

Differential expression of solute carrier family 11a member 1 and inducible nitric oxide synthase 2 in skin biopsies from leprosy patients

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ABSTRACT

Background: Leprosy is a chronic granulomatous infection caused by *Mycobacterium leprae*, an intracellular parasite that resides within macrophages and cannot be eliminated effectively. Solute carrier family 11a member 1 (Slc11a1) and inducible nitric oxide synthase (iNOS), both expressed in macrophages, play major roles in host defense against several intracellular pathogens. However, the roles of these molecules in natural infection with *M. leprae* remain unknown. **Objective:** We aimed to investigate the expression of Slc11a1 and iNOS in macrophages (CD68+ cells) infiltrating skin lesions in leprosy. **Methods:** Skin biopsies from 48 Mexican patients of leprosy [(33 lepromatous (LL), 15 tuberculoid (TT)] and from 10 healthy controls, were subjected to immunohistochemistry to determine expression of CD68, Slc11a1 and iNOS. **Results:** We found a high expression of Slc11a1 and iNOS in most lepromatous leprosy samples. In tuberculoid leprosy samples, Slc11a1 expression was moderate or low, and that of iNOS was almost always low. In addition, Slc11a1 and iNOS expression levels were positively associated with bacillary loads in lepromatous leprosy lesions ($P = 0.05$). **Conclusions:** These observations suggest that *M. leprae* infection promotes the expression of Slc11a1 and iNOS in macrophages and that lepromatous leprosy can occur despite this response.

Key words: Immune response, macrophage, solute carrier family 11a member 1, inducible nitric oxide synthase, leprosy, lepromatous, tuberculoid, Slc11a1, NRAMP1, iNOS

INTRODUCTION

Leprosy is a chronic infection of skin and peripheral nerves due to *Mycobacterium leprae*. Although its prevalence has decreased, the incidence remains relatively static,^[1] and it is still an important public health problem in some countries around the world. Based on immunological, histopathological and microbiological parameters, leprosy has been classified as lepromatous leprosy, borderline lepromatous,

borderline-borderline, borderline tuberculoid and tuberculoid leprosy.^[2] At opposite ends of the disease spectrum, tuberculoid leprosy typifies a resistant response that restricts growth of the pathogen while lepromatous leprosy represents susceptibility to disseminated infection. These presentations correlate respectively with levels of cell-mediated and humoral immune responses against *M. leprae*.^[3]

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Although infiltration by macrophages is prominent across the disease spectrum, they are well-differentiated and rarely contain bacteria in tuberculoid leprosy, whereas lepromatous leprosy is characterized by abundant intracellular bacilli and foamy macrophage differentiation, the latter due to the accumulation of host- and pathogen-derived lipids.^[4]

In the phagosome, pathogens are normally subjected to massive attack by reactive oxygen and nitrogen intermediates.^[5] This is achieved by two macrophage-specific enzymatic pathways viz., phagocyte oxidase and inducible nitric oxide synthase (iNOS).^[6,7] Macrophage-mediated innate resistance to intracellular pathogens like *Mycobacterium* is affected by a dominant gene named the natural resistance-associated macrophage protein 1 (NRAMP1), known as solute carrier family 11a member 1 (Slc11a1) or Ity/Lsh/Bcg. The function of Slc11a1 as an iron (Fe²⁺) and divalent cation transporter is still controversial. It either increases transphagosomal Fe²⁺ transport, catalyzing the Haber–Weiss/Fenton reaction to generate the highly toxic hydroxyl radical essential for macrophage bactericidal activity, or it reduces the intraphagosomal availability of Fe²⁺ and other divalent cations which are critical for the invading pathogens to survive phagosomal damage.^[8]

We have in previous studies demonstrated a high expression of NRAMP1 in granulomas from cattle naturally infected with *M. bovis*. We also found co-expression of NRAMP1 and iNOS in macrophages from granulomatous lesions in bovines naturally infected with *Mycobacterium avium* subspecies paratuberculosis (Johne's disease).^[9,10] In the present study we investigated the co-expression of Slc11a1 and iNOS in infiltrating macrophages (CD68+ cells) in skin biopsies from leprosy patients. CD68, associated with lysosomal glycoproteins, is a specific marker for human monocytes and macrophages.^[11]

METHODS

The study was carried out at the Dermatology Institute of Jalisco, "Dr. José Barba Rubio," Guadalajara Jalisco México, from 2010 to 2013, and approved by the Research Ethics Committee in Biomedical Sciences (Guadalajara University, Guadalajara, Jalisco, Mexico and the Dermatology Institute of Jalisco "Dr. José Barba Rubio," Guadalajara Jalisco México). The study was in accordance with the guidelines of the

Mexican official standards (Norma Oficial Mexicana) and the World Medical Association Declaration of Helsinki.^[12]

Patients and tissue samples

Tissues samples were obtained from 48 patients with leprosy: 33 cases of lepromatous leprosy and 15 of tuberculoid leprosy, categorized in accordance with the Ridley and Jopling classification, and 10 healthy controls. Patients had not received treatment when biopsies were taken for this study and the time of duration of the disease was unknown in all patients.

Skin samples were fixed in 4% formalin and embedded in paraffin. Sections obtained were stained with hematoxylin/eosin and Kinyoun stains, the latter to detect acid-fast bacilli (bacilli staining red and cells, blue). Histopathologic characterization, carried out by pathologists experienced in evaluating leprosy, was also based on the criteria of Ridley and Jopling.^[13]

Immunohistochemical staining

Serial sections were processed to detect Slc11a1, iNOS and CD68 by immunohistochemistry. Sections were deparaffinized with successive immersions in 100% xylene, 100% ethanol, 96% ethanol and 70% ethanol for 10, 10, 5 and 5 minutes respectively.^[14] Endogenous peroxidase activity was inactivated by incubating with peroxidase blocking reagent (S2001, DAKO) for 10 minutes. The slides were treated to 10 mM citrate buffer (pH 6) and heated to 121°C for 15 minutes. Next, blockade with 50 µl of 1% Bovine Serum Albumin (BSA) (Sigma, USA) in Tris-buffered saline with Tween 20 (TBST) buffer (50 mM, Tris-HCl, 150 mM NaCl, 0.1% Tween 20) for 5 minutes was carried out at room temperature. Sections were then incubated overnight with 40 µl of anti-NRAMP1 (Slc11a1) (Santa Cruz Biotechnology Inc., Cat num. sc-20113) diluted 1:100 (200 µg/µl), anti-iNOS2 (BD Transduction Lab., Cat num. 610332), diluted 1:100 (250 µg/µl), or anti-CD68 (Dako Cytomation, Denmark A/S, M0876) diluted 1:75 (32 mg/L), all diluted in TBST buffer. Subsequently, sections were washed with TBST and each incubated with one drop of secondary antibody conjugated with horseradish peroxidase (K4061, DAKO) for 60 minutes at room temperature. After washing, the sections were each incubated with one drop of chromogenic diaminobenzidine substrate (K3468, DAKO) for 15 minutes at room temperature. They were then counterstained with Mayer's hematoxylin and mounted with an

aqueous-based mounting medium (Vectamount AQ). All sections were developed in parallel with a negative control, where the primary antibody was omitted.

Microscopic analysis

Specimens were analyzed with an optical microscope (Carl Zeiss, Göttingen, Germany) with the ×10, ×20, ×40 and ×100 objectives. Images from the ×40 objectives were captured with an AxioCam MRm digital camera (Carl Zeiss) and recorded with AxioVs40 V 4.8.2.0 software (Carl Zeiss). Fluorescence intensities were rated on an arbitrary scale as follows: low (5–15 cells), moderate (15–50 cells) and high (>50 cells).

Statistics

P values were calculated based on a one-way Chi-square (χ^2) test when patients’ biopsies were compared with controls, and values ≤ 0.05 were considered significant.

RESULTS

The average age of patients was 58 ± 16 years, 41% were females and 49% males and there were 33 lepromatous leprosy (LL) patients and 15 tuberculoid leprosy (TT) patients. Biopsies were classified according to the histology [Table 1]. Since 87% of leprosy patients in Mexico are at the lepromatous pole,^[15] the number of tuberculoid leprosy samples analyzed in this study was relatively low.

Characteristic lesions of leprosy were observed in hematoxylin and eosin–stained slides (not shown). Lymphocytes, epithelioid macrophages and giant cells of the Langhans type were observed throughout the granulomas in tuberculoid leprosy, and foamy macrophages were found in lepromatous leprosy. Kinyoun staining revealed few AFB in tuberculoid leprosy and many bacilli in lepromatous leprosy (not shown). CD68 was expressed by macrophages and macrophage-derived cells including foam cells in lepromatous leprosy [Figure 1c and d], and by giant cells and epithelioid cells in tuberculoid leprosy [Figure 2c and d]. The CD68 immunoreactivity was moderate to strong in both polar forms of leprosy, while no signal was detected in the healthy subjects’ skin biopsies.

A semi-quantitative estimation of the immunolabeling of cells with the anti-Slc11a1 or anti-iNOS antibodies is presented in Table 2. Both were detected in all leprosy

biopsies with varying intensities. The percentage of immunolabeled cells was classified as low: (5–15%), moderate: (16–50%), or high: more than 50%. The number of Slc11a1-labeled foamy macrophages and the intensity of labeling was very high in most of the lepromatous leprosy samples [Figure 1e and f]. However, immunolabeling of Slc11a1 was moderate

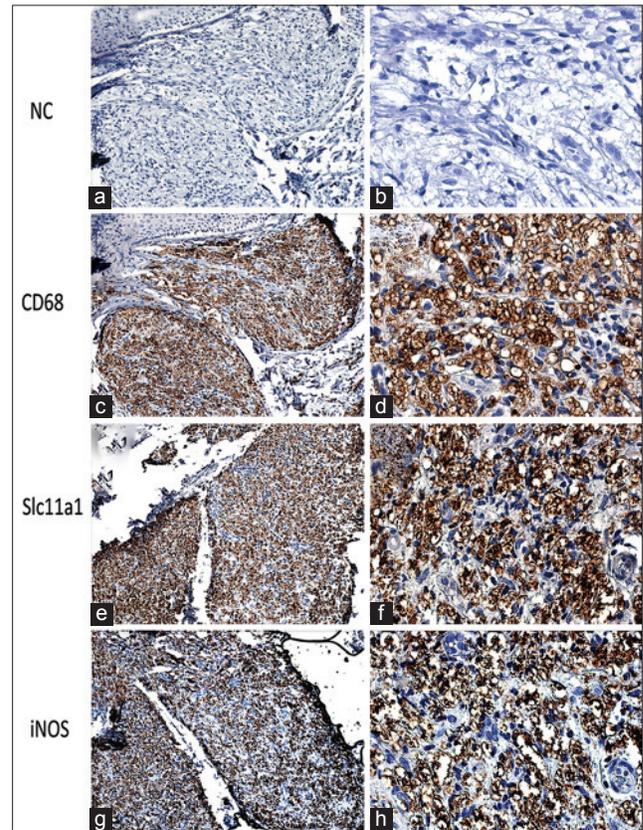


Figure 1: Expression of CD68 (c and d), (e and f) Slc11a1, and (g and h) iNOS in skin biopsies of lepromatous leprosy; (a and b) negative control with primary antibodies omitted. Immunoperoxidase stain, ×200 (a, c, e, g), ×400 (b, d, f, h)

Table 1: Histological features in biopsies from leprosy patients studied

Histological characteristics	n (%)	
	LL Total=33	TT Total=15
Clear subepidermal zone	20 (60)	2 (13)
Epithelioid cells	0 (0)	15 (100)
Non-vacuolated giant cells	1 (3)	15 (100)
Histiocytes/foamy macrophages	31 (94)	0 (0)
Lymphocytes	25 (8)	11 (73)
Bacillary load		
Scarce	0 (0)	15 (100)
Abundant	33 (100)	0 (0)
Dermal nerves infiltrated by bacilli	8 (24)	0 (0)

LL: Lepromatous leprosy, TT: Tuberculoid leprosy

and low in 11 and 3 of the tuberculoid leprosy samples respectively [Figure 2e and f], and high in only one. Immunolabeling of iNOS too followed a similar pattern, being high in 29 of the lepromatous leprosy cases, moderate in 3 and low in only 1 case. In contrast, the expression of iNOS was very low in 11 of the tuberculoid leprosy samples and moderate in the other 4 [Figure 2]. There was a direct relationship between the intensity of Slc11a1 and iNOS immunostaining and the type of leprosy [Table 2; $P = 0.05$].

Biopsies from the 10 healthy controls exhibited little or no immunostaining [Figure 3].

Table 2: Expression of Slc11a1 and iNOS in leprosy skin biopsies

Skin biopsies	Total	Immunolabeling n (%)					
		Low		Moderate		High	
		Slc11a1	iNOS	Slc11a1	iNOS	Slc11a1	iNOS
LL	33	1 (3)	1 (3)	3 (9)	3 (9)	29 (96)	29 (96)
TT	15	3 (20)	11 (73)	11 (73)	4 (26)	1 (6)	0 (0)

Percentage of immunolabeled cells - low: 5-15%, moderate: 16-50%, high: More than 50%. LL: Lepromatous leprosy, TT: Tuberculoid leprosy, Slc11a1: Solute carrier family 11a member 1, iNOS: Inducible nitric oxide synthase

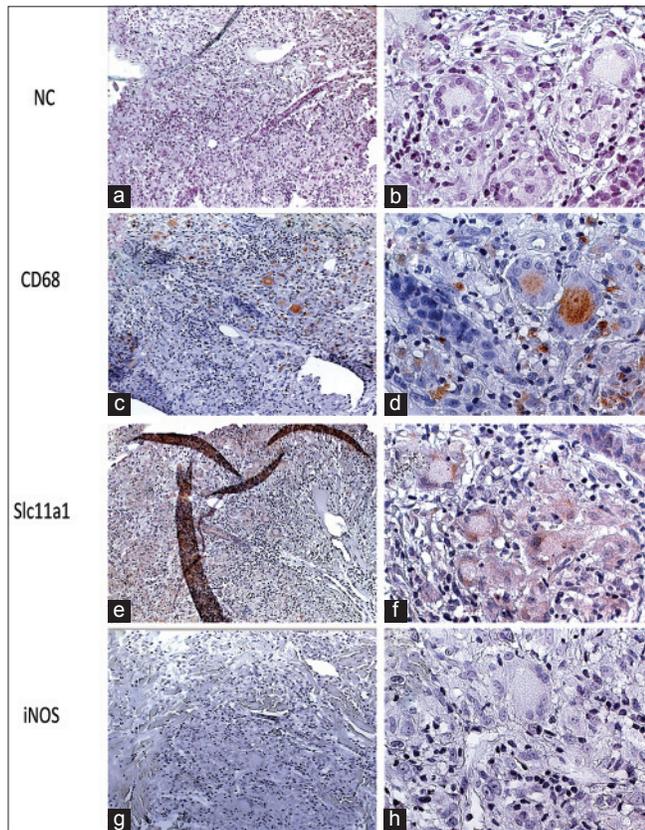


Figure 2: Expression of CD68 (c and d), (e and f) Slc11a1, and (g and h) iNOS in skin biopsies of tuberculoid leprosy; (a and b) negative control with primary antibodies omitted. Immunoperoxidase stain, x200 (a, c, e, g), x400 (b, d, f, h)

DISCUSSION

A recent study showed high expression of iNOS in 78% of leprosy biopsies,^[16] a finding which may be associated with bactericidal or bacteriostatic capacity. In the present study, we found that the bacterial load was directly related to the intensity of immunostaining for iNOS and Slc11a1, but not to that for CD68.

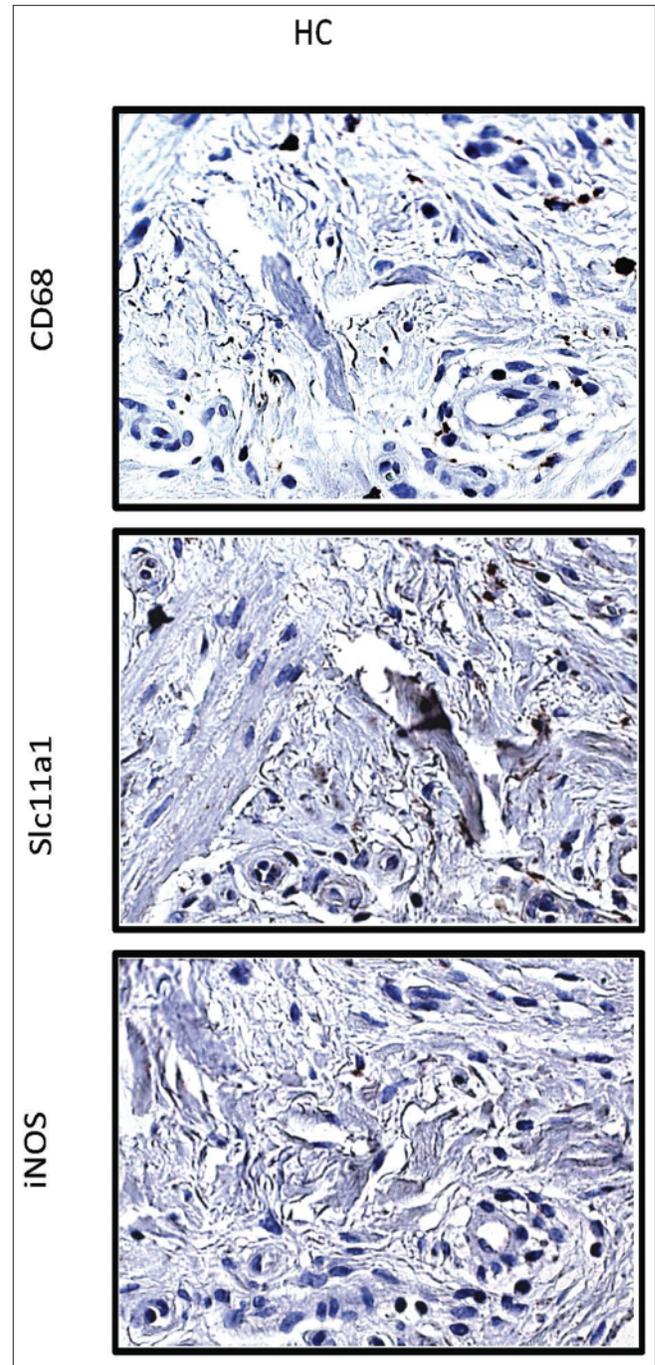


Figure 3: Expression of CD68, Slc11a1, and iNOS in biopsies of healthy skin. Immunoperoxidase stain, x200

Different subpopulations of macrophages display different phenotypes with some having a high capacity for fighting other infections, but low antimycobacterial activity.^[17,18] Patients of tuberculoid and lepromatous leprosy were found to have macrophages that were unable to lyse live *M. leprae*.^[19] A previous analysis of macrophages infiltrating leprosy lesions had also shown altered regulation of phagocytosis and antimicrobial responses.^[20]

Tests of the *M. leprae*-killing ability of resting and gamma interferon-activated macrophages from normal subjects and patients with leprosy suggested that the innate ability of macrophages might be of greater importance in conferring protection against *M. leprae* infection than lymphocyte-mediated activation.^[21] It is likely that infiltrating macrophages in lepromatous leprosy are defective in processing *M. leprae* and antigen presentation.^[22] These defects may in turn reduce the secretion of interferon-gamma and tumor necrosis factor- α and favor a Th2 immune profile as the disease progresses.^[23] Host genetic factors might be important in controlling mycobacterial infections regardless of the bacterial genotype or strain.^[24] Though studied a decade ago, no linkage between leprosy susceptibility and the human *Slc11a1* gene was found in some multi-case leprosy families.^[25] More recently, tuberculoid, but not lepromatous leprosy, was reported to be associated with the *Slc11a1* locus.^[26]

Our results suggest that *M. leprae* infection might upregulate the expression of Slc11a1 and iNOS in lesional foamy macrophages in lepromatous leprosy. This could be mediated by cytokines in the lesional microenvironment. It could also be that iNOS expression is upregulated by Slc11a1. It has been reported that murine macrophages carrying functional Slc11a1 display increased nitric oxide synthesis that is abrogated by iNOS inhibitors while iNOS production is markedly diminished in mice with mutated Slc11a1.^[27]

In view of our findings, NRAMP1 and iNOS could be considered part of the arsenal of antimycobacterial molecules expressed in leprosy lesions. However, upregulated Slc11a1 and iNOS expression may not be sufficient to eliminate the pathogen in lepromatous leprosy. Other important mechanisms in signaling, phagosome formation and maturation contributing

to the bactericidal activity of macrophages were not included in this work. Further studies including several cell markers and examining both skin biopsies and serum samples are needed. Genetic analyses looking at associations between allelic variants of genes codifying Slc11a1 and iNOS and different types of leprosy may help to elucidate their role further.

CONCLUSION

Our observations suggest that *M. leprae* infection promotes the expression of Slc11a1 and iNOS by human macrophages. Their expression being particularly strong in foamy cells suggests that these molecules may be involved in the host defense against *M. leprae*.

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Conflicts of interest

There are no conflicts of interest.

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