern of cytokines.<sup>1,2</sup>

Nitric oxide (NO) and inducible nitric oxide synthase (iNOS) play an important role in the elimination of *Leishmania*. Interferon (IFN)- $\gamma$  drives macrophage activation and parasite death via NO, whereas interleukin (IL)-4 and IL-10 inhibit NO production by murine macrophages.<sup>3</sup> Recently, iNOS has been demonstrated in lesions of human cutaneous leishmaniasis<sup>4,5</sup> In this study, we analysed the local production of iNOS and its relationship with Th1/Th2 cytokine expression in lesions of patients with ATL.

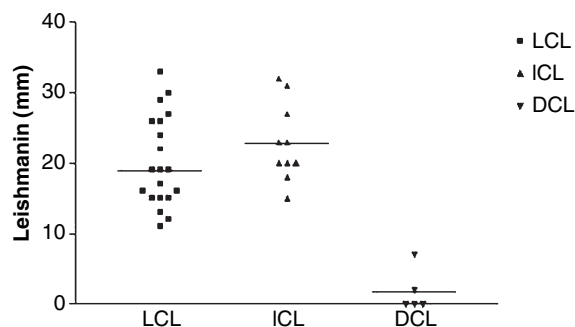
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## Report

Patients with LCL ( $n = 20$ ), ICL ( $n = 10$ ), and DCL ( $n = 5$ ), were diagnosed using established criteria. Leishmanin skin tests were performed by the intradermal injection of autoclaved promastigotes from *L. (L) pifanoi*. Skin biopsies were embedded in a cryopreservation resin, snap-frozen and stored in liquid nitrogen. Cryostat-frozen sections were prepared for immunostaining, performed as previously described.<sup>2</sup> Cells were counted using a light microscope connected to a video monitor, calibrated to determine the number of cells/mm<sup>2</sup>. Only cells with a visible nucleus and showing red immunostaining were counted as positive. Percentages of each phenotype were calculated based on previous counts of the nucleated cells in haematoxylin/eosin sections that showed about 6000 cells/mm<sup>2</sup> of infiltrate in LCL and ICL patients and about 4000 cells/mm<sup>2</sup> in DCL patients. Comparisons between groups were made with nonparametric Mann-Whitney test and Fisher's exact test. Correlations between variables were analysed using the Spearman rank coefficient.



**Figure 1** Size of leishmanin intradermal skin tests in patients with localized (LCL), intermediate (ICL) and diffuse (DCL) cutaneous leishmaniasis.

The leishmanin test was strongly positive in LCL (mean  $\pm$  SEM 20.2  $\pm$  1.5 mm) and ICL biopsies (22.9  $\pm$  1.8 mm), whereas in DCL the intradermal test was negative (1.8  $\pm$  1.3 mm) (Fig. 1).

Immunohistology showed higher numbers of iNOS+ cells in the granulomas of LCL and ICL biopsies (1373  $\pm$  77 and 1222  $\pm$  190 cells/mm<sup>2</sup>, respectively) compared with DCL (631  $\pm$  160 cells/mm<sup>2</sup>) (Table 1, Fig. 2). iNOS expression was also observed in keratinocytes of LCL and ICL specimens (2655  $\pm$  337 and 2916  $\pm$  263 cells/mm<sup>2</sup>, respectively) and in epidermal dendritic cells of ICL (Fig. 2, Table 2).

LCL granulomas showed a preponderance of IFN- $\gamma$  and IL-12 with significant differences ( $P \leq 0.05$ ) and a low density of transforming growth factor (TGF)- $\beta_1$ + cells. ICL lesions showed a mixed pattern of cytokines with a slight increase of IL-4+ and IL-10+ cells. In contrast, in DCL granulomas, TGF- $\beta_1$  expression was most prominent, although IL-4+ and IL-10+ cells were more abundant than IFN- $\gamma$ + and IL-12+ cells (Table 1). In LCL and ICL epidermis, a high percentage of keratinocytes expressed IL-12 (51.50% and 70.76%, respectively) and a portion expressed IFN- $\gamma$  in these lesions (14.83% and 10.63%, respectively) (Fig. 2, Table 2). Low densities or absence of IL-12+ and IFN- $\gamma$ + dendritic cells were observed in LCL and ICL epidermis

(Table 2). The correlation was positive and significant between IFN- $\gamma$  and IL-12 in granulomas of LCL ( $r = 0.7364$ ;  $P = 0.0001$ ) and ICL ( $r = 0.8810$ ;  $P = 0.0072$ ). A similar result was obtained between iNOS and IL-12 in LCL granulomas ( $r = 0.7619$ ;  $P = 0.0062$ ). Moreover, ICL patients showed a positive and significant correlation between iNOS and IFN- $\gamma$  ( $r = 0.8810$ ;  $P = 0.0368$ ), and iNOS and IL-12 ( $r = 0.7619$ ;  $P = 0.0368$ ).

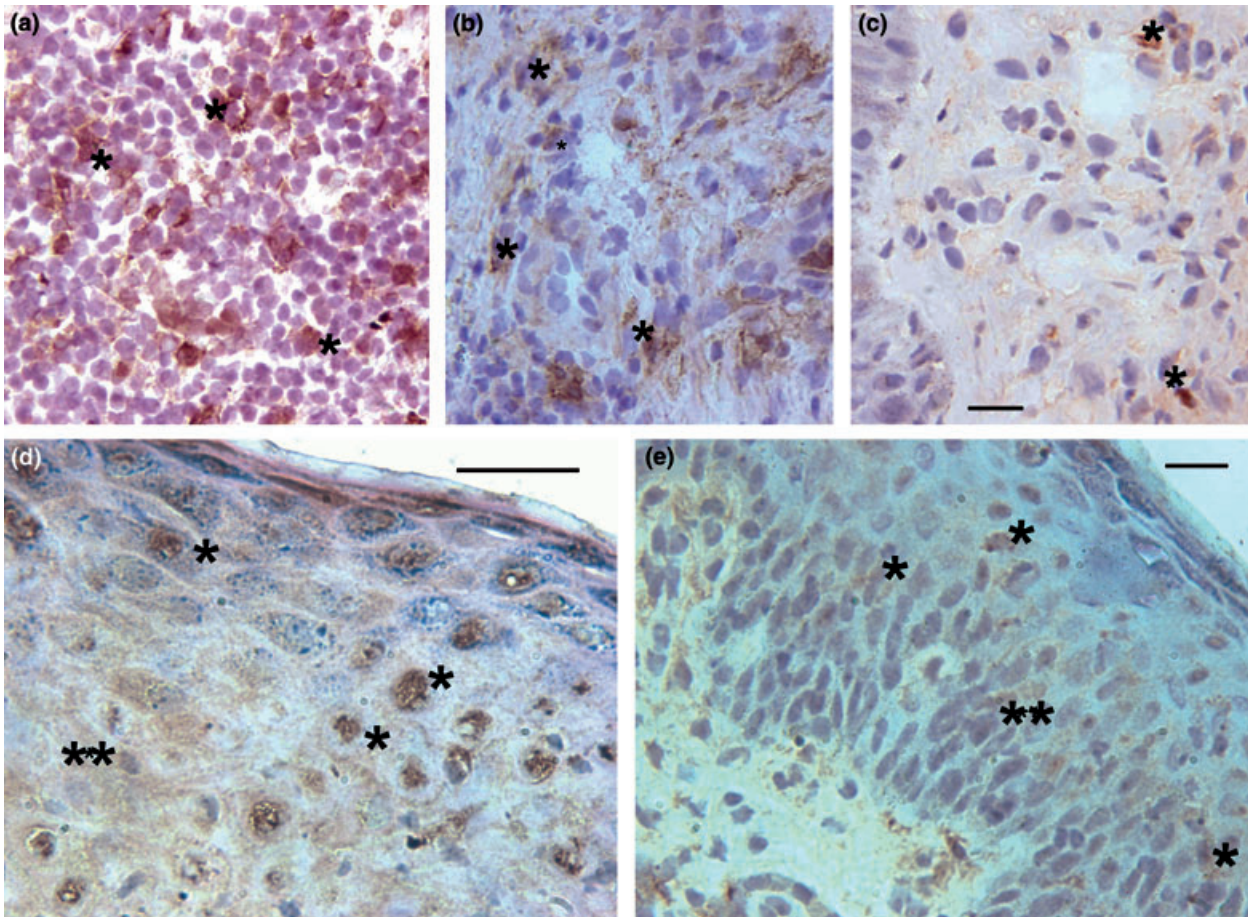
Our iNOS results in the granulomas of ATL coincide with those of Qadoumi *et al.*,<sup>5</sup> showing that iNOS expression is augmented in LCL and diminished in DCL, where parasite burdens are high and very low, respectively. We have also shown that ICL lesions with few parasites have similar iNOS expression to LCL. The role of iNOS activity in the epidermis is unknown. However, NO may serve as a barrier against bacterial invasion and UVA radiation.<sup>6</sup>

Th1 cytokines are involved in NO production, especially IFN- $\gamma$  and TNF- $\alpha$ , which act synergistically to stimulate NO synthesis.<sup>7</sup> This may explain the positive correlation observed between iNOS and IFN- $\gamma$  production in LCL and ICL, and the decreased iNOS expression in DCL lesions due to low production of IL-12 and IFN- $\gamma$ . The positive correlation between IL-12 and IFN- $\gamma$  further confirms the strict bidirectional relationship that exists between these cytokines. IL-12 induces the transcription of IFN- $\gamma$  by NK cells stimulating the differentiation of naive T cells to Th1 cells, and IFN- $\gamma$  produces increased expression of IL-12 receptors by macrophages.<sup>8</sup> The absence of IL-4 during the early stage of *Leishmania* infection triggers an increase in IL-12 and the development of a Th1 response. After the increase of IL-4, levels of IL-12 decrease and a Th2 response prevails.<sup>9</sup> IL-4 plays a key role in suppressing the production of iNOS, thus immobilizing macrophages to destroy parasites. IL-4 expression is not sufficient to develop Th2 responses, which depend on other cytokines that prevent macrophage activation, such as IL-10. DCL patients show low levels of IL-12 in the lesions, probably due to the high production of IL-4 and

**Table 1** Inducible nitric oxide synthase (iNOS) and cytokine-producing cells in the granulomas of patients with localised, intermediate and diffuse cutaneous leishmaniasis

	LCL	ICL	DCL	$P \leq 0.05$
iNOS	1373 $\pm$ 77 (22.88 $\pm$ 1.28)	1222 $\pm$ 190 (20.36 $\pm$ 316)	631 $\pm$ 160 (1051 $\pm$ 2.66)	LCL–DCL, ICL–DCL
IFN- $\gamma$	1151 $\pm$ 78 (19.18 $\pm$ 1.30)	791 $\pm$ 146 (13.18 $\pm$ 2.43)	434 $\pm$ 242 (7.23 $\pm$ 4.03)	LCL–DCL
IL-12	1326 $\pm$ 77 (22.10 $\pm$ 1.28)	831 $\pm$ 155 (13.85 $\pm$ 2.58)	400 $\pm$ 275 (6.66 $\pm$ 4.58)	LCL–DCL
IL-10	495 $\pm$ 51 (8.25 $\pm$ 0.85)	1120 $\pm$ 126 (18.66 $\pm$ 2.10)	863 $\pm$ 474 (14.38 $\pm$ 7.90)	LCL–ICL
IL-4	501 $\pm$ 66 (8.25 $\pm$ 0.85)	1064 $\pm$ 105 (17.73 $\pm$ 1.75)	606 $\pm$ 179 (10.10 $\pm$ 2.98)	LCL–ICL
TGF- $\beta_1$	636 $\pm$ 105 (10.60 $\pm$ 1.75)	998 $\pm$ 134 (16.63 $\pm$ 2.23)	1161 $\pm$ 348 (19.35 $\pm$ 5.80)	LCL–ICL

Values are expressed as cells/mm<sup>2</sup> (mean)  $\pm$  standard error of the mean (respectively, % of the designated cells  $\pm$  SEM).



**Figure 2** iNOS positive cells (\*) in the granulomas of patients with cutaneous leishmaniasis: (a) LCL; (b) ICL; (c) DCL. Scale bar = 10  $\mu$ m. (d) Keratinocytes (\*) and dendritic cells (\*\*) expressing iNOS in the epidermis of LCL. Scale bar = 5  $\mu$ m. (e) Keratinocytes (\*) and dendritic cells (\*\*) producing IFN- $\gamma$  in the epidermis of LCL. Scale bar = 10  $\mu$ m.

**Table 2** Inducible nitric oxide synthase (iNOS) and cytokine-producing cells in the epidermis of patients with localized and intermediate cutaneous leishmaniasis

	LCL	ICL	$P \leq 0.05$
iNOS			
Keratinocytes	2655 $\pm$ 337 (88.50 $\pm$ 11.23)	2916 $\pm$ 263 (97.20 $\pm$ 8.76)	NS
Dendritic cells	Absent	6.3 $\pm$ 6.3 (0.21 $\pm$ 0.21)	NS
IL-12			
Keratinocytes	1545 $\pm$ 521 (51.50 $\pm$ 17.36)	2123 $\pm$ 680 (70.76 $\pm$ 22.66)	NS
Dendritic cells	29 $\pm$ 19 (0.96 $\pm$ 0.63)	Absent	NS
IFN- $\gamma$			
Keratinocytes	445 $\pm$ 185 (14.83 $\pm$ 6.16)	319 $\pm$ 239 (10.63 $\pm$ 7.96)	NS
Dendritic cells	13 $\pm$ 13 (0.43 $\pm$ 0.43)	63 $\pm$ 63 (2.10 $\pm$ 2.10)	NS
IL-10			
Keratinocytes	166 $\pm$ 134 (5.53 $\pm$ 4.46)	327 $\pm$ 154 (10.90 $\pm$ 5.13)	NS
Dendritic cells	9 $\pm$ 6 (0.30 $\pm$ 0.20)	25 $\pm$ 25 (0.83 $\pm$ 0.83)	NS
TGF- $\beta$ 1			
Keratinocytes	142 $\pm$ 97 (4.73 $\pm$ 3.23)	Absent	NS
Dendritic cells	49 $\pm$ 23 (1.63 $\pm$ 0.76)	Absent	NS

Values are expressed as cells/ $\text{mm}^2$  (mean)  $\pm$  standard error of the mean (respectively, % of the designated cells  $\pm$  SEM). NS, not significant.

IL-10. Although in this study we did not analyse disease progression, we did observe increased levels of IL-4+ and IL-10+ cells compared with IL-12+ cells in the lesions of ICL and DCL patients. The higher density of IL-12+ and IFN- $\gamma$ + epidermal cells in LCL patients may result in activation of Th1 responses against the parasite.

We observed high levels of TGF- $\beta$ 1 in DCL compared to LCL lesions, showing a relationship between the susceptibility to infection and TGF- $\beta$ , as previously described.<sup>10</sup> The low production of TGF- $\beta$ 1 by LCL granulomas may be important for the regulation of the immune response, limiting macrophage activation once the parasitic infection is controlled. The results also demonstrate that LCL granulomas have high numbers of iNOS+, IL-12+ and IFN $\gamma$ + cells associated with a Th1 immune response, which drives lesion healing. In contrast, DCL patients have fewer numbers of iNOS+ cells associated with a Th2 response, which maintain a tolerogenic state incapable of controlling the infection, leading to parasite dissemination in the skin.

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