LEPROSY REACTIONS: NEW KNOWLEDGE ON PATHOPHYSIOLOGY, DIAGNOSIS, TREATMENT AND PREVENTION

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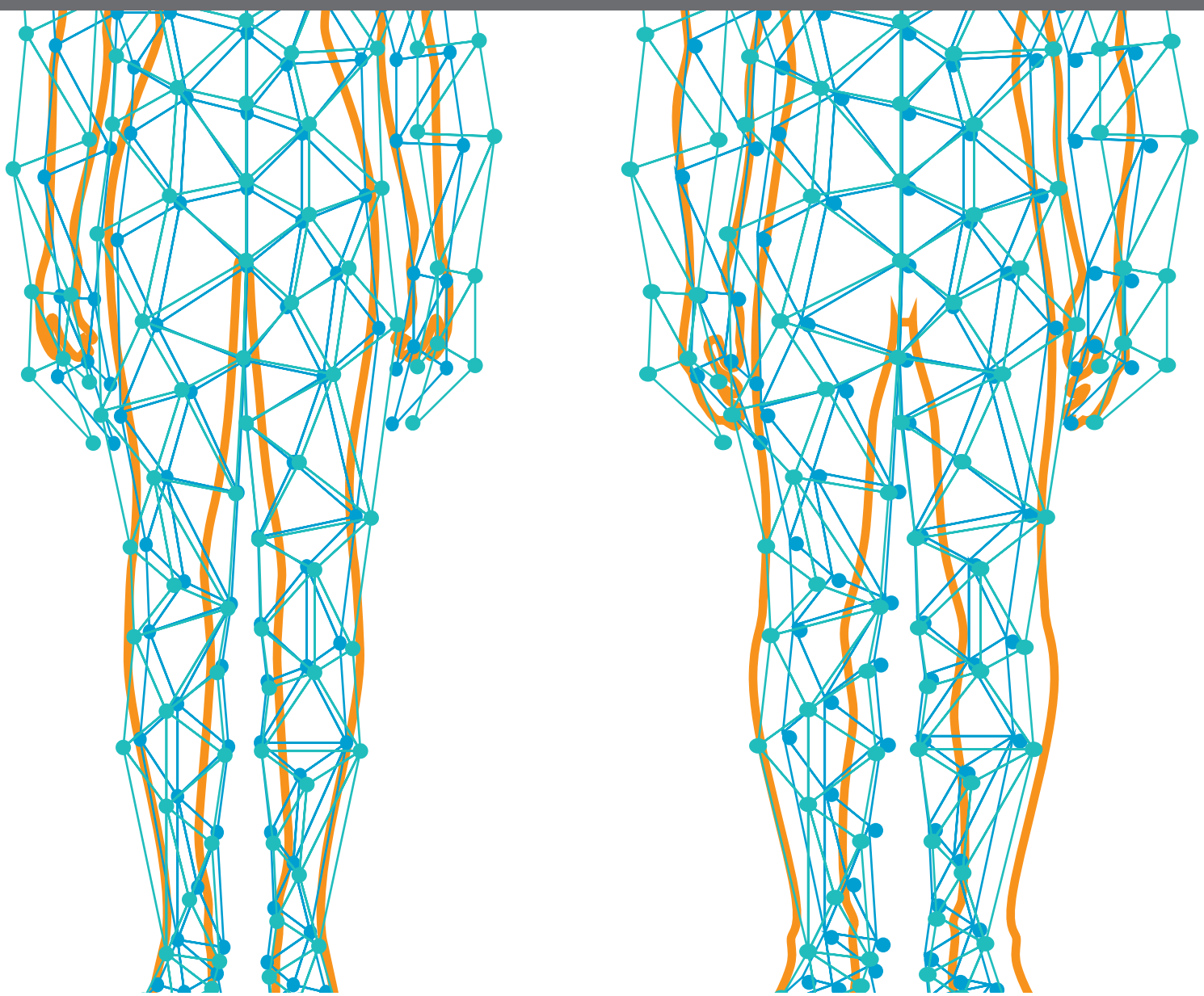

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LEPROSY REACTIONS: NEW KNOWLEDGE ON PATHOPHYSIOLOGY, DIAGNOSIS, TREATMENT AND PREVENTION

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Editorial: Leprosy reactions: New knowledge on pathophysiology, diagnosis, treatment and prevention

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Editorial on the Research Topic
Leprosy reactions: New knowledge on pathophysiology, diagnosis, treatment and prevention

Leprosy reactions may occur during the clinical course of the disease and are associated with an improvement in neural damage that contributes to the deformities and incapacities due leprosy (1–4). Early diagnosis of leprosy and the identification of prognostic factors related to the outcome of leprosy reactions are pivotal for reducing morbidity related to the disease (5–7). The mechanisms related to reactional outcome remain unknown. This is the central idea behind this Research Topic. Here, we selected outstanding papers that evaluated aspects related to early diagnosis of Hansen's disease, predictive markers of reactions and aspects related to diagnosis and treatment.

There is no gold standard test for leprosy diagnosis and the difficulty in distinguishing subclinical or asymptomatic infected individuals from those exhibiting active disease makes leprosy diagnosis essentially based on well-defined clinical signs and symptoms. Clinical examination is important to find out if the patients have any signs of skin or nerve damage. Slit skin smear (SSS) and histopathology are simple and important but require well-trained professionals and may not be available in resource constrained settings. The molecular diagnosis appeared as an adjunct strategy and the literature shows the efficacy of PCR of *M. leprae* DNA in difficult-to-diagnose cases. In this context, Lima et al.(a) measured the accuracy and performance among SSS and PCR of dermal scrapings stored on filter paper and anti-PGL-I serology for leprosy diagnosis showing that PCR combined with serological tests allows for a more sensitive and accurate diagnosis when compared to SSS alone. Since there are not many satisfactory immunoassay methods for leprosy diagnosis, Lima et al.(b) evaluated the use of serology against the mammalian cell-entry 1A (Mce1A) protein that is present in the cell wall of *M. leprae* and is associated with the entry of the bacillus into nasal epithelial cells and skin cells. They demonstrated that the Mce1A antibody profile can be an excellent diagnostic and therapeutic follow-up

method to be used in Hansen's disease. Although serological tests using PGL-I have been used with limitations as a positive test cannot be used as a stand-alone diagnostic test, Antunes et al. demonstrated that anti-PGL-I serology at diagnosis is the most important prognostic factor for leprosy reactions after starting MDT. In this scenario, these data offer knowledge that can be applied in the development of new diagnostic strategies.

Clinical diagnosis of the neural forms of the disease is a challenge. Pure neural leprosy (PNL) is a clinical form in which dermatological signs are absent, but it is less well-understood than the dermatological forms of the disease. Pitta et al. evaluated the occurrence of reactions in PNL patients from a leprosy reference center as well as the occurrence of neuropathic pain. They demonstrated that PNL patients have more neuritis than those with classical leprosy skin reactions and that there is no association between acute neuritis and neuropathic pain. In another perspective, Feitosa et al. described that the pain that occurs in both leprosy reactional patients and patients with fibromyalgia may be a challenge in primary health care and although the leprosy reactional state is not a risk factor for fibromyalgia it can act as a confounder. The correct diagnosis of neuritis and leprosy neuropathy is essential to reduce disability and it certainly has an impact on public health.

The infection of peripheral nerves and neural damage, especially in response to reactional episodes, are hallmarks of leprosy. However, although there is no consensus about the involvement of the bacilli in neural damage, Junqueira de Souza et al. demonstrated that viable and dead bacilli differentially modulate the biology of Schwann cells, which can have implications for the ongoing neuropathy seen in leprosy patients. The understanding of the mechanisms associated with the neural damage will contribute to the development of more effective strategies of control for leprosy neuropathy and its complications. In this context, Pena et al. described the armadillo as a model for *M. leprae*-induced peripheral nerve injury that can provide insights toward the understanding of nerve function impairment progression.

Type 2 reaction or Erythema Nodosum Leprosum (ENL) is an acute and systemic inflammatory episode that may affect patients with the multibacillary form of leprosy. The pathogenesis of ENL is not fully understood and Rosa et al. performed an RNAseq to evaluate the overall gene expression in samples from ENL patients. They demonstrated that type 1 interferon is associated with ENL pathogenesis and that thalidomide, a drug used for ENL management in Brazil, can modulate the expression of several genes of the type 1 IFN pathway, suggesting that this pathway may be targeted for the design of specific, safer, and effective drugs against ENL. In order to elucidate the adaptive immune pathways associated with ENL, Gomes de Castro et al. characterized phenotypically and functionally CD4+ an CD8+ T cells *ex vivo*, comparing cells from non-reactional multibacillary patients with patients with type 1 or type 2 reactions. They observed a decrease in CD4+TGF-β+ Treg and CD8+TGF-β+ Treg in leprosy multibacillary patients during both reactional episodes, suggesting that the onset of reactional episodes involves the downregulation of Treg cells.

Thalidomide is teratogenic and some patients present a chronic and severe ENL which represents an extra challenge for the clinicians. Mendes et al. described their experience with four patients in which anti-TNF therapy was used with successful results, suggesting that anti-TNF may be used as an alternative in patients with chronic and severe ENL who do not respond to traditional treatment.

Reactional episodes are complex, but the understanding of the mechanisms associated with the onset and management of leprosy reactions is pivotal to reduce the morbidity associated with the disease (8, 9). Here, we discuss different aspects regarding reactional episodes, since diagnosis, immunomodulation, therapeutics, and new models to study nerve function impairment are linked to understanding and treating these debilitating episodes. All 10 papers published highlighted certain aspects of leprosy reactions but also point to the potential gaps in our understanding of the mechanisms associated with the establishment of leprosy reactional episodes.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Leprosy Reactions and Neuropathic Pain in Pure Neural Leprosy in a Reference Center in Rio de Janeiro – Brazil

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Introduction: Leprosy reactions are complications that can occur before, during, or after multidrug therapy (MDT) and are considered a major cause of nerve damage. Neuritis is an inflammatory process that causes nerve function impairment associated with pain and tenderness along the nerve. Neuritis can be found in both type 1 and type 2 reactions and may also be the sole manifestation of a leprosy reaction. The objective of this study is to describe the incidence of leprosy reactions and its association with neuropathic pain in pure neural leprosy (PNL) patients.

Methods: We selected 52 patients diagnosed with PNL and 67 patients with other clinical forms of leprosy. During the MDT the patients visited the clinic monthly to take their supervised dose. The patients were instructed to return immediately if any new neurological deficit or skin lesions occurred during or after the MDT.

Results: Of the PNL patients, 23.1% had a leprosy reaction during or after the MDT, while this was 59.7% for patients with the other clinical forms of leprosy. There was an association between having PNL and not having any reaction during and after the MDT, as well as having PNL and having neuritis after the MDT.There was also an association between having previous neuritis and having neuropathic pain in the other clinical forms of leprosy group, although this association was not present in the PNL group.

Discussion: Our data suggest that PNL is a different form of the disease, which is immunologically more stable. In addition, PNL patients have more neuritis than the classical leprosy skin reactions. In PNL there was no association between acute neuritis and neuropathic pain, suggesting that these patients may have had silent neuritis. Understanding and identifying neuritis is essential to reduce disability and the impact on public health.

Keywords: leprosy, pure neural leprosy, leprosy reactions, neuritis, neuropathic pain

INTRODUCTION

Leprosy is usually known for its skin lesions, but neural abnormalities are another hallmark of the disease and the basis of leprosy-associated disability (1). Despite advances in the treatment of leprosy with the introduction of multidrug therapy (MDT) in the 1980s by the WHO, leprosy and its related disability are still prevalent worldwide (2).

Leprosy reactions are complications that can occur before, during, or after the MDT and are considered a major cause of nerve damage (3–6). They represent acute changes in the host immune response to *Mycobacterium leprae* and are thought to occur in 30–50% of all leprosy patients (2, 3). They are classically categorized in two subgroups according to the clinical and immunological presentation. Type 1 reactions usually have the development of an inflammatory response in the skin or nerves and are thought to occur in borderline leprosy patients, whose immunological status is unstable. Type 2 reactions are known to cause painful erythematous subcutaneous nodules as well as systemic symptoms, occur mainly in lepromatous leprosy, and are thought to be primarily humoral mediated (5, 6).

In the clinical evaluation, neuritis is an inflammatory process that affects the nerves and causes nerve function impairment associated with pain and tenderness along the nerve (3, 5, 6). It can be found in both type 1 and type 2 reactions and may also be the sole manifestation of a leprosy reaction, as an isolated neuritis without skin lesions (2, 4, 7–9). Nerve function impairment without pain and tenderness has been described as silent neuritis and may be present in a proportion of leprosy patients associated or not with skin reactional episodes (10). The presence of demyelinating features in nerve conduction studies (NCS) has already been described as occurring during neuritis episodes (6, 11, 12).

Silent or acute neuritis can result in nerve damage that may ultimately lead to the fearful disabilities (2). Neuropathic pain is characterized as a neural pain associated to dysfunction of the peripheral or central nervous sensory system that can be a consequence of neuritis (13). It has been estimated that there is an annual prevalence of 15% of neuropathic pain in leprosy patients and, although not usually included in the disability evaluation, this has a huge impact on the patients' quality of life (13).

There are few data regarding leprosy reactions in patients with pure neural leprosy (PNL), a rare form of the disease that presents with nerve function impairment without skin lesions (14). The objective of this study is to describe the incidence of leprosy reactions and its association with neuropathic pain in leprosy patients.

PATIENTS AND METHODOLOGY

The patients in our study were selected from the database of the Souza Araujo Outpatient Clinic, at the Oswaldo Cruz Institute, a referral center for leprosy in Rio de Janeiro, Brazil. The study was approved by the Research Ethics Committee of the institution. We selected the 52 patients diagnosed with PNL between 1998 and 2016 that had neurological examination performed by a neurologist at the moment of diagnosis and at the end of the MDT, as well as NCS before the diagnosis. All of the PNL patients were evaluated by dermatologists who excluded the presence of skin lesions and were submitted to a nerve biopsy for diagnosis. The biopsied nerve was chosen accordingly to clinical and NCS impairment The methodology and criteria used for the histopathological diagnoses were those described by Antunes et al. (15). The PCR for *M. leprae* DNA and the detection of the antibodies against phenolic glycolipid-I (PGL-1) were done as the procedures described in Jardim 2003 and Jardim 2005, respectively (14, 16). All of the patients registered in the outpatient clinic in 2013 with the diagnosis of other clinical forms of leprosy were also selected. The diagnosis of these 67 patients was based on the positivity of slit-skin smear and skin biopsies, and the classification was made by a dermatologist according to the Ridley-Jopling system: tuberculoid (TT), borderline-tuberculoid (BT), borderline-borderline (BB), borderline-lepromatous (BL), lepromatous (LL), and undetermined. At the moment of the diagnosis the grade of disability of the patients was evaluated by a physiotherapist with experience in leprosy and based on WHO recommendations (17).

For treatment purposes, the patients with PNL were classified as either paucibacillary (PB) or multibacillary (MB) depending on the presence of bacilli in the slit-skin smears until 2005. After that, our reference center began to classify PNL patients as MB when acid-fast bacilli (AFB) were detected in the nerve biopsy.

During the MDT the patients had monthly visits to the clinic when they would take their supervised dose. A type 1 reaction was defined as an increased inflammation of existing lesions with or without new non-painful lesions and/or edema of the extremities. A type 2 reaction was defined as the sudden appearance of inflamed papules, nodules, and plaques sensitive to palpation. Leprosy reactions were always evaluated by a dermatologist and a type 1 or type 2 leprosy reaction was only considered in the presence of skin lesions. All of the patients with neural symptoms were evaluated by a neurologist and when neuritis was suspected the patient was submitted to NCS using the procedures described by Vital et al. (18). The patient was considered as having neuritis when there was pain and tenderness of the nerve together with demyelinating features like reduced motor conduction velocities, presence of conduction block, or abnormal temporal dispersion. The patients were instructed to return immediately in the case of any new neurological deficit or skin lesions during or after the MDT. All of the patients with leprosy reactions were treated with corticosteroids. Neuropathic pain was always evaluated by a neurologist, and its diagnosis was based on the presence of pain in a neuro-anatomically plausible area with confirmed negative or positive sensory signs (13).

Statistical analysis was performed by the Pearson's chi-square and Fisher's exact tests for categorical variables and Mann-Whitney test for continuous variables using the *Statistical Package for the Social Sciences* (SPSS) v16.0. A significance level of 5% was adopted.

TABLE 1 | Number of patients with pure neural leprosy and other clinical forms with leprosy reactions and neuritis during the multidrug therapy.

		PNL	Other clinical forms	Total
No leprosy reaction		**46 (88.5%)**	33 (49.3%)	79
Leprosy reaction	Type 1 reaction	2 (3.8%)	**14 (20.9%)**	16
	Type 2 reaction	0	7 (10.4%)	7
	Neuritis	**3 (5.8%)**	2 (3.0%)	5
	Type 1 reaction with neuritis	1 (1.9%)	7 (10.4%)	8
	Type 2 reaction with neuritis	0	4 (6.0%)	4
	Total	52	67	119

PNL, Pure neural leprosy.

TABLE 2 | Number of patients with pure neural leprosy and other clinical forms with leprosy reactions and neuritis after the multidrug therapy.

		PNL	Other clinical forms	Total
No leprosy reaction		**45 (86.5%)**	45 (67.2%)	90
Leprosy reaction	Type 1 reaction	0	6 (9.0%)	6
	Type 2 reaction	0	**9 (13.4%)**	9
	Neuritis	**6 (11.5%)**	0	6
	Type 1 reaction with neuritis	1 (1.9%)	5 (7.5%)	6
	Type 2 reaction with neuritis	0	2 (3.0%)	2
	Total	52	67	119

PNL, Pure neural leprosy.

TABLE 3 | Number of patients with pure neural leprosy and other clinical forms of leprosy that developed acute neuritis during the evaluation period and number of patients that developed neuropathic pain.

			Acute neuritis		*p*-value
			No	Yes	
PNL	Neuropathic pain	No	32 (61.5%)	7 (13.4%)	0.697
		Yes	**10 (19.2%)**	3 (5.7%)	
Other clinical forms	Neuropathic pain	No	49 (73.1%)	5 (7.4%)	<0.001
		Yes	5 (7.5%)	**8 (11.9%)**	

PNL, Pure neural leprosy.

RESULTS

For this study, 52 patients with PNL and 67 patients with other clinical forms of leprosy were selected. Of the PNL patients, 98.1% (51 patients) were treated with PB-MDT and 1.7% (1 patient) were treated with MB-MDT. In the other clinical forms group, 50.7% (34 patients) were treated with PB-MDT and 49.3% (33 patients) with MB-MDT. In the PNL group, the mean age of patients at diagnosis was 47 years and the median was 46 years; while in the other clinical forms group, the mean age of the patients was 42 years and the median was 39 years ($p = 0.086$). In the PNL group, 73.1% (38 patients) were male and 26.9% (14 patients) were female; in the other clinical forms group, 58.2% (39 patients) were male and 41.2% (28 patients) were female ($p = 0.097$). The patients with other clinical forms were classified as follows: 3% as TT (2 patients); 43.3% as BT (29 patients); 10.4% as BB (7 patients); 19.4% as BL (13 patients); 20.9% as LL (14 patients); and 3% as undetermined. The mean follow-up period for the PNL group was 188 months (ranging from 47 to 260 months) and for the other clinical forms group the mean follow-up period was 93 months (ranging from 89 to 98 months).

Reactional Episodes

Patients with PNL and those with other clinical forms of leprosy were compared in relation to the presence of reactions during and after the MDT. Of the PNL patients, 12 (23.1%) had a leprosy reaction during or after the MDT, while 40 patients (59.7%) of the other clinical forms group had a leprosy reaction. Pearson's chi-square confirmed that there was an association of having other clinical forms of leprosy and having leprosy reactions during or after the MDT ($p < 0.001$). The greater incidence of reactions during or after the MDT was present for the BB, BL and LL forms ($p < 0.001$). When comparing the PNL group with the TT and BT groups there was no difference in the incidence of leprosy reactions ($p = 0.606$). The number of patients in each group during the MDT is shown in **Table 1** and after the MDT in **Table 2**.

Fisher's exact test confirmed that there was association between having PNL and not having any reaction and an association between having other clinical forms of leprosy and having type 1 and type 2 reactions during and after the MDT ($p<0.001$). Fisher's exact test also confirmed an association between having PNL and having neuritis after the MDT ($p < 0.001$). There was no association between any clinical form and having type 1 and type 2 reactions together with neuritis.

Neuropathic Pain

At the moment of leprosy diagnosis, in the PNL group 11.5% (6 patients) had neuropathic pain, while this was only 2.9% (two patients) for the other clinical forms group. Despite this, Fisher's exact test did not show statistically significant association between the clinical form and the presence of neuropathic pain at diagnosis ($p = 0.078$).

The mean time between the beginning of the MDT and the emergence of neuropathic pain was 32 months and the median was 8 months (min 0, maximum 156 months) in the PNL group. In the other clinical forms group, the mean period was 12 months and the median was 8 months (minimum 0, maximum 53 months) ($p = 0.254$).

In terms of neuropathic pain, 13 patients (25%) in the PNL group and 13 patients (19.2%) in the other clinical forms group presented this after the MDT. In the PNL group, Fisher's exact test confirmed an statistically significant association between

having more than one nerve affected at neurological examination and developing neuropathic pain ($p = 0.044$). Fisher's exact test confirmed an association between having previous neuritis during or after the MDT and having neuropathic pain in the other clinical forms of leprosy group. The association was not present in the PNL group. The number of patients with neuritis and the patients that developed neuropathic pain in both groups are reported in **Table 3**.

Histopathological Correlations

When evaluating the nerve biopsies of the PNL group that were made for the diagnosis, 7.6% (four patients) had the presence of AFB and epithelioid granuloma. Fisher's exact test did not show statistically significant association between these histopathological signs and neuritis during or after the MDT ($p = 1.000$ and $p = 0.450$) or with neuropathic pain at diagnosis ($p = 1.000$).

All of the PNL patients and 62 of the patients with other clinical forms had their degree of leprosy-related disability evaluated at diagnosis. Of these, 41 (78.8%) of the PNL patients and 25(40.3%) of the patients with other clinical forms had disabilities. Person's chi-square confirmed an association between having PNL and having any degree of disability ($p < 0.001$).

DISCUSSION

Leprosy reactions are inflammatory episodes related to changes in the immune response of the patients and are thought to be one of main causes for nerve damage in leprosy (2). PNL is a form of the disease characterized by the absence of skin lesions and a negative slit-skin smear (14, 19). PNL is a still poorly understood form of the disease and there are few data regarding the presence of leprosy reactions in these patients.

Leprosy reactions are present in 30–50% of all leprosy patients and can present in any moment of the disease (2, 8). In our sample, the groups with other clinical forms of leprosy had a frequency of leprosy reactions within the range that is described in the literature. However, the frequency was much lower in the PNL group, which could suggest that this is a different form of the disease. This difference was only observed when specifically comparing the PNL group to the BB, BL, and LL patients. This data could suggest that PNL patients may be similar to the patients in the tuberculoid pole, the TT and BT patients. The classification system proposed by Ridley and Jopling is based on the immune response to the bacilli and does not include PNL (20). This greater immune response to the bacilli could explain the fact that most PNL patients, despite having neuropathy symptoms for a long time before diagnosis, do not have episodes of pain and tenderness along the nerve. A type 2 leprosy reaction is usually observed in patients in the lepromatous pole of the disease and associated with a higher burden of bacilli in slit-skin smear (2, 8). None of the patients in the PNL group had type 2 leprosy reactions, even when they had more extension of nerve lesions.

Both type 1 and type 2 reactions are associated with nerve damage and neuritis, but neuritis can also appear alone as an isolated neuritis (2, 4, 7–9). In our sample, in the other clinical forms of leprosy, neuritis alone was an uncommon finding, but in the PNL group it was more common than the neuritis associated with a type 1 reaction. It has been described that genetic variability may be responsible for the variable clinical phenotypes of leprosy (1, 2). Our data suggest that the reactional episodes in PNL patients are also limited to the peripheral nerve. It is not known what causes the disease to stay restricted to the nerve at presentation, but the same mechanisms may be responsible for this in the reactional episodes. It has also been described that while the MDT is capable of killing the *M. leprae*, it still leaves dead bacterial cells within the nerve (7). These fragments may be responsible for triggering new episodes of neuritis, especially in the PNL group where the disease was present only in the nerve.

The term neuritis has been used to describe nerve function impairment associated with nerve pain and tenderness associated to demyelinating features in the NCS (3, 6, 11, 12). However, the existence of silent neuritis, where the patient has nerve function impairment without pain, has been well-described in the leprosy literature (10). The majority of patients in our sample did not present clinical signs and symptoms of acute neuritis. If we consider that all of the PNL patients have nerve function impairment caused by the disease, associated or not with nerve pain and tenderness, we could suggest that the majority of these PNL diagnosed patients may have had silent neuritis prior to the diagnosis.

Neuropathic pain is described as increased pain sensitivity or spontaneous pain caused by lesions or diseases involving the somatosensory system (21). In our sample, in both groups, neuropathic pain was more common after the MDT, as was previously suggested by other authors (22). Our data also suggest that in the other clinical forms of leprosy group there is an association between previously having neuritis and having neuropathic pain, since these patients were diagnosed based on skin lesions and most of them did not have any neural symptom prior to the diagnosis. This is unlike the patients with PNL, who have nerve function impairment and therefore may be susceptible to neuropathic pain.

The prevalence of neuropathic pain in our PNL group was lower than the 60% prevalence described in diabetic neuropathy (23). However, the neuropathy severity is thought to be one of the risk factors for neuropathic pain in diabetic neuropathy (23). The statistically significant association between having more than one nerve clinically affected and developing neuropathic pain may also suggest that this may also be true in PNL. Since most of our PNL patients have a small number of affected nerves, this could be one of the explanations for the lower prevalence of neuropathic pain.

It has been suggested that the presence of both AFB and epithelioid granuloma in the same biopsy specimen may indicate reactional neuritis (15, 24). In our PNL patient group, only 7.6% had these features in the nerve biopsy, which can be explained by the fact that they did not have acute neuritis at the moment when the biopsy was conducted. The lack of association between the histopathological neuritis and the presence of neuropathic pain at the diagnosis may be considered another sign that acute neuritis is not the only

mechanism involved in the generation of neuropathic pain in PNL.

Although leprosy reactions are thought to be one of the greatest causes for disability in leprosy (2, 3), patients with PNL had a greater burden of disability than the other clinical forms despite the lowest incidence of leprosy reactions. This could be explained by the fact that in all of these patients the *M. leprae* initially targets the Schwann cell within the peripheral nerve. Although the host immune response has a critical role in the neural damage in leprosy, it has already been described that the *M. leprae* itself may initiate nerve damage, even in the absence of the host inflammatory response (1, 7).

The term neuritis has been used in numerous ways in the leprosy literature, sometimes describing the acute episode, as in a leprosy reaction, and sometimes as the silent nerve impairment caused by the disease (13). Limitations are present in this study, including the fact that the data is retrospective. Nevertheless our study showed that in PNL during reactional episodes, acute neuritis is usually easily diagnosed, but silent neuritis is still under recognized. The identification and understanding of silent neuritis is of great importance as it may help reduce the physical disability, and could also the pain-related disability that may have economic and social impacts.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee of the Oswaldo Cruz Foudation (Fiocruz). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

IP: conceptualization, data curation, investigation, formal analysis, and writing. MH: conceptualization, data curation, formal analysis, and writing. RV, LA, CS, AS, and SA: investigation. ES: conceptualization and investigation. MJ: conceptualization, investigation, writing, and supervision. All authors contributed to the article and approved the submitted version.

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Gene Expression Profile of *Mycobacterium leprae* Contribution in the Pathology of Leprosy Neuropathy

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Peripheral neuropathy is the main cause of physical disability in leprosy patients. Importantly, the extension and pattern of peripheral damage has been linked to how the host cell will respond against *Mycobacterium leprae* (*M. leprae*) infection, in particular, how the pathogen will establish infection in Schwann cells. Interestingly, viable and dead *M. leprae* have been linked to neuropathology of leprosy by distinct mechanisms. While viable *M. leprae* promotes transcriptional modifications that allow the bacteria to survive through the use of the host cell's internal machinery and the subvert of host metabolites, components of the dead bacteria are associated with the generation of a harmful nerve microenvironment. Therefore, understanding the pathognomonic characteristics mediated by viable and dead *M. leprae* are essential for elucidating leprosy disease and its associated reactional episodes. Moreover, the impact of the viable and dead bacteria in Schwann cells is largely unknown and their gene signature profiling has, as yet, been poorly explored. In this study, we analyzed the early differences in the expression profile of genes involved in peripheral neuropathy, dedifferentiation and plasticity, neural regeneration, and inflammation in human Schwann cells challenged with viable and dead *M. leprae*. We substantiated our findings by analyzing this genetic profiling in human nerve biopsies of leprosy and non-leprosy patients, with accompanied histopathological analysis. We observed that viable and dead bacteria distinctly modulate Schwann cell genes, with emphasis to viable bacilli upregulating transcripts related to glial cell plasticity, dedifferentiation and anti-inflammatory profile, while dead bacteria affected genes involved in neuropathy and pro-inflammatory response. In addition, dead bacteria also upregulated genes associated with nerve support, which expression profile was similar to those obtained from leprosy nerve biopsies. These findings suggest that early exposure to viable and dead bacteria may provoke Schwann cells to behave differentially, with far-reaching implications for the ongoing neuropathy seen in leprosy patients, where a mixture of active and non-active bacteria are found in the nerve microenvironment.

Keywords: leprosy, *Mycobacterium leprae*, peripheral nervous system, Schwann cell, host-pathogen interaction

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INTRODUCTION

Leprosy neuropathy is a chronic neurological condition, caused by the infection of the nerve by its etiologic agents, *Mycobacterium leprae* and *Mycobacterium lepromatosis* (1–5). *M. leprae* infection provokes early pathological changes in the host cell that are, to some extent, associated with the late degenerative appearance of the infected nerves (6, 7). Schwann cells, the glial cells of the peripheral nervous system, are the preferable host for *M. leprae* entry, persistence, and replication within the nerve (8–11). Therefore, much attention has been given to the molecular and cellular alterations driven by leprosy bacilli once inside Schwann cells in order to identify the underlying reasons that culminate in the severe neuropathy seen in patients.

The immune response elicited in the nerve microenvironment against the bacilli is a key component that may lead to the distinct clinical manifestations (4). Infected Schwann cells produce a broad panel of inflammatory chemokines and cytokines, that accompanied with cell-mediated immune response, have been linked to the manifestation of neural pain and leprosy neuritis in patients (12–15). Of importance, this immunomodulation profile seen in infected Schwann cells was reported to occur before the reprogramming of the Schwann cells to the immature phenotype, highlighting the crucial role of the immune signaling network in the context of the early stages of *M. leprae* infection (16, 17). Additionally, *M. leprae* infection of Schwann cells has been associated with alterations in the glucose/lactate metabolic pathway (18, 19), lipid/cholesterol accumulation (20, 21), mitochondrial dysfunction (11), and myelin dismantling (22–24). Some of these changes were also confirmed in leprosy patients, and are suggested to cause the ongoing neuropathy and the observed tissue fibrosis and loss of nerve function experienced by leprosy individuals (14, 25). However, while these events may arise from modifications of the host Schwann cell's supportive function of the nerve, the accompanying changes in gene expression profile are largely unknown.

Studies have shown that the phenolic glycolipid 1 (PGL-1), a major *M. leprae* cell wall pathogenic component, is essential for *M. leprae* internalization into Schwann cells and has also been extensively attributed to induce pathology *in vitro* and in experimental infectious models (10, 23, 26). The understanding of the immunopathogenic mechanisms related to nerve damage in leprosy patients is pivotal for the development of new therapeutic strategies to control leprosy neuropathy. The treatment of nerve damage with steroids is effective but at least 40% of patients relapse and require a further course of steroids (27, 28).

Because leprosy neuropathy is an intricate complex disease, in which both viable and dead *M. leprae* may have a detrimental role for disease progression, it is necessary to fully understand and decipher the contribution of viable and dead bacteria in altering Schwann cell biology. For example, it was suggested that dead bacilli, unlike viable *M. leprae*, make Schwann cells susceptible to attack by killer cells (29). Moreover, dead bacteria and its components, such as lipoarabinomannan (LAM), were also reported to cause neural damage via modulation of the autophagic flux (30) and the complement attack of the nerve (31).

Despite these observations, the early effects of viable and dead *M. leprae* on the global Schwann cell gene expression profile that may be linked to primary neural leprosy are still largely unknown. Therefore, in the present study, we analyzed the expression profile of transcripts involved in neuropathy, glial cell plasticity, nerve repair, and the inflammatory network in leprosy and non-leprosy nerve biopsies and after challenging Schwann cells with viable and dead *M. leprae* independently. Our utmost goal was to provide novel evidence of how viable and dead bacteria modulate Schwann cell gene expression responses along with a detailed statistical correspondence to several histopathological findings commonly observed in nerve biopsies from leprosy and non-leprosy patients.

MATERIALS AND METHODS

Human Nerve Biopsy

Nerve biopsy specimens from eight patients diagnosed with pure neural leprosy (PNL) were obtained from volunteers recruited at the Souza Araujo Outpatient Unit (Leprosy Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation) (**Table 1**). Nerve biopsy fragments, as well their nerve sections, were available for histopathological staining and PCR analysis. For the present study, patients with PNL were selected who did not present any sign of nerve endoneurial fibrosis to ensure the chosen nerve specimens were in the early stages of leprosy neuropathy progression. This selection was made after analyzing the nerve section stained with the hematoxylin and eosin and Gomori trichrome stains under a light microscope following previously published protocol (6). Exclusion criteria were patients with coinfection, metabolic comorbidities such as diabetes, and signs of endoneurial fibrosis, pregnant women and patients under 18 years. For the control group, nerve biopsy specimens from three individuals who underwent brachial plexus surgery were kindly donated by the University Hospital Clementino Fraga Filho (HUCFF-UFRJ). This study was approved by the Oswaldo Cruz Foundation Ethics Committee (number of purports: 2.227.887).

Schwann Cell Culture

The human Schwann cell line, ST88-14, was used in the present study for the *in vitro* assays. Prior to the assays, cells were cultured in RPMI media (Gibco BRL, Grand Island, NY, USA) supplemented with 1% penicillin, 1% streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. The cells were maintained in a controlled environment at 37°C and 5% CO_2. For the assays, ST88-14 cells were suspended in culture medium without penicillin-streptomycin and cultured at a density of 5×10^5 cells/well on six-well culture plates. The cell culture was infected with viable *M. leprae,* gently donated by Lauro de Souza Lima Institute (Sao-Paulo, Brazil) or stimulated with dead (gamma-irradiated) *M. leprae*, obtained through BEI Resources (#NR-19326), at a multiplicity of infection (MOI) of 50 bacilli/cell (50:1). After 24 h of incubation, supernatants were harvested and kept frozen at −20°C until quantification of inflammatory chemokines and cytokines. Additionally, Schwann cell cultures were subjected to total RNA extraction procedures.

TABLE 1 | Clinical data from PNL patients included in the present study ($n = 8$).

Age (years)	Gender	Leprosy clinical form	Leprosy reaction	Multidrug therapy	Physical disability level
67	female	PNL	RR + Neuritis	No	0
26	male	PNL	RR + Neuritis	No	0
34	female	PNL	No	No	0
48	male	PNL	RR + Neuritis	No	0
47	female	PNL	RR	No	2
22	male	PNL	RR	No	2
22	female	PNL	No	No	0
48	female	PNL	No	No	0

PNL, Pure Neural Leprosy; RR, Reverse Reaction.

RNA Extraction and RT-qPCR Array

Schwann cell cultures and nerve biopsy fragments were mechanically grinded and resuspended in 1 mL TRIzol (Gibco BRL) and RNA was obtained following the manufacturer's orientations and stored at −70°C until use. After, 10 ng of total RNA was reverse-transcribed to cDNAs using the Superscript III kit (Invitrogen, Carlsbad, CA, USA) and then amplified using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) or TaqMan assays (ThermoScientific). The GeneQuery™ Human Schwann cell PCR Primer library array kit (Realtime Primers, Elkins Park, PA, USA #GK096) was used to profile total gene expression in Schwann cells and human nerve biopsies. The full list of genes is available on https://www.sciencellonline.com/genequerytm-human-schwann-cell-biology-qpcr-array-kit.html" (accessed on 27th Jan 2022). The TaqMan Fast Universal PCR Master Mix and Human TaqMAn MGB-Probe assays (ThermoScientific), were used to determine mRNA expression of TNF (HS-99999043_m1), IL-23A (HS-0037334324_m1), CCL2 (HS-00234140_m1) and CXCL10 (HS-0017042_m1). The RT-qPCR array was performed in triplicate, and the amplifications were carried out in the ViiA7 Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA). The ΔΔCT method (32) was used to analyze the obtained data after normalization using the endogenous control of the housekeeping gene *RPL13,* for SYBR Green analysis, or normalized using the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; HS-02758991_g1), for TaqMan assays.

Enzyme-Linked Immunosorbent Assay

For cytokine/chemokine release evaluation, the supernatants from control ST88-14 cultures and *M. leprae* (viable or dead) infected ST88-14 cultures were harvested after 24 h and stored at −20°C until use. The following inflammatory mediators (TNF, TGF-β, IL-6, IL-8, IL-12, IL-10, MCP-1/CCL2, and IP-10/CXCL10) were quantified by ELISA technique following the manufacturer's orientations (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

Analyses of the experiments were performed by unpaired *t*-test, Kruskal-Wallis test or one-way ANOVA. For all statistical analyses the value of $p \leq 0.05$ was considered significant. Statistical analyses were performed using the GraphPad Prism version 8.0 software (GraphPad Software, San Diego, CA, USA). Alternatively, a correlogram graph was generated to evaluate the correlation between histopathological characteristics in nerve fragments and the pattern of gene expression. A Pearson's correlation was applied to each pair of variables present in the data. The correlation value varies between −1 and 1, with negative values implying the existence of negative correlation and positive values implying positive correlation. The strength of the correlation is measured by the proximity of the value to 1 or −1, with values closer to these suggesting stronger correlation. Each regression was performed using the gene expression as the response variable and initially both PCR and Acid Fast Bacilli (AFB) as covariates. Each coefficient had its statistical significance tested by the *t*-test for regression coefficients. In the cases where one of the variables was not statistically significant, the model was fitted again using only the statistically significant covariate. Finally, the model's goodness of fit was evaluated using R^2, a statistical measure that evaluates how much of the variation on the response variable is explained by the covariates.

RESULTS

Gene Profiling Analysis of Leprosy and Non-leprosy Nerve Biopsies

In order to identify the molecular pathways related to primary neural leprosy (PNL), a Schwann cell biology PCR array was performed comparing gene expression in nerve fragments from PNL patients and non-leprosy controls. As illustrated in **Figure 1A**, differentially expressed patterns of genes related to peripheral neuropathy, Schwann cell plasticity/reprogramming, and nerve support could be observed. Regarding neuropathy-related genes, some targets were statistically elevated in nerves from PNL patients, such as *HLA-DRB1*, *APOB*, and *WNK*, while others were downregulated, including *HLA-DQB1* and *PLP1*. Interestingly, *HLA-DRB1*, previously reported to influence leprosy susceptibility (33), was upregulated 60-times more in leprosy nerves when compared to non-leprosy nerves. *APOB* and *WNK* were also augmented in leprosy nerves, by 10- and 4-times more, respectively.

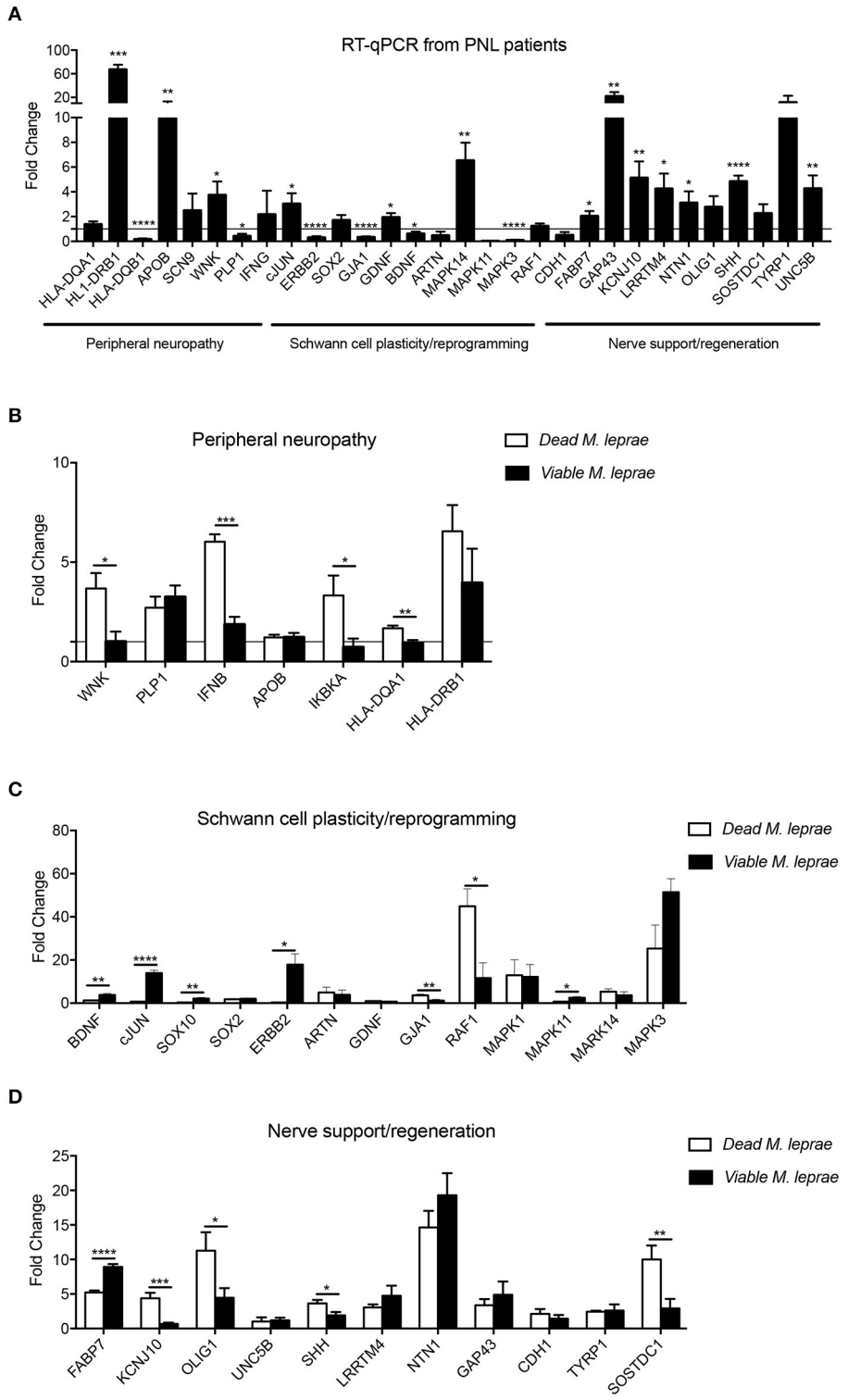

FIGURE 1 | Expression pattern of genes involved in Schwann cell biology. Gene expression profile analysis of upregulated and downregulated mRNA transcripts from signaling pathways related to peripheral neuropathy, Schwann cell plasticity and regeneration support. Analysis were performed in human nerve biopsies from leprosy (**A**, black bars) and non-leprosy individuals (**A**, horizontal line) and in human Schwann cells **(B-D)** infected with dead *M. leprae* (white bars) and viable *M. leprae* (black bars); results are presented as mean ± SD from three to eight normalized independent biological replicates; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

By examining the mRNA profile involved in Schwann cell dedifferentiation, we observed that *cJUN*, *GDNF*, and *MAPK14* were statistically upregulated, while *ERBB2*, *GJA1*, *BDNF*, and *MAPK3* were significantly decreased in nerves from PNL patients. Although neurotrophins, such as BDNF, are required for axon regeneration, here we found that *BDNF* was downregulated in PNL patients, which is in agreement with a previous report that investigated neurotrophin expression in leprosy infection (34).

In summary, our PCR array analysis remarkably showed that most genes involved in nerve regeneration were significantly increased in leprosy nerve biopsies, possibly suggesting a continuous balance of nerve degeneration and an attempt to regrow during the course of infection *in vivo*.

Viable and Dead *M. leprae* Distinctly Modulate Schwann Cell Functional Genes

Neural damage in leprosy has been associated with the role played by viable and dead bacteria after being in contact with Schwann cells (10, 35). Therefore, we were interested in exploring the early effect of *M. leprae* infection on Schwann cell biology genes. For that, we infected human Schwann cells with viable and dead *M. leprae* and screened changes in the mRNA levels after 24 h of infection using the Schwann cell biology PCR array. As observed in **Figures 1B-D**, dead *M. leprae* increased the expression of *WNK*, *IFNB*, *IKBKA*, and *HLA-DQA1* (genes related to neuropathy), in addition to *GJA1* and *RAF1* (for Schwann cell reprogramming) and *KCNJ10*, *OLIG1*, *SHH*, and *SOSTDC1* (for neural regeneration). Interestingly, viable *M. leprae* appeared to modulate genes related to Schwann cell plasticity and dedifferentiation, such as *BDNF*, *cJUN*, *SOX10*, *ERBB2*, and *MAPK11*. In summary, this first set of analysis points to the notion that dead *M. leprae* induces greater expression of peripheral neuropathy and nerve regeneration support genes whereas viable *M. leprae* acts by modulating genes related to Schwann cell plasticity and dedifferentiation. We have summarized this gene intersection in a Venn diagram (**Figure 2**).

Inflammatory Network Analysis Suggests That Dead and Viable *M. leprae* Increase CCL2 Expression

We next aimed to analyze the inflammatory network profile in leprosy and non-leprosy nerve biopsies, as well as *in vitro*, using Schwann cells challenged with viable and dead bacteria. The human nerve analysis indicated that *IL23*, *TNF*, *CXCL10*, and *CCL2* were increased in PNL patients when compared with control biopsies (**Figure 3A**). In addition, when we analyzed changes in Schwann cells *in vitro*, we observed that dead *M. leprae* induced a higher expression of *TNF*, *CXCL10*, and *IL6* in Schwann cells when compared with those infected with viable bacilli (**Figure 3B**). Furthermore, cells infected with viable *M. leprae* increased *IL23* and *CCL2* expression when compared to non-stimulated cultures. These results support the notion that dead bacteria are likely involved in the induction of a pro-inflammatory profile, suggesting that such pro-inflammatory mediators, in the context of neural involvement shown by the biopsies, are induced by dead bacteria present at the site of infection.

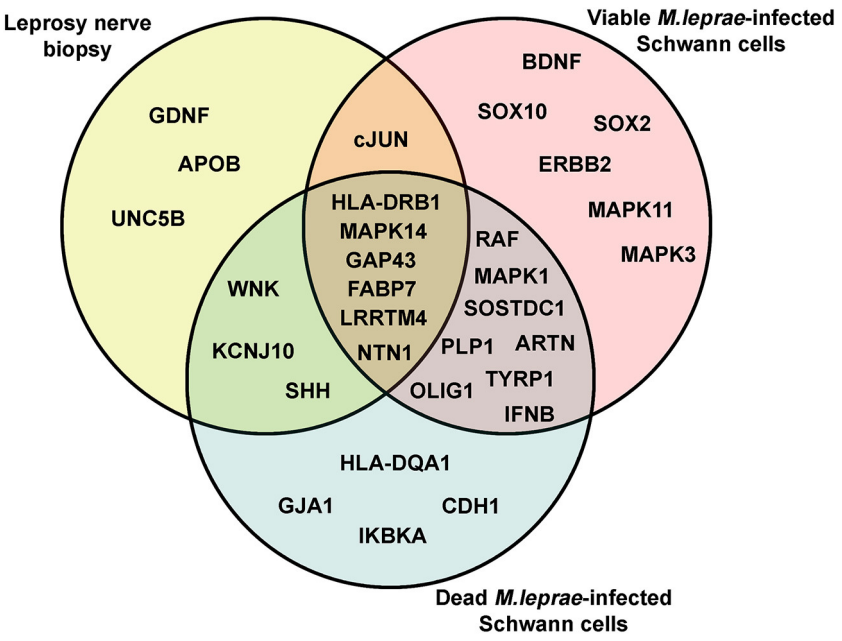

FIGURE 2 | The Venn diagram of differentially upregulated genes related to Schwann cell biology genetic signature in leprosy. The Venn diagram was used to identify clusters of overlapping and non-overlapping mRNA transcripts, upregulated in leprosy nerve biopsies and in Schwann cells infected with viable and dead *M. leprae*.

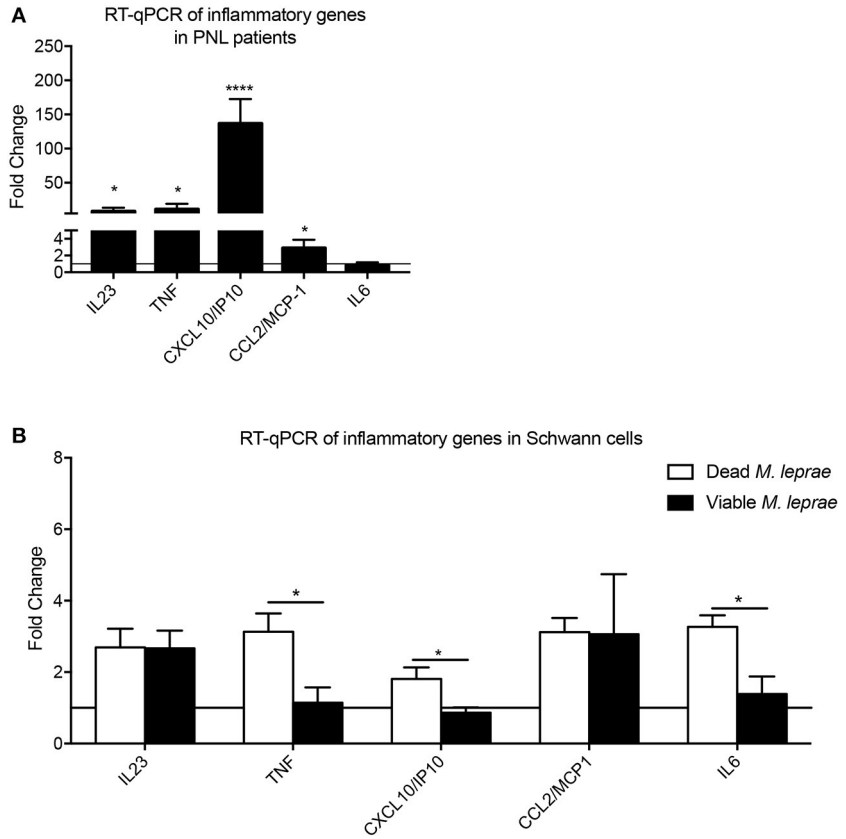

FIGURE 3 | Analysis of inflammatory network in leprosy infection. Fold-change measurement of inflammatory cytokines/chemokines mRNA transcripts in human nerve biopsies from leprosy (**A**, black bars) and non-leprosy patients (**A**, horizontal line) and in human Schwann cells **(B)** infected with dead *M. leprae* (white bars) and viable *M. leprae* (black bars); results are presented as mean ± SD from three to eight normalized independent biological replicates; $^{*}p < 0.05$, $^{****}p < 0.0001$.

Viable and Dead *M. leprae* Promote a Distinct Inflammatory Response in Schwann Cells

We substantiated our transcriptional analysis by evaluating the cytokine network production in Schwann cells challenged with viable and dead bacilli. For that, we performed analysis of cytokine levels in 24-h supernatants, and found that dead *M. leprae* caused an increase in the TNF, IL-8, MCP-1/CCL-2, and CXCL-10 levels in comparison to non-stimulated control cultures (**Figure 4**). Conversely, viable *M. leprae* infection led to increased TGF-β, IL-8, IL-6, IL-10, MCP-1/CCL2, and CXCL-10 in comparison to non-stimulated controls. We also observed a statistical reduction in TNF levels after viable M. leprae infection when compared to dead stimulated cultures and controls. Together, these results suggest that dead *M. leprae* induces pro-inflammatory mediators in human Schwann cells, whereas viable *M. leprae* preferably promote anti-inflammatory cytokines like IL-10 and TGF-β and reduced TNF production (**Figure 4**).

CCL2 Correlates With Decreased Fiber in Histopathology, AFB and PCR From Nerve Fragments

We next applied a correlation matrix to analyze a potential relationship of histopathological findings and changes in gene profiling of Schwann cells in order to evaluate top molecular signature candidates during the early stages of nerve damage in leprosy patients. The overall nerve pathological findings are listed on the y-axis of **Figure 5**. Using this correlogram, we observed that, onion bulb axons, axonal regeneration, demyelination, and Schwann cell proliferation were positively correlated with *SHH*, *TYRP1*, *ERBB2*, and *MAPK14* genes. With regards to remyelination appearance, this was positively correlated with *NTN1*, *OLIG1*, and *UNC5B* genes. Foamy macrophages correlated with *IL6* and granulomas were positively correlated with *GDNF*, *GJA1*, and *SHH* (**Figure 5**). *CCL2* was negatively correlated with lymphocytic, perineurial, and epineurial infiltrate and positively correlated with decreased fibers and Schwann cell proliferation (**Figure 5**).

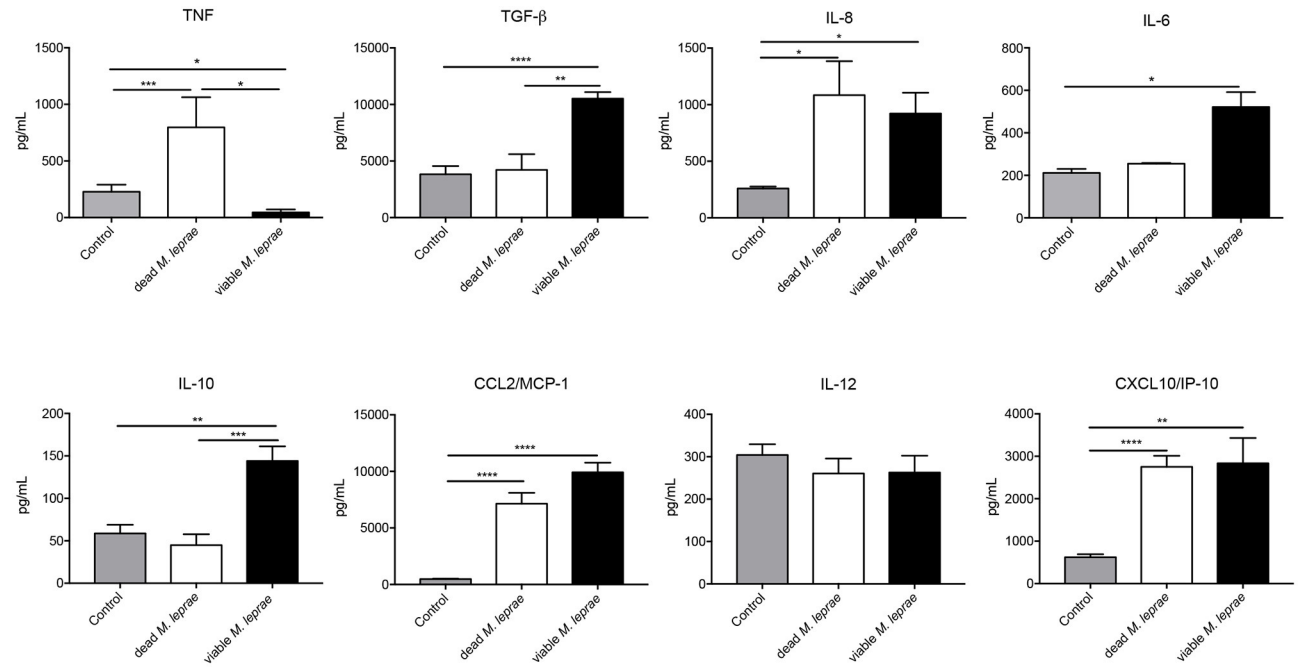

FIGURE 4 | Differentially pattern of cytokine/chemokine release after viable and dead *M. leprae* infection in human Schwann cells. Two-side sandwich ELISA showing the pattern secretion of pro- and anti-inflammatory cytokines and chemokines, in human Schwann cells control (gray bars) and after infection with dead *M. leprae* (white bars) and viable *M. leprae* (black bars), at 24 h after stimuli *in vitro*, results are shown as mean ± SD from three to eight normalized independent biological replicates; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

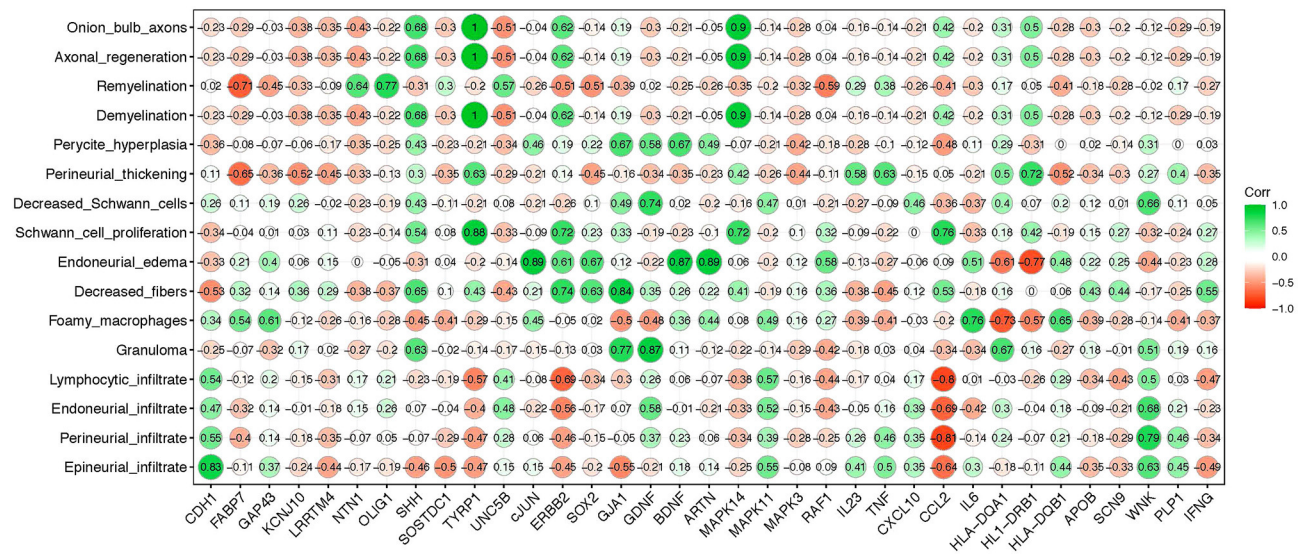

FIGURE 5 | Correlation matrix between leprosy histopathological alterations and gene expression profile in PNL patients. The y-axis indicates major nerve histopathological alterations observed in leprosy patients; the x-axis shows Schwann cell genes evaluated in PNL patients. The green color (closer to 1 value) means stronger correlation while the red color (closer to −1 value) means weaker correlation between the gene expression and the specific histopathological alteration, respectively.

Acid Fast Bacilli (AFB) staining in nerve lesions and positive PCR are two commonly used tools for diagnosing PNL at clinics. Thus, we next searched for a potential association between the presence of the bacilli in nerve and changes in gene expression. It was statistically relevant that the expression of *CCL2* and *CDH1* positively correlated with AFB, while, CCL2 and GDNF expression negatively correlated with PCR (**Table 2**).

TABLE 2 | Linear regression between *M. leprae* PCR and AFB values against the global gene expression.

Variable	Estimative	Standard deviation	*P*-value
CCL2			
Intercept	0.622	1.112	0.5998
FAB	5.247	1.435	0.0147*
PCR	−4.445	1.284	0.0180*
R^2	0.778		
CDH1			
Intercept	0.108	0.253	0.6818
BFAB	1.014	0.412	0.0493*
R^2	0.502		
GDNF			
Intercept	3.119	0.503	0.000815***
PCR	−1.596	0.581	0.033469*
R^2	0.557		

*Among all the genes evaluated, CCL2, CDH1, and GDNF presented a statistical significance with AFB and/or PCR. *$p < 0.05$, ***$p < 0.001$.*

DISCUSSION

The comprehension of Schwann cell-*M. leprae* pathological interactions is vital to fully understand disease progression in experimental models as well as in leprosy patients. Moreover, how viable and dead bacteria affect Schwann cell biology is largely unknown and poorly explored in the field. Therefore, in the present work, we used the *in vitro* host-pathogen interaction model to determine how viable and dead bacteria modify the global gene expression profile as soon as the bacteria comes in contact with the Schwann cells. Furthermore, we extended these early changes in the Schwann cell gene profile in response to infection to the analysis of patient nerve biopsies, establishing a potential link to the early response pattern during the disease progression in leprosy individuals.

We initially performed an exploratory, broad gene expression analysis of Schwann cells challenged with viable or dead bacteria, along with data obtained from nerve biopsies. The following families of genes involved in the nerve response to injury were investigated: (i) peripheral neuropathy, (ii) inflammatory network, (iii) Schwann cell plasticity/dedifferentiation, and (iv) nerve regeneration support.

Among the global gene expression, *HLA-DRB1*, *MAPK14*, *GAP43*, *FABP7*, *NTN1*, and *LRRTM4* were upregulated in nerve biopsies from PNL patients and in viable and dead *M. leprae*-infected Schwann cells, in comparison with the respective control groups (**Figure 2**). It has been described that human leukocyte antigen (*HLA*) alleles affect the host immune response against *M. leprae* (36, 37). *HLA-DRB1* is one of the most upregulated genes in multiple sclerosis, having a special role in inducing demyelination (38). Moreover, this transcript is consistently related to the tuberculoid leprosy (T-Lep) clinical form, while *HLA-DQB1* has a strong relation with the L-Lep clinical form (39). In the present study, *HLA-DQB1* was downregulated in PNL patients, in comparison with the uninfected nerve specimens (**Figure 1A**). Additionally, receptors for the axon guidance molecule, netrin-1 (*NTN1*), are expressed by Schwann cells and play a role in peripheral regeneration and induce the regeneration phenotype (40).

When evaluating the set of genes related to Schwann cell plasticity and dedifferentiation, some serine/threonine kinases, such as mitogen-activated protein kinase 14 (*MAPK14*), widely known for its role at the inflammasome pathways in the neuroglia (41), were found to be upregulated in PNL patients. However, there is a lack of information regarding *MAPK14* in leprosy. *GAP43*, the major protein of the axon growth-cone that plays a role in axon growth (42) and regeneration was also upregulated, which suggests a tentative role for axonal regrowth in the injured infected nerve. *SHH*, *WNK*, and *KCNJ10* were upregulated in leprosy nerve biopsies and in dead *M. leprae*-infected Schwann cells (**Figure 2**). The sonic hedgehog gene (*SHH*), which has been previously reported in nerve damage (43, 44), may also trigger regeneration and induce Schwann cell proliferation, as an attempt to counteract the harm caused by the bacilli (44). *KCNJ10* encodes the inward-rectifying potassium channel (Kir4.1). Alteration of *KCNJ10* gene expression is related to neuropathies, such as Huntington's disease East/SeSAME syndrome, by elevating the extracellular $K^{(+)}$, which consequently leads to abnormal neuron excitability (45, 46). It was demonstrated in mice that inflammation can silence (Kir4.1) channels, leading to hyperalgesia and trigeminal pain (47). Despite the absence of studies relating the *KCNJ10* gene and *M. leprae* infection, it seems this may be a potential pathway to be explored for leprosy neuritis in the future. *cJUN* was the only gene statistically upregulated in PNL biopsies and viable *M. leprae*-infected Schwann cells in comparison to the respective controls (**Figure 2**). *cJUN* is a master Schwann cell regulator involved in the transition of a differentiated phenotype toward a progenitor/stem-cell like stage (48) and has also previously been reported to be upregulated, among other developmental and neural crest genes, during *M. leprae* infection in mouse Schwann cells (35).

PLP1, *SOSTDC1*, *RAF1*, *ARTN*, *OLIG1*, *MAPK1*, *IFNB*, and *TYRP1* were upregulated in viable and dead *M. leprae*-infected Schwann cells, but not in the PNL biopsies. The tyrosinase-related protein 1 (*TYRP1*) plays a role in the melanin biosynthetic pathway, being mostly expressed by melanocytes (49). It has been documented that leprosy patients may present hyper or de-pigmented lesions due to a defective transfer of melanin (50). The melanocytes have been recently associated with the innate immune response, by producing inflammatory cytokines, such as IL-1β (50). But the role of the *TYRP1* pathway in the peripheral nerve requires further investigation. It was documented that interferon beta (*IFNB*) is increased during cell stress conditions, but the lack of this protein leads to neuroinflammation (51, 52). *IFNB* is essential to start the cell fate pathway driven by *NUPR1*, a gene signature that contributes to a progressive infection in human cells (53). Oligodendrocyte transcription factor 1 (*OLIG1*) is an important protein of the remyelination phenotype, usually upregulated after a disturbance in the cell microenvironment (54). It is well described that during diabetic neuropathy, the lack of insulin

leads to the demyelination phenotype due to a downregulation of *OLIG1* (54). On the other hand, in this study, some genes like *MAPK3* (*ERK1*), an important extracellular signal-regulated kinase and Schwann cell migration and proliferation molecule (55) was downregulated in PNL patients. Likewise, ErbB2, a well-known demyelination inducer and Connexin43—*GJA1*—were also significantly reduced in PNL patients. Despite the lack of information about *GJA1* in leprosy, this has been described in neural impairment, like in the pathogenesis of Alzheimer's disease, where GJA1 downregulation leads to reduced levels of ApoE (56, 57).

While we know that the polarity of leprosy can result from the host's inflammatory response profile, multiple features of neurological involvement may also occur. For example, polymorphisms in certain genes such as IL10, ninjurin 1 and TNF have been associated with leprosy susceptibility (58–60).

In the present work, we were able to correlate the pattern of histopathological impairments with alteration in the gene expression profile induced by *M. leprae* infection. The correlogram analysis demonstrated, for the first time, a statistically significant correlation between the gene signature from the early stages—before fibrosis—and the histopathological alterations of the nerve damage in leprosy neuropathy. Regarding the histopathological features, *TYRP1*, *SHH*, and *MAPK14* expression were strongly correlated to onion bulb axons, axon regeneration, demyelination, and Schwann cell proliferation, while *MCP1/CCL2* was strongly negatively correlated with nerve inflammatory infiltrates: epineurial, endoneurial, perineurial, and lymphocytic infiltrates. It is important to realize that the weak correlation with endoneurial, perineurial, and epineurial fibrosis was due to the inclusion criteria for this study, which selected patients with no signs of fibrosis.

The biserial correlation demonstrated that the monocyte chemoattractant protein 1 (*MCP1/CCL2*) was positively correlated with AFB and negatively correlated with PCR, which strongly indicates the correlation of increased expression of this gene by viable bacteria. This finding corroborates a previous study by our group, which showed activation of the ESX-1 mycobacterial system by the viable *M. leprae*, leading to the activation of the *OASL* gene and the induction of *CCL2*, impairing the host bactericidal response, which was not observed with dead *M. leprae* stimuli in THP-1 cells (61). More than that, independently, Schwann cells are responsible for the triggering response of nerve damage through initiating the clearance of the debris by myelinophagy, followed by macrophage recruitment, which is especially regulated by CCL2 (62). In this sense, we see the relevance of *CCL2* gene activation not only as a protective mechanism in the maintenance of mycobacterial viability, but also as a biological marker indicative of positive AFB. CCL2 has been described as one of the innate immunity genes immediately activated in the context of infection *in vitro* (35). Thus, despite the relevant findings, there is still a way to go in terms of understanding the role of this chemokine in infection and nerve damage during leprosy.

To strengthen the data obtained from the gene signature of *M. leprae* infection, we evaluated the immunomodulatory profile secreted by Schwann cells without infection and challenged with viable and dead *M. leprae*. We observed a pro-inflammatory profile elicited by the dead bacilli, while an anti-inflammatory microenvironment appeared to be promoted by infection with the viable bacteria. Studies have shown that macrophage infection by viable *M. leprae* leads to a regulatory T cell response rather than a cytotoxic T cell response, which contributes to the persistence of the infection in the host (63, 64). This characteristic is already well demonstrated in patients with L-lep, which leads to a high bacillary load. In the present work, we demonstrated that Schwann cells also behave in a way to induce the anti- or pro-inflammatory phenotype according to the bacilli viability. Since in reactional episodes there is a mixture of viable and dead bacilli, it is interesting to point out the role of the Schwann cells, and not only the macrophages, as modulators of the reaction process in the nerve, which can even lead to leprosy neuritis (14).

CONCLUSION

In the present study, we identified the early impact of viable and dead bacteria, independently, on modifying the gene expression profile of human Schwann cells *in vitro*. We also described a molecular signature associated with neural damage in early stages of pure neural leprosy from patients (i.e., *HLA-DRB1*, *MAPK14*, *GAP43*, *FABP7, NTN1*, and *LRRTM4*). Leprosy is a complex intricate disease and the identification of this genetic profiling may contribute for the fully understanding of leprosy neuropathy pathogenesis with the long-term goal of identifying these pathways as targets for the development of effective therapeutic strategies. We acknowledge the relatively limited number of human nerve samples, as in our experimental design we narrowed our analysis to nerve biopsies of leprosy patients with no signs of neural fibrosis and at the initial stages of neural damage. Therefore, future work with additional nerve samples from leprosy individuals, at distinct clinical stages, are important to advance this host-pathogen interactions and associated genetic analysis in larger cohorts. In summary, these results open new perspectives for the understanding of the genetic signature of neural commitment in leprosy disease.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Oswaldo Cruz Foundation Ethics Committee (number of purports: 2.227.887). The Ethics Committee waived the requirement of written informed consent for participation.

AUTHOR CONTRIBUTIONS

BS, RP, and BM designed and carried out experiments, collected and interpreted data, and prepared illustrations. BS, GS, RP, and BM analyzed the data. BS, MM, GS, PS-R, MOM, MJ, ES, RP, and BM reviewed and edited the manuscript. ES, RP, and BM conceptualized and designed experiments, supervised the study, interpreted data, and obtained funding. BS, RP, and BM wrote the manuscript. PS-R provided viable *M. leprae*. All authors read and approved the final version of the manuscript.

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BRIEF RESEARCH REPORT
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Can Leprosy Reaction States Mimic Symptoms of Fibromyalgia? A Cross-Sectional Analytical Study

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Leprosy causes significant pain in affected patients, especially those experiencing reactional states. Fibromyalgia is characterized by widespread pain and is often accompanied by fatigue. Confusion between the clinical manifestations of fibromyalgia and those of leprosy reactions is possible at the primary care level, the first contact with the health system in most cases. We aimed to determine whether the presence of leprosy reactional states is related to the development of signs and symptoms included in the case definition of fibromyalgia and establish recommendations for obtaining the correct diagnosis. We performed a cross-sectional study in which the main independent variable was the presence of any leprosy reactional state and the primary dependent variable was the diagnosis of fibromyalgia according to the 2016 Revisions of the 2010/2011Fibromyalgia Provisional Criteria of the American College of Rheumatology. Forty-three patients were included in the study. Twenty-eight (65.12%) patients had a type I reactional state, only 1 (2.33%) had an isolated type II reactional state, and 5 (11.63%) had both type I and type II reactional states. Only 2 patients who suffered from cooccurring type I and II reactional states obtained sufficient scores for the diagnosis of fibromyalgia. Although diffuse pain was common in leprosy patients, none of the types of reactional states were associated with a higher frequency of criteria for fibromyalgia. We can conclude that a leprosy reactional state is probably not a risk factor for fibromyalgia but can act as a confounder, as tender points may be similar in both diagnoses. In patients diagnosed with fibromyalgia, leprosy must be considered in the differential diagnosis in endemic regions.

Keywords: leprosy, fibromyalgia, dermatology, rheumatology, diagnosis

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INTRODUCTION

Leprosy is an infectious disease with chronic progression and intense immunological manifestations caused by *Mycobacterium leprae* and *Mycobacterium lepromatosis* (1). The disease mainly affects the skin and peripheral nerves. Early multidrug therapy is the best way to prevent transmission and morbidity (2–5). Leprosy reactions are acute immune-mediated episodes that are responsible for morbidity in most cases (6).

Fibromyalgia is present in 2-8% of the population (7, 8). The disease is characterized by widespread pain and is often accompanied by fatigue, memory problems, and sleep disturbances. After osteoarthritis, fibromyalgia is the second most common rheumatic disease (9). Its diagnostic criteria were originally published in 1990, and the symptoms emphasized were chronic generalized pain accompanied by tender points (7).

There seem to be no similarities between infectious diseases, such as leprosy, and chronic diseases, such as fibromyalgia. However, leprosy is a chronic disease that causes significant pain in affected patients and in advanced stages, especially in those experiencing reactional states (10). Chronic inflammation and the social stigma associated with leprosy also induce frequent constitutional symptoms (11). The importance of these two very different pathologies with many characteristics in common (12) will be addressed in this paper.

Patients who develop fibromyalgia usually have a lifelong history of chronic pain throughout the body (13, 14). They are more likely to have a history of headaches, dysmenorrhea, temporomandibular disorders, joint disorders, chronic fatigue, irritable bowel syndrome and other functional gastrointestinal disorders, interstitial cystitis, endometriosis, and other regional pain syndromes than those without fibromyalgia (7). Pain in the neck, back, shoulders, pelvic girdle, and hands is frequent; however, any part of the body can be affected. Leprosy can be associated with chronic pain due to immune-mediated neuropathy or direct action of the bacillus and thus can possibly be confused with fibromyalgia.

The main goal of this study was to determine whether the presence of leprosy reactional states is related to the development of signs and symptoms included in the case definition of fibromyalgia. Additionally, we aimed to monitor the frequency of leprosy symptoms that overlap with fibromyalgia and finally establish recommendations for the differential diagnoses of these two diseases to prevent any delay in diagnosis of leprosy.

METHODOLOGY

Study Design, Location and Circumstances of the Study

We performed a cross-sectional analytical study in which the main independent variable was the presence of any leprosy reactional state. The primary dependent variable was the diagnosis of fibromyalgia according to the 2016 Revisions to the 2010/2011 Fibromyalgia Diagnostic Criteria composed of the following diagnostic instruments (scales): the widespread pain index (WPI), symptom severity scale (SSS) and fibromyalgia severity (FS) score, which is the sum of the WPI and SSS (14). This is an updated version of the provisional criteria of the American College of Rheumatology (ACR) 2010 and the 2011 self-report modification for survey and clinical research and is widely used for fibromyalgia diagnosis. According to validation assessments, the criteria have an adequate sensitivity and specificity for the diagnosis and classification of fibromyalgia. The complete form can be found in **Supplementary File 1** (14). Patients were consecutively recruited in 2020 and 2021 at the University Hospital of Brasília (HUB), which is a tertiary hospital for the care of patients with dermatological and rheumatological diseases.

Inclusion Criteria

We included patients who received a formal diagnosis of leprosy according to the criteria of the World Health Organization (WHO), who were suspected of experiencing an active reactional state and who had not been diagnosed with fibromyalgia at the beginning of leprosy treatment according to the assessment instruments described elsewhere (1). The diagnosis of leprosy was always made by a dermatologist based on the criteria of the WHO (15), and at least one of the following findings was required for diagnosis: (1) skin lesion with altered sensitivity; (2) compatible peripheral neurological alterations, including asymmetric neural thickening, motor deformities, or hypoesthetic areas; (3) parasitological finding of *M. leprae* on smear examination or skin biopsy (performed for all patients in our center). The differential diagnosis of each included patient was systematically searched in our center by specialized clinical evaluation and complementary exams such as ultrasonography, electroneuromyography, and magnetic resonance imaging of the axial skeleton.

Exclusion Criteria

We excluded patients from indigenous communities, and those who did not sign the consent form were excluded following local ethical restrictions.

Main Independent Variable Assessment

In all the included patients, the presence of a leprosy reactional state was evaluated by a dermatologist with a specialist title provided by the Brazilian Society of Dermatology. The classification of reactional states was also at the discretion of this expert and followed the WHO guidelines.

Primary Dependent Variable Assessment

The primary dependent variable was the diagnosis of fibromyalgia in the study population comprising only leprosy patients. Fibromyalgia was diagnosed according to the definition by Wolfe et al. (14). A patient fulfilled the diagnostic criteria for fibromyalgia if the following three conditions were met. (1) Part I of the WPI was equal to 7 and the parts IIa and IIb of the SSS were equal to 5, or the WPI ranged from 4 to 6, with an SSS score of 9. (2) Generalized pain, defined as pain in at least 4 of 5 regions, was present; pain in the jaw, chest, and abdomen was not included in the definition of generalized pain. (3) Symptoms were present for at least 3 months. A diagnosis of fibromyalgia was considered valid regardless of other diagnoses, except for leprosy in this specific case. A diagnosis of fibromyalgia does not exclude the presence of other clinically important diseases. Each variable and classification was analyzed for an association with the presence and type of any reactional state. We also considered the total sum of points (FS score) from the classification form for comparison.

Additional Variables

Additional factors were adjusted for when analyzing the above-described association, such as sex, age, personal history, family history, pathological history (mainly psychiatric illness), medication use, and social demographics. Leprosy classification was performed according to the Madrid classification, considering mainly the profile of skin lesions, to reduce the number of classification types and to improve the analytical approach (16). Leprosy classifications were as follows: indeterminate, tuberculoid, borderline, lepromatous, and pure neural leprosy. Joint and enthesis count, a clinical evaluation considered for the monitoring of disease activity of several rheumatic diseases, was also analyzed for an association with the presence of leprosy reactional states (17).

Sample Size

Our target population was all patients with leprosy and with suspected active reactional states in the recruitment center.

Statistical Analysis

After conducting interviews during clinical consultations, the data were anonymized to guarantee the confidentiality and identity of the patients. Initially, central tendency and dispersion values were calculated. The Analysis relied on a univariate approach followed by subgroup analysis, including the subanalyses of type I and II reaction states. Statistical analysis was performed with the "survminer" package of R version 0.4.9. (https://CRAN.R-project.org/package=survminer) (R Studio: Integrated development for R. R Studio, PBC, Boston, MA; URL http://www.rstudio.com/ by the free program R Core Team (2020); R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). Statistical significance was defined as a p-value < 0.05 and a 95% confidence interval (CI).

Ethical Aspects

The project was approved by the Ethics and Research Committee of the Faculty of Medicine of the University of Brasília (UnB), identification number CAAE: 07539519.2.0000.5558. All patients who agreed to participate in the study signed an informed consent form.

RESULTS

Forty-three patients were included in the study. The mean age of the participants was 46.81 years old (standard deviation = 13.39 years); 22 (51.16%) patients were female. The characteristics related to leprosy are shown in **Table 1**. Borderline leprosy was the most frequent form, affecting 30 (69.77%) patients. Eleven patients were using alternative treatment. Twenty-eight (65.12%) patients had a type I reactional state when evaluated, only 1 (2.33%) had an isolated type II reactional state and 5 (11.63%) had both type I and type II reactional states. The medications used to treat reactional episodes are shown in **Table 1**. None of the included patients presented associated rheumatologic or neurologic conditions that could explain diffuse pain episodes.

TABLE 1 | Clinical variables related to leprosy classification and treatment.

Variable	Frequency (*n*)(%)
Leprosy classification	
Borderline	30(69.77%)
Lepromatous	7(16.28)
Pure neural leprosy	6(13.95%)
Treatment	
Standard multidrug therapy	32(74.42%)
Alternative treatment	11(25.58%)
Reactional state	
Type I	28(65.12%)
Type II	1(2.33%)
Types I and II	5(11.63%)
No reactional state	9(20.93%)
Medications for reactional states	
Prednisone	30(69.77%)
Thalidomide	4(9.30%)
Pentoxifylline	2(4.65%)

n, number of patients.

Analysis of the Primary Dependent Variable

In the present study, only two patients who were simultaneously affected by types I and II reactions obtained sufficient scores for the diagnosis of fibromyalgia according to the adopted criteria. None of the types of reactional states were associated with a higher frequency of criteria for fibromyalgia. Multivariate analysis was not performed due to the small number of patients with a diagnosis of fibromyalgia, the primary dependent variable.

Although only two patients had a complete diagnostic criterion of fibromyalgia, only two patients had no points scored in the WPI and SSS scores. All remaining patients had at least one unspecific sign or symptom that could be found in patients with fibromyalgia. Among all the patients, the median part I WPI was 1.00, with an interquartile range of 2.00. The median (interquartile range) numbers of part IIa and part IIb WPIs were 2.00 (6.00) and 1.00 (1.50), respectively. The median total score (FS score) in the studied population was 4.00, with an interquartile range of 8.00. The median joint count was 0 in the total population, with an interquartile range of 3.00. The median and interquartile range of the enthesis count was 0. The analysis of the scores obtained from the assessment tool is shown in **Table 2**.

DISCUSSION

Leprosy is essentially a disease of the peripheral nerves and is the most frequent cause of treatable peripheral neuropathy in endemic countries (6, 18, 19). In the initial phase of the disease, invasion of the dermal and superficial peripheral nerves occurs, resulting in reduced thermal, pain and tactile sensitivity, often preceded by autonomic changes, such as lack of sweating and hair loss. Subsequently, neuropathic pain and deformity can occur as a result of an immune-mediated reaction targeting

TABLE 2 | Comparison between the evaluation of the Widespread Pain Index (WPI) (part I), the Symptom Severity Score (SSS) (parts IIa and IIb) and the fibromyalgia severity (FS) score with the presence of leprosy reactional states.

	Type I reaction Median (interquartile range)		
Criteria	**Yes**	**No**	***p*-value**
WPI part I	1.00(2.00)	0.50(1.75)	0.690
SSS part IIa	2.00(6.00)	2.00(5.00)	0.596
SSS part IIb	1.00(1.00)	2.00(1.00)	0.013
FS score	4.00(8.00)	3.50(8.75)	0.839
Joint count	0(3.00)	0(2.50)	0.766
Enthesis count	0(0)	0(0)	0.816
	Type II reaction Median (interquartile range)		
Criteria	**Yes**	**No**	***p*-value**
WPI part I	0(0)	1(2.75)	0.028
SSS part IIa	2.00(1.00)	3.00(6.00)	0.485
SSS part IIb	1.00(1.50)	1.00(1.00)	0.895
FS score	3.50(4.00)	4.0(8.00)	0.408
Joint count	0(0)	0.50(3.00)	0.149
Enthesis count	0(0)	0(0)	0.878
	Simultaneous type I and II reactions Median (interquartile range)		
Criteria	**Yes**	**No**	***p*-value**
WPI part I	1.00(2.00)	0(1.00)	0.493
SSS part IIa	2.50(6.00)	1.00(5.00)	0.258
SSS part IIb	1.00(1.00)	2.00(1.00)	0.033
FS score	5.00(8.00)	3.00(8.00)	0.539
Joint count	0.50(3.00)	0(1.00)	0.526
Enthesis count	0(0)	0(0)	0.968

WPI, Widespread Pain Index; SSS, symptom severity scale; FS, fibromyalgia severity.

neural structures (5). Although the leprosy case detection rate is considered very low in developed countries such as the United Kingdom (2 new cases in 2019) and the United States of America (no new cases in 2019), in tropical countries and in countries in development such as Brazil (27.863 new cases in 2019) and India (114.451 new cases in 2019) this is a relevant public health problem (20). In addition, crescent migration waves show that leprosy can be a possible reality in any world region.

In the clinical evaluation of a leprosy patient, a history of pain and/or paraesthesia in the areas corresponding to the affected nerves, as well as the sensation of numbness in extremities or other specific areas of the skin, are important symptoms (21, 22). In addition, acute manifestations induced by reactional states may develop, resulting in progression to chronic neuropathy with persistent pain (23). When chronic neuropathy persists, complications such as osteomyelitis and plantar perforating disease can be aggravating factors. The present study, which was performed in a tertiary hospital located in a leprosy-endemic country, demonstrated that most patients were affected by type I leprosy reactions (33 patients), and only 6 had type II leprosy reactions. A minority of the evaluated patients did not develop a reactional state.

Fibromyalgia syndrome is characterized by pain throughout the body, especially in the musculature (24, 25). Symptoms such as fatigue, non-restorative sleep, memory and attention disorders, anxiety, depression and intestinal disorders are associated with fibromyalgia pain. Since pain is the primary manifestation in fibromyalgia, fibromyalgia can be confused with leprosy, which is also characterized by pain as an initial symptom. Although most leprosy patients included in this study were experiencing reactional states, only two patients had a clinical picture compatible with fibromyalgia. However, most leprosy patients reported multiple points of pain according to the tool, showing that confusion between these 2 diseases may occur during non-specialized evaluations.

Leprosy and its reactions can mimic many dermatological, rheumatological, and neurological diseases (26–28). The most characteristic sign of leprosy is skin hypoesthesia or anesthesia, while peripheral neuropathy is also an important manifestation of this infectious disease. In some cases, no skin changes are recognized, and these cases are considered pure neural leprosy. The real incidence of leprosy without skin manifestations is unknown, but this type of leprosy is probably underdiagnosed. There are many rheumatological and neurological diseases that induce changes in peripheral sensitivity. The data from the present study show some similarities between leprosy reactions and fibromyalgia that can be confused especially by poorly trained providers, but the applied tool seems to be adequate for the differentiation of the two conditions. This means that both conditions are possibly confused only in cases in which clinical examination is not proper. Specific educational interventions must be directed to healthcare providers at the primary care level and in underdeveloped areas where leprosy is endemic.

Fibromyalgia is a disease with a certain degree of difficulty in definitive diagnosis, as no definitive laboratory standard exists. Our results clearly showed that confusion between the clinical manifestations of fibromyalgia and those of leprosy reactions is possible (12). Fibromyalgia is a common disease with a prevalence of more than 20% prevalence in rheumatology clinics, even in regions where leprosy is not endemic; this suggests that relevant epidemiological information, especially a history of household contact with a leprosy patients or migration from an endemic country, must be collected and considered (8). Fibromyalgia is characterized by tendon and muscle pain, adynamia, paraesthesia, and non-specific symptoms, but it does not manifest as neuropathy. Changes in the sensitivity of peripheral nerves should be investigated in patients with suspected fibromyalgia, especially in regions where leprosy is endemic. This is the most important clue for differential diagnosis (12). Healthcare providers must be aware that both diseases can occur concurrently, with overlapping or mimicking signs and symptoms.

Although our entire target population was evaluated, the size of our study population is a probable limitation for the assessment of causal association. Future longitudinal studies

may use the present data for the calculation of associations. Additionally, data were collected with the use of a questionnaire; therefore, a certain degree of subjectivity is expected. This is a constant limitation for the evaluation of diseases that have no laboratory or gold standard diagnostic criteria (3, 5).

The present study allows a better understanding of the relationship between leprosy and fibromyalgia and thus contributes to a better understanding of the control of chronic pain in leprosy; this study also contributes to the analysis of the differential diagnosis of these two diseases. Rheumatologists and clinicians must be careful when evaluating and diagnosing fibromyalgia, as it is important to consider the possibility of a diagnosis of leprosy, which can be a confounder, in patients in endemic countries.

In conclusion, a leprosy reactional state is not a risk factor for fibromyalgia but can act as a confounder. Tender points are common in both diagnoses. Several chronic pain points were present in patients with leprosy and leprosy reactions, but only 2 patients met the diagnostic criteria for fibromyalgia according to the tool. New prospective studies must be conducted to better elucidate this issue. Finally, patients diagnosed with fibromyalgia must be actively evaluated for leprosy during the differential diagnosis in regions where leprosy is endemic.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Faculdade de Medicina da Universidade de Brasília. All patients who agreed to participate in the study signed an informed consent form.

AUTHOR CONTRIBUTIONS

MF and SC: investigation. GS, MF, CG, and LM: formal analysis. GS, MF, LM, CG, and SC: resources. GR and CG: visualization, writing—original draft, and writing—review and editing. CG: conceptualization, data curation, funding acquisition, methodology, project administration, software, supervision, and validation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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The Type I Interferon Pathway Is Upregulated in the Cutaneous Lesions and Blood of Multibacillary Leprosy Patients With Erythema Nodosum Leprosum

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In leprosy patients, acute inflammatory episodes, known as erythema nodosum leprosum (ENL), are responsible for high morbidity and tissue damage that occur during the course of *Mycobacterium leprae* infection. In a previous study, we showed evidence implicating DNA-sensing *via* TLR9 as an important inflammatory pathway in ENL. A likely important consequence of TLR9 pathway activation is the production of type I interferons (IFN-I) by plasmacytoid dendritic cells (pDCs), also implicated in the pathogenesis of several chronic inflammatory diseases. In this study, we investigated whether the IFN-I pathway is activated during ENL. Blood samples and skin lesions from multibacillary patients diagnosed with ENL were collected and the expression of genes of the IFN-I pathway and interferon-stimulated genes were compared with samples collected from non-reactional multibacillary (NR) patients. Whole blood RNAseq analysis suggested higher activation of the IFN-I pathway in ENL patients, confirmed by RT-qPCR. Likewise, significantly higher mRNA levels of IFN-I-related genes were detected in ENL skin biopsies when compared to NR patient lesions. During thalidomide administration, the drug of choice for ENL treatment, a decrease in the mRNA and protein levels of some of these genes both in the skin and blood was observed. Indeed, *in vitro* assays showed that thalidomide was able to block the secretion of IFN-I by peripheral blood mononuclear cells in response to *M. leprae* sonicate or CpG-A, a TLR9 ligand. Finally, the decreased frequencies of peripheral pDCs in ENL patients, along with the higher TLR9 expression in ENL pDCs and the enrichment of $CD123^{+}$ cells in ENL skin lesions, suggest the involvement of these cells as IFN-I producers in this type

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of reaction. Taken together, our data point to the involvement of the pDC/type I IFN pathway in the pathogenesis of ENL, opening new avenues in identifying biomarkers for early diagnosis and new therapeutic targets for the better management of this reactional episode.

Keywords: immunopathogenesis, leprosy reaction, type I interferons, plasmacytoid dendritic cells, erythema nodosum leprosum

INTRODUCTION

Interferons constitute a broad class of cytokines, classified into types I, II, and III, performing multifaceted roles. Type I IFNs make up the largest family of interferons, which, in humans, is composed of thirteen IFN-α subtypes and one copy of IFN-β, IFNε, IFNκ, and IFNω each. IFN-α along with IFN-β are the most abundant and well characterized (1). Although best known for their overall antiviral activity, IFN-α and IFN-β are now recognized for their roles as modulators in the immune response against parasites, bacteria, and fungi (2, 3), as well as in the immunopathogenesis of a number of inflammatory and autoimmune diseases (4, 5). All type I IFNs bind to the same ubiquitously- expressed type I IFN receptor (IFNAR), composed of the IFNAR1 and IFNAR2 subunits (5). The type I interferons signaling pathway culminates in the induction of hundreds of interferon-stimulated genes (ISGs) that mediate antiviral effects and other biological activities, leading to the emergence of what is called an *interferon signature* (6).

The role of IFN-I in the pathogenesis of systemic lupus erythematosus (SLE) and other inflammatory diseases (7, 8), including the potential use of this pathway in controlling disease symptoms, has been extensively explored over the last few decades (9–11). It is now well established that type I interferons (most related studies have explored IFN-α/β functions) crucially affect different subsets of innate and adaptive immune cells that can exacerbate inflammation and lead to immunopathology and tissue damage (5, 12, 13). Increasing evidence also points to plasmacytoid dendritic cells (pDCs) as the major source of IFN-α/β in these diseases *via* the recognition of extracellular self-nucleic acids such as nucleic acid-immune complexes and extracellular neutrophil traps (NETs) by way of the toll-like receptors 7 (TLR7) and 9 (TLR9; 5, 9, 10, 14). The pathogenic role of pDCs-derived IFN-I has been especially emphasized in inflammatory diseases with cutaneous manifestations during which these cells accumulate in the skin lesions (15–18).

In about 40% of leprosy patients, acute inflammatory episodes, known as leprosy reactions, may occur before, during, or after multidrug therapy (MDT). These inflammatory episodes complicate the course of *Mycobacterium leprae* infection and are responsible for high morbidity and tissue damage (19, 20). Thus, the early diagnosis and treatment of leprosy reactions are highly relevant since these episodes exacerbate nerve damage and may also be a major cause of patient hospitalization and death. Most are classified as either type 1 reaction/reverse reaction (RR) or type 2 reaction/erythema nodosum leprosum (ENL). ENL only affects the multibacillary clinical forms: borderline lepromatous (BL) and lepromatous leprosy (LL; 20, 21). ENL is characterized by the sudden appearance of small, reddened skin nodules, in most cases accompanied by systemic symptoms like fever, malaise, iritis, arthritis, neuritis, and lymphadenitis, in addition to peripheral nerve impairment (20). The severity of ENL varies and is classified according to the clinical symptoms of the patient as mild, moderate, or severe (22). Frequently recurrent, it often requires long-term treatment with oral corticosteroids (20, 21, 23). However, an effective alternative drug is thalidomide. This medication has been shown to be extremely beneficial within a short period of time (24, 25). However, due to its teratogenic effects, its use is tightly restricted and only allowed in Brazil and a few other countries (23, 25).

In a previous report, evidence was presented that implicated DNA sensing *via* TLR9 as an important inflammatory pathway in ENL. Higher TLR9 expression levels in skin lesions and blood cells together with higher circulating levels of endogenous and *M. leprae*-derived TLR9 ligands were detected in these patients (26). In the present study, the hypothesis under review was that TLR9 activation by autologous and/or *M. leprae* DNA induces the production of type I interferons by pDCs, perhaps contributing to ENL immunopathogenesis in a way similar to what is observed in other inflammatory diseases with cutaneous manifestations.

MATERIALS AND METHODS

Subjects and Clinical Specimens

The study population consisted of leprosy patients referred to the Souza Araujo Outpatient Clinic, a Reference Center for Leprosy Diagnosis and Treatment, Oswaldo Cruz Foundation (FIOCRUZ) in Rio de Janeiro, RJ, Brazil. Age- and ethnic-matched healthy volunteers were also recruited among the Leprosy Laboratory staff at the same institution to serve as healthy donors (HD). Each patient was clinically assessed by way of detailed clinical and dermatological examinations, after which WHO–recommended MDT for leprosy patients was administered. Bacteriological examinations of slit-skin smears were done to determine the bacilloscopic index. None of the participants displayed any infectious or chronic inflammatory co-morbidities such as HIV, syphilis, hepatitis, cancer, or diabetes. The present study was approved by the FIOCRUZ Committee for Ethics in Research (CAAE 56113716.5.0000.5248). Written informed consent was obtained from all participants prior to enrollment and sample collection.

Leprosy patients were diagnosed and categorized according to the Ridley and Jopling classification scale (27) as BL or LL. ENL diagnosis was primarily based on the occurrence of nodular skin lesions with or without fever and peripheral nerve pain and/or

nerve dysfunction. Clinical samples were collected from three different groups of patients: (i) The NR group – BL/LL patients with no signs of leprosy reaction at diagnosis; (ii) The ENL group – patients at the onset of reaction and before thalidomide or corticosteroid administration, none of whom had been treated with corticosteroid and/or thalidomide for at least 4 months prior to recruitment; and (iii) The ENL_{Thal} patients recruited for a reevaluation at day 7 after starting thalidomide at 100–300 mg/day. Punch biopsies (6 mm in diameter) from active skin lesions, containing both epidermis and dermis, were obtained at diagnosis. These specimens were split in parts and later used for histopathological, molecular, and Western blot analyses. Whole blood samples were collected from leprosy patients and HD for *in vitro* stimulation assays and *ex vivo* analysis. The baseline characteristics of the patients enrolled in the study are shown in **Supplementary Tables 1–5**.

RNAseq Analysis

Total RNA from 2.5 mL of fresh blood collected in the PAXgene Blood RNA Tube (Qiagen, Germany) was extracted using the PAXgene blood RNA kit (Qiagen), as instructed by the manufacturer. RNA quality was assessed *via* Agilent tapeStation 2200 (Agilent, United States), with the sole inclusion of RNAs with RIN > 8. A polyA-enriched complementary DNA (cDNA) library was obtained using the NEBNext Poly(A) mRNA magnetic isolation module (New England Bioscience, United States) and NEBNext Ultra II Directional RNA for Illumina kit (New England Bioscience). RNA sequencing was performed on the NextSeq550 Illumina platform with 75 paired-end cycles (Illumina, United States). The raw RNAseq dataset is readily available at Gene Expression Omnibus (GSE198609). For the initial read quality control, FastQC v.0.11.8[1] and MultiQC v.1.9 (28) were utilized. To remove adapter and poly-X sequences and trim the first 10 bases at 5'-end, fastp v.0.21.0 was applied. Pre-processed reads were quantified against the human transcriptome (GRCh38p.12)[2] with the Salmon v.1.4. pipeline *via* the quasi-mapping method with the –gcBias and –seqBias flags set and other default settings. Transcript quantification was summarized into ENSEMBLE genes with tximport v.1.12.0 (29) and biomaRt v.2.40.5 (30). Differential gene expression analysis was performed with DESeq2 v.1.24.0 (31); and *p*-values were adjusted by the Benjamini and Hochberg method to control the false discovery rate (FDR; 32). Fold-changes were moderated with the "ashr" adaptive estimator (33). Genes were considered differentially expressed if $|\log_2 FC| \geq 0.585$ and FDR ≤ 0.1. For heatmap and clustering, the normalized expression matrix was transformed by way of the shifted logarithm (base 2). Next, gene expression was standardized to mean zero and unit standard deviation while the hierarchical clustering of genes and the heatmap were generated by the R package pheatmap v.1.0.12 with the Pearson correlation coefficient as the distance metric (34). Gene set variation analysis (GSVA) was used to summarize the multiple gene expressions of a given pathway into a sample-wise representative score. Genes were retrieved from Gene Ontology's "type I interferon signaling" (GO: 0060337), "type I interferon production" (GO:0032606), and Reactome's "interferon alpha/beta signaling" (R-HSA-909733.5) pathways. Patient information is described in **Supplementary Table 1**. The statistical significance of GSVA scores was tested separately for within- and between-patient comparisons. Within patient comparisons, the significance between scores was tested *via* a non-parametric permutation procedure using the R package exactRankTests v.0.8-34. Independent samples were tested by the exact Independence test implemented in the coin v.1.4-2 package. All *p*-values reported are two-tailed and nominal.

Real Time RT-qPCR

Total RNA from whole blood cells was isolated by way of the PAXgene Blood RNA kit (Qiagen) according to the manufacturer's recommendations. Total RNA was obtained from skin samples, cDNA was synthesized, and real-time polymerase chain reactions (quantitative RT-PCR) were performed as previously described (35). Oligonucleotide sequences used in the RT–qPCR assays are displayed in **Supplementary Table 6**. The reactions were incubated in the StepOnePlus® real-time PCR equipment (Applied Biosystems, United States) as described (36). For each sample, the cycle- threshold (CT) means of the genes of interest (*IFNB*, *IFNAR1*, *IFI16*, *TBK1*, *EIF2AK2,* and *MX1*) were normalized by the CT mean of the reference gene *RPL13a* (ThermoFisher Scientific, United States). The relative gene expression analysis was performed utilizing the $2^{-\Delta CT}$ method for each target gene (37).

Immunohistochemical and Immunofluorescence Stainings

Histology of skin tissue from NR and ENL patients was carried out as reported previously (38, 39). Standard staining with hematoxylin and eosin (H&E) was done in sections obtained from paraffin-embedded tissue for morphological analysis. For immunohistochemical procedures, tissue sections taken from frozen samples were incubated overnight in 1% normal goat serum (NGS, Sigma-Aldrich, United States) at 4°C with primary anti-IFI16 human monoclonal antibodies (sc-8023, 1G7 clone, Santa Cruz Biotechnology, United States) or anti-CD123 (306002, Biolegend, United States), both at 1:50 dilution, and polyclonal anti-IRF3 (sc-9082, FL-425, Santa Cruz Biotechnology) at 1:100 dilution. An AEC (3-amino-9-ethylcarbazole) solution (Vector Laboratories, United States) was used to detect the primary antibodies (as the manufacturer's instructions indicate) and monitored under a microscope for a maximum of 20 min to avoid overstaining. The sections were then counterstained with Mayer's Hematoxylin and mounted with aqueous Mounting Medium (Cell Marque, United States). The primary antibodies were omitted in the negative control slides. Samples were analyzed on a Nikon Eclipse E400 brightfield optical microscope (Nikon Instruments Inc., United States); and a minimum of ten random images per sample were evaluated. For immunofluorescence analysis, sections were incubated overnight with primary mouse monoclonal antibodies

[1]https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[2]http://refgenomes.databio.org/v2/asset/fa159612d40b1bedea9a279eb24999b3d27145f9dd70dcca/salmon_index/splash?tag=default

against IFN-α (21100, MMHA-2 clone, PBL Assay Science, United States), at 1:100 dilution at 4°C, followed by incubation with secondary goat anti-mouse conjugated with Alexa Fluor 594® (A-11005, Invitrogen, United States) at 1:500 dilution for 1.5 h at room temperature. The nuclei were evidenced by staining with 4'-6'-diamidino-2-phenylindole (DAPI; Molecular Probes, United States) while the slides were mounted with Prolong Gold Antifade (Invitrogen) and analyzed *via* an Axio Observer.Z1 fluorescence microscope equipped with the Colibri.2 illumination system (Carl Zeiss, Germany).

Western Blot

Biopsied skin lesions were processed for protein extraction in accordance with TRIzol® Reagent protocol (Thermo Fisher Scientific). Proteins isolated from the phenol-ethanol supernatant were dialyzed against 0.1% sodium dodecyl sulfate (SDS, Sigma-Aldrich) as previously described (40). The amount of protein in the samples was measured by the Bradford method with the Pierce Coomassie reagent (Thermo Fisher Scientific). Total protein was separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to the nitrocellulose membrane (G.E. Healthcare Life Sciences, United States), and incubated overnight at 4°C with rabbit polyclonal anti-MX1 antibody (13750-1-AP, Proteintech, United States; 1:1,000 dilution), followed by HRP-conjugated anti-rabbit IgG (A16104, Thermo Fisher Scientific; 1:5,000 dilution). Loading control was assessed *via* GAPDH quantification. Membranes were incubated for 2 h with mouse monoclonal anti-GAPDH antibody (sc-32233, 6C5 clone, Santa Cruz Biotechnology; 1:1,000 dilution) followed by HRP-conjugated, anti-mouse IgG (A16072, Thermo Fisher Scientific; 1:10,000 dilution). Protein bands were detected by chemiluminescence using the Amersham ECL Western Blotting Kit (G.E. Healthcare Life Sciences). Relative protein levels were analyzed *via* ImageJ software.

Flow Cytometry Analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by gradient centrifugation using Ficoll-Paque (G.E. Healthcare Life Sciences). Purified cells were suspended in PBS containing 0.5 M ethylenediamine tetraacetic acid and 10% BSA treated with Fc receptor blocking (human TruStainFcX; Biolegend) and then labeled with the extracellular antibody cocktail for 30 min at 4°C. The following fluorochrome-conjugated human antibodies were used for identification of circulating pDCs: Lineage-FITC (αCD19: 302206, HIB19 clone, Biolegend; αCD20: 302304, 2H7 clone, Biolegend; αCD56: 304606, MEM-188 clone, Biolegend; αCD3: 300306, HIT3a clone, Biolegend; αCD14: 347493, MφP9 clone, BD Bioscience, United States and αCD16: 555406, 3G8 clone, BD Bioscience), αCD123-PerCP/Cy5.5 (306016, 6H6 clone, Biolegend), αCD11c-APC/Cy7 (3372118, Bu15 clone, Biolegend), and αBDCA4-APC (354506, 12C2 clone, Biolegend). After extracellular labeling, the cell suspension was fixed with a 2% paraformaldehyde solution. For experiments in which intracellular staining was performed, the pDCs were identified similarly to what has been described above, except for the exclusion of antibodies that make up the lineage cocktail and the replacement of αBDCA4-APC by αBDCA2-BV421 (354212, 201A clone, BD Bioscience).

Next, fixed cells were permeabilized with 2% saponin and incubated with intracellular antibodies against TLR9 (ab134368, 26C593.2 clone, Abcam, United States) and TLR7 (ab28048, 4F4 clone, Abcam) conjugated with Alexa Fluor® 488 and Alexa Fluor® 647, respectively. Cells were assessed using BD FACSAria™ flow cytometer (BD Bioscience); and the resulting data were analyzed by FlowJo V10 software (BD Bioscience, United States).

In vitro Stimulation Assays

2×10^6 PBMCs from healthy individuals were stimulated with 0.5 μM CpG-A (ODN-2216, InvivoGen, United States) or 20 μg/mL of *M. leprae* whole cell sonicate (NR-19329, BEI Resources, United States). In parallel, 50 μg/mL of thalidomide (ab120032, Abcam) were added or not to the cultures, which were kept at 37°C in a 5% CO_2 atmosphere for 24 h. Supernatants were harvested and stored at −20°C until TNF and IFN-I measurements.

Biological Assay of Type I IFN

Type I interferon levels were quantified using the Hek-Blue™ IFNα/β (hkb-ifnab, InvivoGen) biological assay. Twenty microliters per well of conditioned supernatants from stimulated PBMCs or synthetic IFNα/β (Biosintetica, Brazil), utilized to build the standard curve, were added to a 96-well cell culture plate (Corning, United States). A volume of 180 μL from a cell suspension containing 2.8×10^5 Hek-Blue cells/mL was added to each well; and the cultures were maintained at 37°C in a CO_2 atmosphere for 24 h. Afterward, 20 μL of the supernatants were transferred to another plate and 180 μL of QUANTI-Blue™ (InvivoGen) were added to the wells. The plates were incubated at 37°C for 15 min in the dark. The optical density was determined at 620 nm with the BioTek Eon™ Microplate Spectrophotometer (BioTek, United States).

Enzyme-Linked Immunosorbent Assay

IFN-β detection was performed in serum samples using the immunoenzymatic sandwich VeriKine Human IFN Beta ELISA kit (41410, PBL assay science), according to the manufacturer's protocol. TNF levels in conditioned supernatants from stimulated PBMCs were measured by the Human DuoSet kit (DY210, R&D Systems, United States) as recommended. The reading of optical densities was measured *via* the SpectraMax® 190 Absorbance Microplate Reader (Molecular Devices, United States); and the data were analyzed by SoftMax® Pro Software version 5.3.

Statistical Analysis

Comparisons between two groups were assessed by the two-tailed Student *t* test for normally distributed data. Comparisons between more than two groups with normally distributed data were addressed *via* variance analysis (ANOVA) using Bonferroni's correction for multiple testing. Alternatively, the non-parametric Mann Whitney test was chosen to

analyze unpaired data and the Wilcoxon test, for paired data. Non-normal comparisons among more than two groups were performed *via* the Kruskal–Wallis test with Dunn's multiple comparison *post hoc* test. Statistical analysis was done *via* GraphPad Prism version 9.0.0 (GraphPad Software, United States); and the adopted statistical significance level was $p < 0.05$.

RESULTS

Type I IFN Signaling Is Upregulated in the Whole Blood of Erythema Nodosum Leprosum Patients

Since ENL is frequently accompanied by systemic symptoms, an assessment of the IFN-I pathway in the blood compartment was performed. RNAseq data comparing the whole blood of ENL and NR patients revealed an increased enrichment of type I IFN genes in the former patients when compared to the latter, in whom, respectively, the reactome IFN-α/β pathway (median difference = 0.3, $p = 0.12$) presented a median pathway score of −0.09 and −0.39. Using the gene ontology Type I IFN annotation, the same comparison resulted in median scores of 0.32 and −0.27, respectively, for ENL and NR (median difference = 0.59, $p = 0.067$; **Figure 1A**). The *IFITM1* (a 1.85-fold increase), *GBP2* (a 1.6-fold increase), and *IFITM2* genes (a 1.62-fold increase) were the top three upregulated IFN I-related genes found in the ENL whole blood samples (**Figure 1A**). Interestingly, the genes associated with IFN I production such as *TLR8* and *TLR9* were also positively regulated in whole blood cells of ENL patients (**Supplementary Table 7**). Moreover, the analysis of paired samples from ENL patients before treatment of reaction (ENL) and on day 7 of thalidomide administration (ENL_{Thal}) points to an overall decreasing trend in the IFN-I pathway scores for both datasets during treatment (**Figure 1A**). The expression of three type I IFN-regulated genes (*EIF2AK2, MX1,* and *IFI16*) and two IFN-I pathway genes (*TBK1* and *IFNAR1*) was further explored by RT-qPCR analysis (**Figure 1B**). The ISGs were selected based on their upregulation in other diseases with a type I IFN signature in the blood (41–44). Despite the observation of heterogeneous behavior among ENL patients, significantly higher mRNA levels of *EIF2AK2, MX1, IFI16,* and *TBK1* were detected in ENL when compared to those of NR patients (with respective p values of 0.0387; 0.0358; 0.0331; and 0.0514). The RT-qPCR analysis of the *EIF2AK2* and *MX1* genes in paired samples of ENL and ENL_{Thal} did not show a clear decrease in the expression of these genes in blood cells after 7 days of thalidomide treatment (**Figure 1C**). *EIF2AK2* mRNA levels were reduced in four patients after treatment while no change was observed in the remaining three. Similar behavior was observed regarding the *MX1* gene (**Figure 1C**). Clinical data of the patients enrolled in the experiments shown in **Figures 1A–C** can be seen in **Supplementary Table 1**.

IFN-β concentration (expressed as pg/mL) was then quantified in the serum samples of NR ($n = 8$) and ENL patients ($n = 18$). Although no statistically significant differences were observed, the median IFN-β serum levels were roughly 1.7-fold higher in ENL than in NR patients [876.8 (IQR: 251.9–2058) versus 521.5 (IQR: 169.1–765.4), respectively, $p = 0.1961$] (**Figure 1D**). To verify whether the IFN-β serum levels observed in ENL patients are affected by thalidomide, a longitudinal follow-up of these patients was performed during treatment. A decrease in IFN-β serum levels was observed in 9 out of the total 13 patients at day 7 of thalidomide treatment (**Figure 1E**; $p = 0.0574$). Clinical data of patients enrolled in the assays in **Figures 1D,E** are shown in **Supplementary Table 2**. In summary, these results suggest the upregulation of the IFN-I signature in the peripheral blood of ENL patients and the tendency to decrease this upregulation during thalidomide treatment.

Type I IFN Pathway Genes Are Upregulated in Erythema Nodosum Leprosum Skin Lesions

To investigate whether type I interferons play a role in ENL immunopathogenesis on the reaction site, the mRNA levels of the IFN-I-related genes previously evaluated in the blood were then analyzed in ENL skin lesions by RT-qPCR. For comparison, cutaneous lesions of NR patients, previously described as already exhibiting an IFN-β signature (45), were also studied and the mRNA levels of the *IFNB* gene, monitored. Clinical data of patients enrolled in this analysis are shown in **Supplementary Table 3**. In contrast to NR, the majority of ENL skin lesions showed detectable levels of all genes investigated. Although a heterogeneous expression profile was observed among patients, significantly higher levels of mRNA for the *EIF2AK2* ($p = 0.0131$), *MX1* ($p = 0.0469$), *IFNB* ($p = 0.0004$), *IFNAR1* ($p = 0.0001$), and *TBK1* ($p = 0.0150$) genes were detected in ENL versus NR lesions (**Figures 2A–E**). The expression of the *IFI16* gene was also noted, presenting similar levels in both groups of patients ($p = 0.9824$; **Figure 2F**). These initial data suggest that the type I IFN pathway is activated in ENL skin lesions in that IFN-I-associated genes showed higher expression levels than those found in the NR lesions.

The IFN-I Pathway Is Downregulated During Thalidomide Treatment in Erythema Nodosum Leprosum Skin Lesions

Since thalidomide is known to drastically ameliorate ENL cutaneous and systemic symptoms after a brief period of treatment (24, 25) the next step involved investigating the effect of this drug on the expression of type I IFN-associated genes/molecules in ENL lesions of leprosy patients during thalidomide therapy. For this purpose, at day 7 of thalidomide treatment, ENL skin biopsies were collected for analysis (ENL_{Thal} group) and compared with the pre-treatment lesions. Clinical data of the patients enrolled in this section of the study are shown in **Supplementary Table 4**.

Firstly, the transcriptional expression levels of key genes of the IFN-I pathway were analyzed in a follow-up analysis of ENL skin lesion specimens collected from patients before and after initiating thalidomide therapy. Except for *IFNB* mRNA,

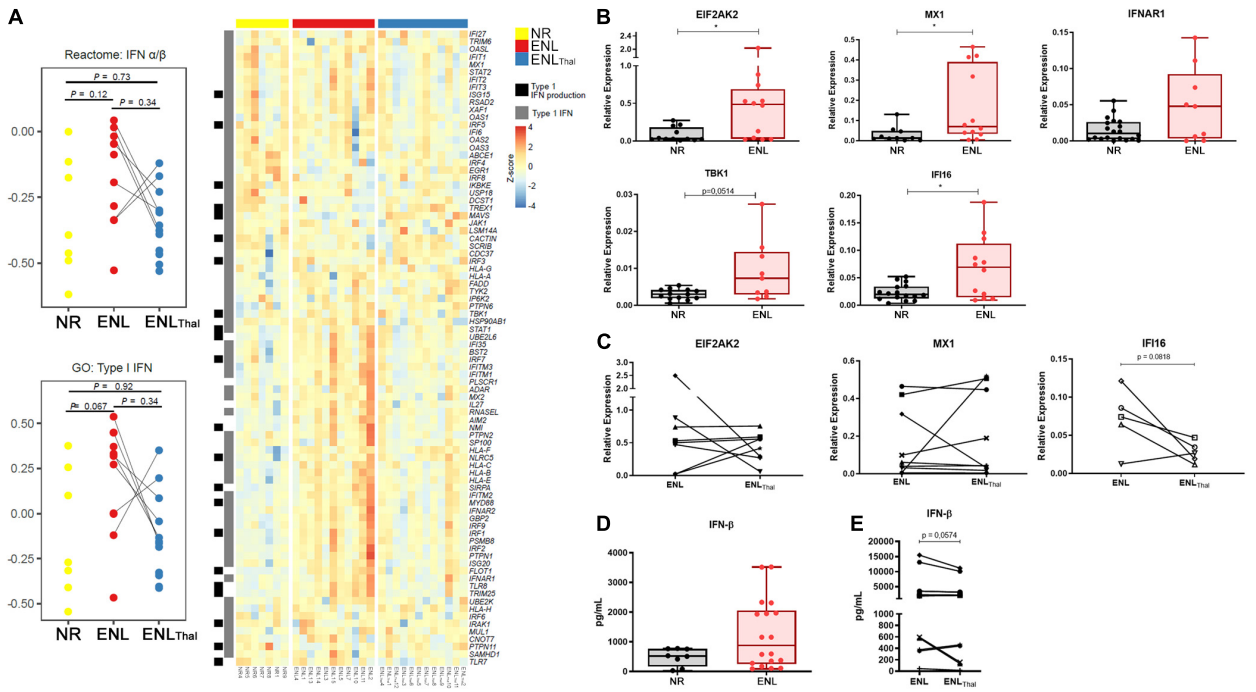

FIGURE 1 | Type I IFN pathway is positively regulated in the blood of ENL patients. Whole blood cells of non-reactional multibacillary leprosy patients (NR – n = 7, *yellow*), ENL patients (ENL – n = 11, *red*), and thalidomide-treated ENL patients (ENL_{Thal} – n = 12, *blue*) were properly collected and RNA was extracted using the PAXgene protocol. RNAseq was performed at NextSeq 550 platform and data analysis was performed using Salmon pipeline, followed by DESeq2 analysis to determine differentially- expressed genes. **(A)** *On the left*: Pathway score for the IFN-I pathway was determined using reactome IFN α/β and gene ontology type I IFN databases. *On the right*: Heatmap including type I IFN genes (*gray*) and Type I IFN production (*black*) from gene ontology database. **(B,C)** RNA from whole blood cells was extracted from NR, untreated ENL, or ENL patients on day 7 of thalidomide therapy (ENL_{Thal}) to validate the gene expression of ISG genes by RT-qPCR. *RPL13* was used as an endogenous control. **(B)** Gene expression levels were determined in NR (*black*) and ENL patients (*red*) for *EIF2AK2* (NR = 14, ENL = 14); *MX1* (NR = 14, ENL = 15); *IFNAR1* (NR = 20, ENL = 9); *TBK1* (NR = 13, ENL = 9), and *IFI16* (NR = 16, ENL = 12). $^*p < 0.05$. **(C)** Follow-up gene expression analysis of *EIF2AK2* (n = 8), *MX1* (n = 9), and *IFI16* (n = 5) in ENL patients before (ENL) and at day 7 of thalidomide treatment (ENL_{Thal}). **(D,E)** IFN-β levels in serum samples of NR and ENL leprosy patients. **(D)** Serum concentrations of IFN-β were evaluated by enzyme-linked immunosorbent assay (ELISA) in NR (n = 8, *black*) and ENL patients (n = 18, *red*). **(E)** Serum concentrations of IFN-β in a longitudinal follow-up measurement of leprosy patients diagnosed with ENL and after 7 days of thalidomide treatment (n = 13). Box plots show median, interquartile range, sample minimum and maximum. Each dot represents a donor.

which displayed a highly heterogeneous patient response, our data showed a decrease in the mRNA levels of *MX1*, *IFNAR1*, *TBK1*, *EIF2AK*, and *IFI16* in most patients under thalidomide treatment. Yet, only *MX1* (p = 0.0127; **Figure 3A**) attained a statistically significant difference.

Next, a longitudinal follow-up analysis of the levels of the MX1 protein by Western blot was performed in ENL and ENL_{Thal} skin lesions. A reduction of about 50% in MX1 protein expression in ENL_{Thal} was detected *via* densitometric analysis (**Figure 3B**). In addition, IFN-α expression was assessed by immunofluorescence staining of the ENL (n = 3) and ENL_{Thal} (n = 3) cryosections of skin lesions. In the dermis of ENL lesions, IFN-α (*green*) expression was found in the inflammatory infiltrate. But, after 7 days of treatment, only a faint signal could be detected (**Figure 3C**). IFI16 protein levels in ENL (n = 4) and ENL_{Thal} (n = 4) tissues were then monitored by immunohistochemistry. To further complement the study, the protein levels of IRF3, a transcriptional factor that regulates the expression of IFN-I genes and a number of ISGs (46), were also analyzed by immunohistochemistry. The *IRF3* gene was one of the upregulated IFN-I-related genes in the RNAseq analysis of ENL peripheral blood (**Figure 1A**). $IFI16^+$ and $IRF3^+$ cells were observed among the inflammatory infiltrates in the ENL dermis (**Figure 3D**). However, a substantial decrease in IFI16 and IRF3 labeling was seen after 7 days of thalidomide intake (**Figure 3D**). Altogether, these data suggest that, in ENL skin lesions, thalidomide treatment leads to the downregulation of the IFN-I pathway.

Thalidomide Inhibits CpG- and *Mycobacterium leprae*-Induced *in vitro* IFN-I Production

To confirm the capacity of thalidomide to inhibit the IFN-I pathway, PBMCs isolated from HD were stimulated for 24 h with CpG-A 2216, a TLR9 agonist, or *M. leprae* whole cell sonicate in the presence or not of thalidomide. IFN-I was measured in the culture supernatants. TNF quantitation was included as a positive control based on the well-established capacity of thalidomide to block *M. leprae*-induced TNF secretion by PBMCs (47). **Figure 4** shows that thalidomide was able to completely block the secretion of IFN-I by the PBMCs of all donors in response to both stimuli

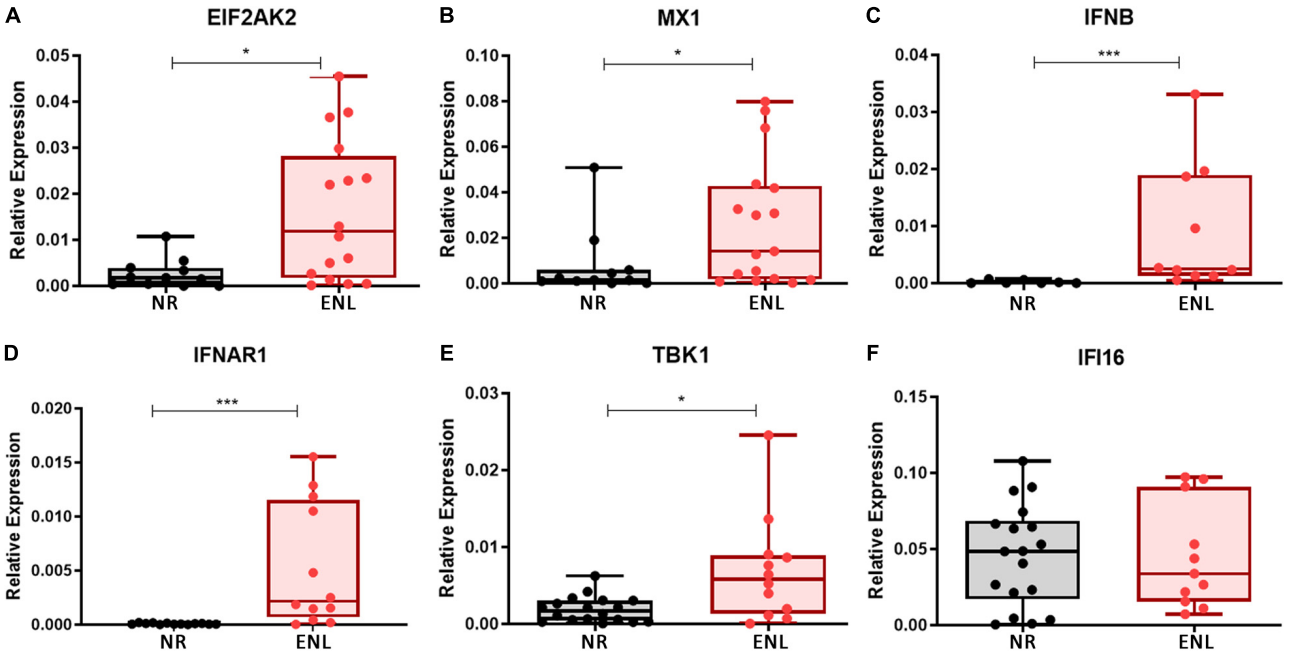

FIGURE 2 | Type I IFN signature genes are upregulated in ENL skin lesions. Skin lesions of non-reactional multibacillary patients (NR, *black*) or ENL patients (ENL, *red*) were processed in TRIzol® reagent for RNA extraction and gene expression was determined by RT-qPCR for the following ISGs: **(A)** *EIF2AK2* (NR = 12, ENL = 16); **(B)** *MX1* (NR = 11, ENL = 17); **(C)** *IFNB* (NR = 7, ENL = 10); **(D)** *IFNAR1* (NR = 13, ENL = 12); **(E)** *TBK1* (NR = 18, ENL = 12); and **(F)** *IFI16* (NR = 18, ENL = 11). *RPL13* was used as an endogenous control. Box plots show median, interquartile range, sample minimum and maximum. Each dot represents a donor. $^{*}p < 0.05$; $^{***}p < 0.001$.

(**Figures 4A,B**). Thalidomide inhibited the production of TNF stimulated by CpG (**Figure 4C**), and as expected, in *M. leprae*-stimulated cells as well (**Figure 4D**).

Involvement of Plasmacytoid Dendritic Cells in Erythema Nodosum Leprosum

Plasmacytoid dendritic cells are able to produce large amounts of IFN-I and, when activated, migrate from the blood to the affected tissues (12). Indeed, the cutaneous infiltration of these cells is a histological hallmark in inflammatory and autoimmune diseases affecting the skin, upon which they imprint an IFN-I signature (15–18). In humans, pDCs express a rather specific set of cell surface markers such as the blood dendritic cell antigen 2 (BDCA-2) and BDCA-4, along with a high expression of CD123, the alpha subunit of the IL-3 receptor (10). Therefore, to investigate whether this particular cell type contributes to the IFN-I signature found in ENL lesions, the frequency of pDCs in the blood of NR and ENL patients in comparison to that of HDs was evaluated. Clinical data of all patients enrolled in these subsequent experiments are shown in **Supplementary Table 5**. **Supplementary Figure 1** shows the flow cytometry gating strategy used to identify circulating pDCs ($Lin^{-}CD11c^{-}CD123^{+}BDCA4^{+}$) among PBMCs and the representative plots of the cell population for each study group. *Ex vivo* flow cytometry analysis showed significantly lower frequencies of pDCs in ENL patients when compared to the healthy donor rates (HD = 0.31%, NR = 0.25%, ENL = 0.20%; HD versus ENL, $p = 0.018$; **Figure 5A**).

Since pDCs are known to produce IFN-I mainly through extracellular nucleic acid recognition *via* TLR7 and TLR9 endosomal receptors (5, 9, 10), the next step aimed to analyze the status of their expression in circulating pDCs. In this *ex vivo* assay, a similar flow cytometry gating strategy was undertaken to identify circulating pDCs, except for the replacement of the anti-BDCA4 by an antibody that recognizes BDCA2 (**Supplementary Figure 2**). TLR9 expression levels in circulating pDCs ($CD11c^{-}CD123^{+}BDCA2^{+}$), obtained by mean fluorescence intensity (MFI) values, were about three-fold higher in ENL pDCs when compared to the pDCs of the NR patients and healthy volunteers (HD = 621, NR = 666.5, ENL = 1,875; NR versus ENL, $p = 0.024$; HD versus ENL, $p = 0.011$). TLR9 MFI values of NR patient pDCs did not differ from those found among the healthy volunteers (**Figure 5B**). In contrast, TLR7 expression presented a significant increase in NR pDCs in comparison to the HD while similar MFI values were observed between the ENL pDCs and their healthy counterparts (HD = 302, NR = 1,803, ENL = 416; HD versus NR, $p = 0.004$; **Figure 5C**). Interestingly, the MFI median values of TLR7 in NR pDCs were overall around four-fold higher relative to those of ENL patients and HD (**Figure 5C**).

The presence of $CD123^{+}$ cells in the skin lesions of NR and ENL patients was analyzed. Skin biopsy specimens stained with H&E confirmed the characteristic inflammatory infiltrate observed in NR lesions, namely, a diffuse dermal inflammatory

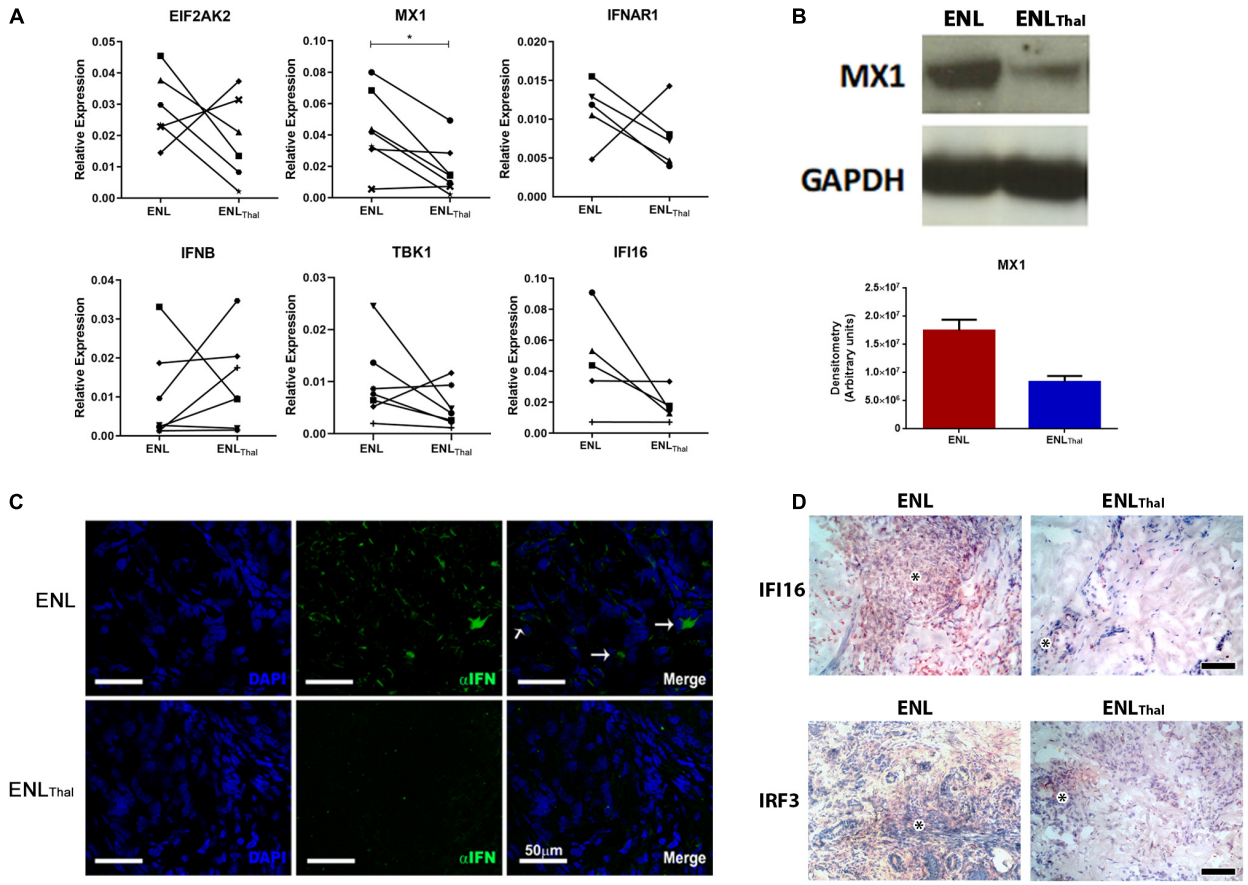

FIGURE 3 | Thalidomide treatment decreases IFN-I pathway activity in skin lesions of ENL patients. **(A)** Follow-up of ENL patients before (ENL) and after 7 days of thalidomide treatment (ENL_{Thal}). Skin lesions were processed in TRIzol® for RNA extraction. Gene expression was determined by RT-qPCR for the following genes: *EIF2AK2* (n = 6), *MX1* (n = 7), *IFNAR1* (n = 5), *IFNB* (n = 7), *TBK1* (n = 7), and *IFI16* (n = 7). *RPL13* was used as an endogenous control. **(B)** *Top*– Longitudinal follow-up of MX1 protein levels by Western blot in skin lesions before (ENL = 3) and on Day 7 of thalidomide treatment (ENL_{Thal} = 3). GAPDH was used as an endogenous constitutive gene. *Bottom* – Densitometry analysis displayed in arbitrary units. **(C)** Immunofluorescence for IFN-α (*green*) of skin lesions of ENL patients (n = 3) and ENL_{Thal} (n = 3). Cell nuclei are in *blue* by DAPI staining. *White* arrows (→) indicate IFN-α labeling in the merge images. Scale bar = 50 μm. **(D)** Representative immunohistochemical analysis of IFI16 and IRF3 proteins in skin lesions of ENL and ENL_{Thal} patients. Inflammatory infiltrate is indicated by the asterisk symbol. The photomicrographs are representative of four patients from each group. Scale bar = 100 μm. *$p < 0.05$.

infiltrate with macrophages and rare polymorphonuclear cells. At the same time, microabscesses with a high concentration of polymorphonuclear cells were observed in ENL lesions (**Figure 5D**, Top). In NR lesions, $CD123^+$ cells were primarily found in the peripheral regions of the dermal inflammatory infiltrates. Conversely, in ENL lesions, marked CD123 staining was more in evidence in microabscesses close to neutrophilic infiltration (**Figure 5D**, Bottom). Altogether, the data generated in this section of the study infer that, in ENL, pDCs may contribute to the production of type I IFNs.

DISCUSSION

The physiopathological mechanisms involved in ENL, a major complication of leprosy, remain elusive. ENL shares similar features with several of the chronic inflammatory and autoimmune diseases that typically intercalate periods of inflammatory activity with periods of remission. In several of these pathologies, type I IFNs have been shown to play a central role as adjuvants by exacerbating inflammation through the activation of both innate and acquired immune pathways (4, 5). Moreover, pDCs activation *via* nucleic-acid recognition by endocytic TLR7/TLR9 has been implicated as an important source of IFN-I in these diseases (5, 9, 10). In the present study, the production of IFN-I during reaction was investigated based on our previous report suggesting that DNA sensing *via* TLR9 constitutes a major innate immunity pathway involved in the pathogenesis and progression toward ENL. The generated data showed higher activation of the type I IFN pathway both in the skin lesions and blood of ENL patients compared to the corresponding data found in NR patients. Upon treatment with thalidomide, the type-I IFN pathway seems to recede in both compartments. Furthermore, *in vitro* assays have confirmed the capacity of thalidomide to block IFN-I production by PBMCs in response to *M. leprae*. Finally, the decreased frequencies

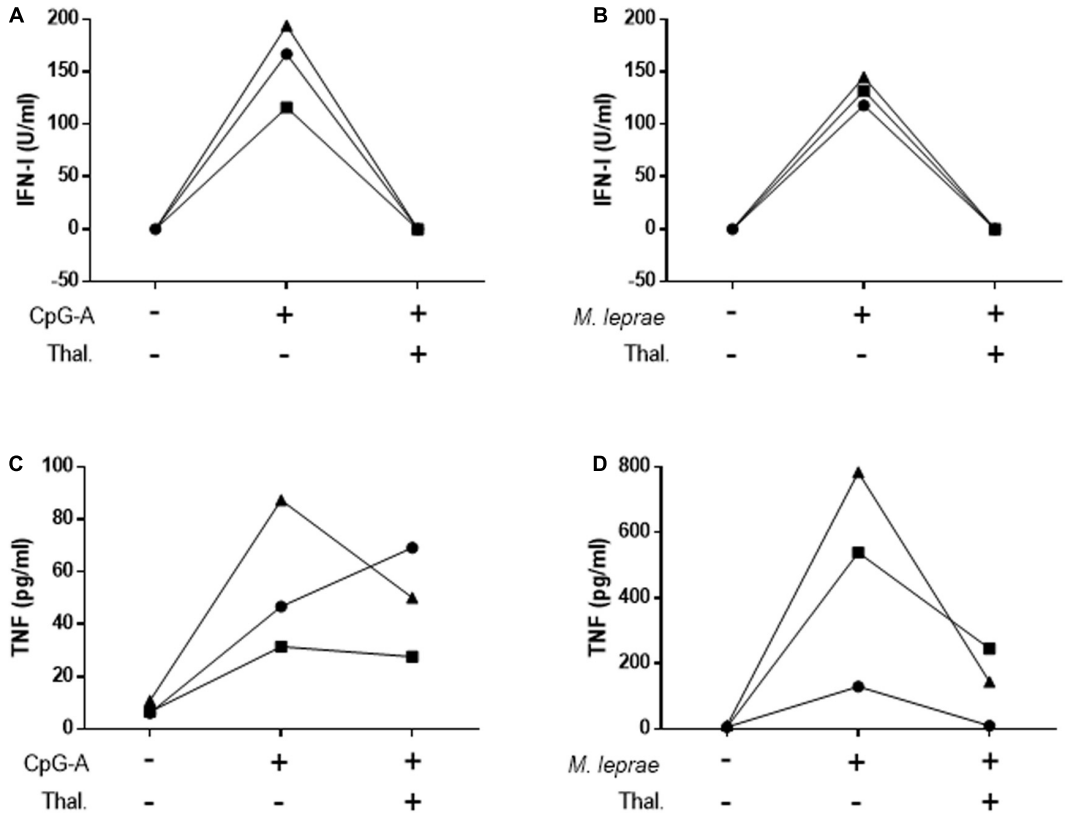

FIGURE 4 | Thalidomide blocks CpG- and *M. leprae*-induced IFN-I secretion by PBMCs. Quantification of IFN-I **(A,B)** and TNF **(C,D)** levels in culture supernatants of PBMCs from three healthy donors stimulated with 0.5 μM CpG-A **(A,C)** or 20 μg/mL *M. leprae* sonicate **(B,D)** and treated or not with 50 μg/mL thalidomide for 24 h. Each individual is represented by a different symbol.

observed in peripheral pDCs, together with their higher TLR9 expression and the presence of CD123 + cells in the ENL skin lesions, imply the involvement of these cells as IFN-I producers during this type of reactional episode.

Although a positive regulation of the type I IFN pathway was detected during ENL, quite heterogeneous behavior was observed among patients with respect to mRNA levels of target genes, the RNAseq profile, and circulating IFN-β levels. A possible reason for this heterogeneity might be traced to the differences each patient encounters regarding the time period covered from the onset of ENL symptoms up to diagnosis and, likewise, in the severity of the reactional episode itself. Another factor that may affect patient response is the time span of MDT treatment since its length may or may not strongly influence the immune status of the patient in any direction (48).

Albeit not a major upregulated pathway in the RNAseq, the observed enrichment in type I Interferon genes in ENL versus NR whole blood correlates with the upregulated IFN I pathway found in whole blood transcriptomics analysis during reversal reaction, the other type of leprosy reaction (49, 50), suggesting that this upregulation could be a common dissonant trait during acute inflammatory reactional episodes. It is noteworthy that IFI16 expression was upregulated in whole blood cells while no difference was observed in ENL skin lesions. Higher anti-IFI16 antibody levels have already been associated with other inflammatory diseases such as SLE (51), rheumatoid arthritis (52), and psoriatic arthritis (53). Besides identifying increased IFI16 gene expression levels in SLE, increased levels have also been reported to be closely related to disease activity (54), pointing to IFI16 as a candidate blood biomarker during an ongoing ENL episode.

Type I interferons play a complex, highly contextually dependent role in infectious diseases, leading to either beneficial or detrimental outcomes for the host. Even during viral infections, in which interferons classically contribute to protection, IFN-I may act by suppressing the immune response, thus favoring a chronic perpetuation of the infection. IFN-I may also be responsible for immunopathology and host morbidity and/or mortality (3). In the case of bacterial pathogens, IFN-I signaling likewise displays diverse effects on host susceptibility; and the mechanisms responsible for these effects are wide and varied (2, 3). In a previous study, the predominance of an IFN-I signature was demonstrated in cutaneous lesions of NR patients (BL/LL) in contrast to the IFN-γ program observed in the self-limiting paucibacillary [tuberculoid (TT) and borderline tuberculoid (BT)] clinical forms of the disease. The authors showed that local IFN-I production, particularly IFN-β produced by infected macrophages, is capable of

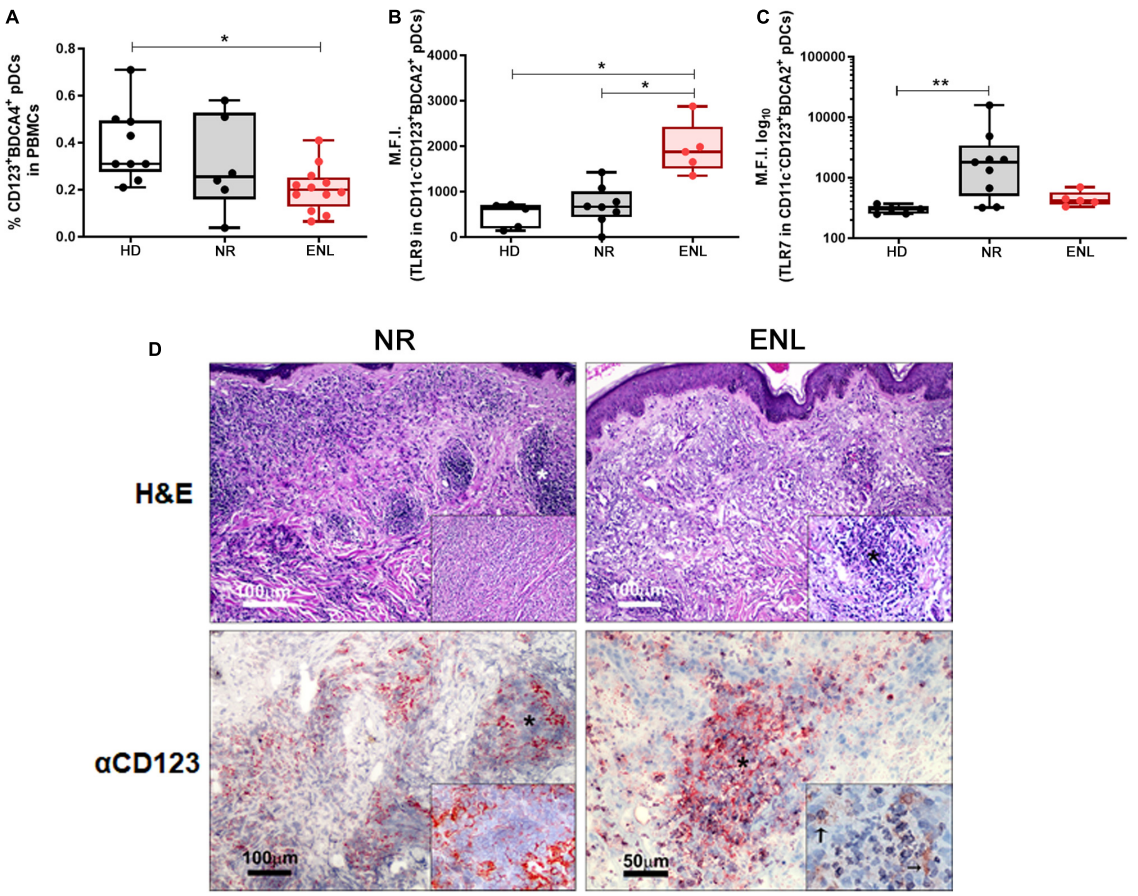

FIGURE 5 | Involvement of plasmacytoid dendritic cells in ENL. **(A–C)** pDC analysis by flow cytometry. **(A)** Frequency of Lin$^-$CD11c$^-$CD123$^+$BDCA4$^+$ pDCs among PBMCs isolated from HD (n = 9), NR (n = 6), and ENL patients (n = 12). TLR-9 **(B)** and TLR-7 **(C)** expression levels in pDCs (CD11c$^-$CD123$^+$BDCA2$^+$) present in PBMCs isolated from HD (n = 5), NR (n = 8), and ENL patients (n = 5). Box plots show median, interquartile range, and sample minimum and maximum. Each dot represents a donor. *$p < 0.05$, **$p < 0.01$. **(D**, *Top*) H&E staining of skin lesions from NR and ENL patients. Asterisks indicate inflammatory infiltrates in the dermis. Insets show dermal inflammatory infiltrate with macrophages and rare polymorphonuclear cells (NR) and a microabscess with a high concentration of polymorphonuclear cells (ENL). **(D**, *Bottom*) CD123 immunohistochemical expression in skin lesions of NR and ENL patients. CD123 expression is mainly observed in the peripheral regions of the dermal inflammatory infiltrates (NR), while microabscesses in ENL lesions show a marked expression of CD123. Inset shows expression of CD123 close to neutrophils (*arrows*). Images are representative of three NR and three ENL patients.

blocking the anti-microbicidal program induced by IFN-γ, allowing for *M. leprae* persistence and multiplication (45). Complementing these data, in a recent report, the present authors demonstrated that *M. leprae* induced IFN-I in infected macrophages and Schwann cells *via* the cyclic cGMP-cAMP synthase (cGAS)-stimulator of the interferon genes (STING) cytoplasmic nucleic acid sensor pathway. Moreover, silencing the 2′-5′ oligoadenylate synthetase-like gene, the ISG that revealed the highest upregulation, decreased the intracellular viability of *M. leprae* together with a concomitant increase in microbicidal mechanisms (36). Thus, the capacity of *M. leprae* to induce an IFN-I program during the early phases of infection has been considered an important event for successful tissue colonization, constituting a crucial mechanism of bacterial pathogenesis (55). A similar detrimental role for the host of IFN-α/β during tuberculosis (TB) has been supported by studies performed on TB patients and in mouse models of infection (3).

To our knowledge, this is the first time that IFN-I pathway activation has been associated with ENL. Type I IFNs affect a broad spectrum of events implicated in the pathogenesis of several inflammatory and autoimmune diseases acting as adjuvants in exacerbating inflammation and tissue damage (5, 12, 13). Thus, it is tempting to speculate that type I IFNs may play a role in ENL pathogenesis through similar mechanisms. Indeed, the capacity of IFN-I to promote DC maturation/differentiation and increase Th1 differentiation while suppressing Tregs may be linked to the presence of a higher percentage of activated CD4$^+$ and CD8$^+$ T cells (56), a lower percentage of CD4$^+$regulatory T-cells (57), and the emergence of an IFN-γ signature in PBMCs of ENL compared to what has been found among non-reactional LL patients (58). Moreover, it has also been shown that IFN-I enhances the formation of neutrophil DNA extracellular traps (NETs; 59, 60), which is in line with our previous findings showing an increase in spontaneous NETs formation in ENL

peripheral neutrophils (61). Lastly, the recently described higher percentage of activated peripheral memory B-cells in ENL patients (62) may to some extent result in the capability of IFN-α/β to promote B-cell activation and antibody production (5, 12, 13).

As often occurs in a number of inflammatory diseases in which type I IFNs play an immunopathological role, the data in the present study suggest that pDCs are important sources of these cytokines in ENL skin lesions. It has been reported that pDCs frequency in the peripheral blood of SLE patients is reduced upon the simultaneous infiltration of these cells in target tissues such as the skin and renal tissue (63–66). Similarly, a significantly lower peripheral frequency of $CD123^+$ $BDCA4^+$ cells and the concomitant detection of $CD123^+$ cells in the inflammatory infiltrate in dermal ENL lesions suggest the migration of pDCs from the blood to the skin during reaction. However, future research is needed to better characterize the IFN-I producer cells as well as the IFN-I subtypes present in ENL skin lesions.

The presence of pDCs in leprosy lesions has been previously described (38). In this study, all $CD123^+$ cells found in the lesions were also shown to express BDCA2 and/or BDCA4, specific markers of these cells. The above authors reported higher concentrations of pDCs in the skin lesions of Type I reactional (RR) patients compared to non-reactional ones, inferring that pDCs may also play a role in this type of reactional episode, probably through the capacity of these cells to enhance both innate and adaptive immune responses *via* numerous mechanisms (18).

Evidence suggesting the involvement of pDCs in the type I signature found in ENL is in accordance with our previous data showing a higher expression of TLR9 in PBMCs and skin lesions besides higher circulating levels of mycobacterial and self-TLR9 ligands in ENL in comparison to the levels associated with NR patients (26, 61). pDCs have the capacity to rapidly produce extraordinary amounts of all type I and type III IFNs, primarily through nucleic acid sensing TLR7/TLR9 receptors. Data point to the central role played by extracellular autologous nucleic acids derived from apoptotic and/or necrotic cells and neutrophils undergoing NETosis as major inducers of IFN-I in pDCs in inflammatory and autoimmune diseases such as SLE (9). As regards ENL, circulating levels of the autologous histone-DNA complex and the mycobacterial histone-like protein (Hlp; likely complexed to bacterial DNA) were observed to be significantly higher, which may function as relevant TLR9 ligands leading to IFN-I production by pDCs during ENL (20). Indeed, after initiating MDT, ENL is most frequently observed in patients with high bacterial loads, coinciding with massive bacterial killing and mycobacterial product release. Thus, the bacterial DNA released from cells after *M. leprae* degradation is likely to act as an ENL trigger.

Furthermore, it has been shown that NETs activate pDCs to produce high levels of IFN-α in a TLR9-dependent manner (67). Additionally, the oxidized mitochondrial DNA present in NETs has been demonstrated to activate pDCs *via* the intracellular nucleic acid sensing cGAS/STING pathway (68). Likewise, recent reports support the idea that neutrophils play a central role in ENL pathogenesis (69). Indeed, in contrast to non-reactional LL/BL skin lesions, neutrophils accumulate inside ENL skin lesions, demonstrating an activated phenotype in the skin and blood (70–73). Of note, our previous study also found that ENL neutrophils underwent increased rates of NETosis (61). Abundant NETs were found in ENL skin lesions and increased spontaneous NETs formation was observed in the peripheral neutrophils of ENL patients. Indeed, neutrophils were found in the microabscesses in the vicinity of $CD123^+$ cells in ENL skin lesions. Thus, it is likely that NETs could play a role in the activation of pDCs to locally produce IFN-I during the emergence of ENL episodes. Conversely, it has been found that IFN-I can stimulate neutrophils to undergo NETosis. Indeed, a link between the presence of an IFN-I signature and neutrophil-mediated pathological damage has been established in several inflammatory manifestations (59). As such, it is possible that this positive feedback between pDCs and neutrophils may be occurring in ENL skin lesions to result in exacerbated type-I IFN production.

An intriguing observation was the opposite behavior displayed by the expression of TLR7 and 9 within the circulating pDCs of ENL versus NR patients. While TLR9 was upregulated in ENL pDCs, TLR7 was more highly expressed in NR pDCs. Since endosomal and cytoplasmic DNA- and RNA-sensing receptors recognize both autologous and pathogen-derived nucleic acids, evidence points to complex interactions maintaining a delicate equilibrium among these receptors to avoid a detrimental response to the host, culminating in inflammation and autoimmunity (74). In this context, a negative regulatory effect of TLR7 on TLR9-mediated signaling in addition to the TLR9 mRNA expression itself have been described (75). In light of these data, it is reasonable to speculate that it is TLR9, and not TLR7, the predominantly activated receptor in ENL pDCs to generate IFN-I, a finding in line with our previous data suggesting that DNA sensing *via* TLR9 is an important inflammatory pathway in ENL (26). Moreover, distinct functional outcomes attributed to TLR9 versus TLR7 activation have also been reported. In mouse models of SLE, e.g., a more pathogenic signal is primarily associated with TLR7- over TLR9-elicited responses (76). Future research is needed to evaluate the potential significance of the distinct phenotypes observed in the TLR7/9 expressions within the pDCs of NR and ENL in leprosy immunopathogenesis.

Thalidomide is considered to be the most effective drug in the treatment of ENL symptoms, with the added benefit of doing so quite rapidly. Several mechanisms have been considered as possible causes of its overall effectiveness. In principle, the capacity of the drug to inhibit TNF production is presumed to be the most important benefit (20). Nevertheless, thalidomide and its derivatives have a broad range of immunomodulatory effects that have also been examined in other diseases. Most of their immunomodulatory effects are associated with their capacity to interact with Cereblon (CRBN), a component of the Cul4A-E3 ubiquitin-ligase complex, thus interfering in the ubiquitination process of a variety of targets (77).

Our data suggest a decrease in type I IFN pathway activity in the skin lesions and blood of ENL patients undergoing thalidomide treatment. Significantly, the complete inhibition by

thalidomide of CpG-A and *M. leprae*-induced IFN-I production in PBMCs *in vitro* imply that the drug could directly inhibit this cytokine production, an observation in accordance with a study showing that thalidomide and its derivatives inhibit TLR-induced IFN-I production in the THP-1 monocytic cell line (78). These authors showed that treatment with lenalidomide, a thalidomide derivative, disrupted the interaction between the rabex-5 protein and CRBN, which is important for inhibiting IFN-I production in this cell line, indicating that this anti-inflammatory property of rabex-5 should be further explored. However, another study by the same authors demonstrated that the inhibition of IFN-I production promoted by thalidomide and its analogs primarily occurred by affecting the TRIF/IRF3 pathway independently of CRBN in peritoneal macrophages of CRBN knockout mice. Conversely, the CRBN knock down in THP-1 monocytes decreased IFN-I production, suggesting a different behavior based on cell type (79). Nonetheless, these reports unquestionably link the immunomodulatory effects of thalidomide with the type-I IFN pathway. In the present study, thalidomide seemed to primarily affect the production of IFN-I by pDCs since the latter accounts for most of the TLR9-induced IFN-I production in PBMCs. The mechanisms and targets that are responsible for thalidomide action in inhibiting IFN-I production by pDCs deserve further exploration.

The development of a safe and effective alternative to both steroids and thalidomide for ENL treatment is an urgent need. The present study pointedly reveals an exacerbation of the type I IFN pathway in the skin lesions and blood of leprosy patients undergoing ENL. The present results also suggest the involvement of ENL pDCs in type I IFN production, the major source of these cytokines in the body. The pDC/IFN-I pathway has been specifically implicated in the pathogenesis of skin manifestations in chronic inflammatory/autoimmune diseases in which an enrichment of pDCs in the inflammatory infiltrate in association with an IFN-I signature has been demonstrated (9, 18). Of note, a recent therapeutic approach in the treatment of cutaneous lupus targeting pDCs has shown promising results (80), which may prove useful in treating ENL as well. Moreover, the data herein generated open new avenues in the process of identifying new biomarkers for early ENL diagnosis that could pave the way toward the better management of reactional patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE198609.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by FIOCRUZ Committee for Ethics in Research. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TRos, MAM, NL, and TRod rationale for the study and manuscript preparation. MOM, RP, VS, LR, and MP designed the study and performed the project supervision. MOM, RP, and MP were responsible for funding acquisition. TRos, MAM, NL, TRod, AD, and FC for performing experiments. AS was responsible for patients' recruitment. HF was responsible for histopathology. TL-C was responsible RNAseq data analysis and graph generation. TRos, MAM, NL, TRod, AD, MG, and TA analyzed the results. MOM, RP, and VS were responsible for manuscript revision. TRos, MAM, NL, TRod, AD, LR, and MP were responsible for writing the original manuscript draft while MOM, RP, and VS were in charge of revising it. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.899998/full#supplementary-material

Supplementary Figure 1 | Flow cytometry gating strategy to identify pDCs in PBMCs. Lumps and doublet cells were excluded using the parameters of frontal dispersion measured by area (FSC-A, forward scatter-area) versus frontal dispersion measured by height (FSC-H, forward scatter-height). Next, the PBMC region was selected by FSC-A and lateral dispersion was measured by SSC-A area (side scatter). In the region of PBMCs, pDCs were identified as lineages (CD3, CD14, CD19, CD20, and CD56)$^{-}$, CD11c^{-}, CD123^{+}, and BDCA4^{+}.

Representative plots of pDCs for each study group are shown by using CD123 and BDCA4 markers. HD, healthy donor; NR, non-reactional multibacillary patient; and ENL, erythema nodosum leprosum.

Supplementary Figure 2 | Gating strategy and representative histograms of TLR9 and TLR7 levels in pDCs. Lumps and doublet cells were excluded using the parameters of frontal dispersion measured by area (FSC-A, forward scatter-area), versus frontal dispersion measured by height (FSC-H, forward scatter-height). The PBMC region was selected by FSC-A and lateral dispersion measured by SSC-A area (side scatter). In the region of PBMCs, pDCs were identified as $CD11c^{-}$, $CD123^{+}$ and $BDCA2^{+}$. The expression levels of TLR9 and TLR7 by pDCs are shown in the representative histograms. HD, healthy donor; NR, non-reactional multibacillary patient, ENL, erythema nodosum leprosum; and FMO, fluorescence minus one.

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Serological Immunoassay for Hansen's Disease Diagnosis and Monitoring Treatment: Anti-Mce1A Antibody Response Among Hansen's Disease Patients and Their Household Contacts in Northeastern Brazil

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Hansen's disease (HD) is an ancient disease, but more than 200,000 new cases were reported worldwide in 2019. Currently, there are not many satisfactory immunoassay methods for its diagnosis. We evaluated antibodies against Mce1A as a promising new serological biomarker. We collected plasma from new cases, contacts, and endemic controls in the city of Parnaíba and treated patients at Carpina, a former HD colony in Piauí state, northeastern Brazil. Receiver operating characteristic (ROC) curves were used to assess the assay thresholds, specificity and sensitivity of the IgA, IgM, and IgG antibodies against α-Mce1A by indirect ELISA and compared it with IgM anti-PGL-I and molecular diagnosis by quantitative polymerase chain reaction (qPCR). Venn diagrams were generated to represent the overlap in the antibody positivity pattern. Multivariate analysis was performed to assess the potential predictor of antibodies for the outcome of having an HD diagnosis. IgA and IgG were positive in 92.3 and 84% of patients, respectively. IgM was negative for all treated patients. IgG had a sensitivity and specificity of 94.7 and 100%, respectively. IgM-positive individuals had a 3.6 chance of being diagnosed with HD [OR = 3.6 (95% CI = 1.1–11.6); $p = 0.028$], while IgA-positive individuals had a 2.3 chance [OR = 2.3 (95% CI = 1.2–4.3); $p = 0.005$] compared

to endemic controls. We found that the Mce1A antibody profile can be an excellent diagnostic method of HD. IgA is an ideal biomarker for confirming contact with the bacillus. IgM has potential in the detection of active disease. IgG antibodies confirm the performance of these serological markers in diagnosis and therapeutic follow-up.

Keywords: serological, biomarkers, Hansen's disease, Mce1A protein, antibodies

INTRODUCTION

HD is a disabling chronic infectious disease caused by *Mycobacterium leprae* that affects the skin and peripheral nerves (1). The disease has high morbidity, mainly due to neural involvement, which can cause permanent physical disabilities and deformities, reinforcing its social stigma (2). In Brazil, during the period without effective therapeutic treatment, HD patients were compulsorily segregated from society into HD colonies (3). The Hospital Colônia do Carpina in Parnaíba-Piauí (PI) was founded in 1931, housing approximately 300 HD patients (4, 5).

The strategy of an early diagnosis and effective treatment with multidrug therapy (MDT) is crucial for HD cure, preventing sequelae and reducing the disease stigma (6). However, the diagnosis of HD is difficult and requires qualified professionals to differentiate it from other dermatological or neurological diseases (7). The current limitations of diagnostic tests, including test accuracy, and the lack of availability of low-cost commercial kits and easy implementation in primary care health units, indicate a need for more effective tests for diagnosis monitoring treatment and assessing household transmission. In addition, there is currently no method that can diagnose all HD clinical forms (8). Thus, the absence of tests that allow for the identification of subclinical infections and mild HD contributes to the progression and spread of the disease and the inability to reach the elimination goals proposed by the World Health Organization (WHO).

There is no laboratory test capable of detecting all clinical forms of HD. The knowledge and skills required for an HD diagnosis, treatment and management by general health workers are unsatisfactory, leading to delayed diagnosis, physical disabilities, socioeconomic impairment, and continued *M. leprae* transmission (9). Bacilloscopy from slit skin smears is the standard laboratory test to detect *M. leprae*, although highly specific, has a low sensitivity and it is performed only in presumed HD cases, and is negative in the majority of initial or neural forms. Serological tests for antibody detection in HD have many limitations in diagnose of all HD clinical forms and discriminating contacts compared to patients (10). The use of cell wall antigens of the bacillus, as serological biomarker has been well-established to detect specific antibodies, such as against PGL-I or protein glycoconjugates. Although anti-PGL-I antibodies serologic evidence has very low sensitivity and low predictive value, its high correlation with high bacillary index and almost completely multibacillary clinical forms, it can be useful in HD exclusion (10, 11). The detection of *M. leprae* DNA in earlobe slit skin smears and other sites using standard PCR or quantitative PCR has also been very useful to detect asymptomatic carriers or complex cases. New screening techniques, including PCR, peripheral nerve ultrasonography and electroneuromyographic are being employed, with a diagnostic serological test in development (12).

However, there is a need for simple, low-cost diagnostic strategies to monitor treatment and assess household transmissions at primary care settings. The mammalian cell-entry 1A (Mce1A) protein, first described in *M. tuberculosis*, is present in the cell wall of *M. leprae* and it is associated with the entry of the bacillus into nasal epithelial cells and skin cells (13, 14). Previous studies have shown the potential of using serum biomarkers such as antibodies against Mce1A in the diagnosis of HD (15). Therefore, because it plays a role in the invasion and maintenance of *M. leprae* infection, Mce1A represents a potential target for the development of new diagnostic tests to diagnose HD, monitor treatment, and screen for contacts of index cases of HD. Thus, our study aimed to evaluate and compare the presence of antibodies against PGL-I and Mce1A among patients newly diagnosed with HD and their contacts in the city of Parnaíba with patients treated for HD and HD residents and inmates of Carpina Hospital. We also explored the utility of IgA, IgM, and IgG anti-Mce1A antibodies and their correlations in HD.

MATERIALS AND METHODS

Setting and Design

A cross-sectional study was conducted at the National Reference Center in Sanitary Dermatology and HD, Clinical Hospital of Ribeirão Preto Medical School (HCFMRP-USP), University of São Paulo, which provides training in HD management for several states of Brazil (MH-Brazil Project). Volunteer subjects were recruited by convenience sampling in March 2016 during a campaign to evaluate contacts of patients in the city Parnaíba, Brazilian municipality in the state of PI, the second most populous city in the state. Treated HD patients living in a former HD colony (Colony of Carpina), PI, Brazil, were also included in this study.

Study Population

After signing an informed consent form, the volunteers were classified into four groups: (1) new cases of HD diagnosed during active search actions in the Parnaíba Municipality (PAR-NC), (2) treated HD patients who were residents of the Carpina Colony Hospital (CAR-TP), (3) household contacts (HHC) evaluated in Parnaíba, and (4) healthy endemic controls (EC) (**Table 1**).

Hansen's Disease Cases

Newly diagnosed HD cases seen at Parnaíba and Carpina were invited to participate in this study. Patients were considered

TABLE 1 | Study population characteristics (N = 82).

	EC n = 20	HHC n = 17	PAR-NC n = 26	CAR-TP n = 19	p-value
Age, years, mean (SD)	29.5 (12.3)	42.8 (16.7)	43.9 (16.9)	58.6 (13.0)	<0.0001[a]
Sex, *n* (%)					
Male	7 (35)	9 (52.9)	17 (65.4)	15 (78.9)	0.03[b]
Female	13 (65)	8 (47.1)	9 (34.6)	4 (21.1)	
Therapeutic scheme, *n* (%)					
PB	–	–	2 (7.7)	1 (5.3)	0.009[b,c]
MB	–	–	24 (92.3)	10 (52.6)	
DDS	–	–	0 (0)	5 (26.3)	
Clinical form, *n* (%)					
I	–	–	1 (3.8)	–	
TT	–	–	1 (3.8)	–	
BT	–	–	1 (3.8)	–	
BB	–	–	13 (50)	–	
BL	–	–	3 (11.6)	–	
LL	–	–	7 (27)	–	
PCR-RLEP test, *n* (%)					
Positive	–	–	15 (57.7)	11 (57.9)	0.09[b,d]
Negative	–	–	3 (11.5)	8 (42.1)	
Ct, mean (SD)	–	–	28·07 (1.065)	30·96 (0.330)	0.03[e]

[a] *Comparison of four groups using the Kruskal–Wallis test.*
[b] *Comparison of the four and two groups using the chi-square test.*
[c] *Data not available for three volunteers in the CAR-TP group.*
[d] *Data not available for eight volunteers in the PAR-HD group.*
[e] *Comparison of qPCR-RLEP positivity between PAR-NC and CAR-TP cells using the t-test.*
EC, endemic controls; HHC, household contacts of HD patients; PAR-NC, new cases of HD in Parnaíba; CAR-TP, patients treated in Colony Carpina; SD, standard deviation; PB, paucibacillary; MB, multibacillary; I, indeterminate; TT, tuberculoid; BT, borderline-tuberculoid; BB, borderline-borderline; BL, borderline-lepromatous; LL, lepromatous; PCR-RLEP, quantitative polymerase chain reaction-specific repetitive element; Ct, cycle threshold; IQR, interquartile range.

eligible for inclusion in the study if their diagnosis was confirmed by clinical evaluation and serological and/or molecular exams. All cases were classified considering the guidelines adapted by Indian Association of Leprologists (16), Ridley and Jopling (17), and Congress of Madrid classifications (18), as follows: indeterminate (I), polar tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL), polar lepromatous (LL); and according to WHO operational criteria [PB (TT) and MB (BT, BB, BL and LL)]. All newly diagnosed patients were referred to a health unit for standard MDT.

Household Contacts

HHC was defined as volunteers residing in the same household with an index case for at least 6 months prior to diagnosis. All HHC and EC were clinically screened for signs or symptoms suggestive of HD and subjected to laboratory analysis with serological examination. Clinical examinations were performed by trained physicians and health professionals at HCFMRP-USP.

Endemic Controls

ECs, representing community contacts, were defined as healthy individuals residing in the city Parnaiba, PI, Brazil an endemic area who had no history of diagnosis or contact with an HD. All participants reported being test-negative for the human immunodeficiency virus and did not diseases or use immunosuppressive drugs.

Serology to Detect IgM Anti-Previous Serologic Test by Enzyme-Linked Immunosorbent Assay

Indirect enzyme-linked immunosorbent assay (ELISA) was used to measure the anti-PGL-I IgM titer of every serum sample according to a previously reported protocol (8).

Detection of *Mycobacterium leprae* DNA by Quantitative Polymerase Chain Reaction

Total DNA extraction of earlobes and at least one elbow and/or lesion SSS sample was performed with the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, cat: 51306) was performed according to the manufacturer's protocol. DNA was used to perform PCR-RLEP according to a previously reported protocol (19).

Serology to Detect IgA, IgM, and Total IgG Anti-Mce1A by Enzyme-Linked Immunosorbent Assay

Quantitative assessment of IgA, IgM and IgG antibodies against the Mce1A protein was performed by indirect ELISA (15). Purified recombinant Mce1A protein was provided by Dr. LW Riley (University of California, Berkeley, CA, United States). Mce1A protein (10 μg/mL) was diluted to 1:1,000 in ethanol, and 50 μL of this solution was dried overnight on polystyrene ELISA well plates (Corning® Costar®, Sigma Aldrich, San Luis, Missouri, United States). The ELISA plates were then blocked with 100 μL of 1% BSA (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and washed with PBS (Laborclin, São José do Rio Preto, SP, Brazil) containing 0.05% Tween 20 (Sigma–Aldrich, St. Louis, MO, United States) (BSA/PBS/T). Frozen serum samples were thawed and diluted 1:100 in BSA/PBS/T. Next, 100 μL of each diluted sample was added to the plates and incubated for 1 h at room temperature (RT) (18–25°C), followed by three washes with PBS/T. Next, 100 μL of 1:10,000, 1:10,000, or 1:25,000 goat-derived anti-human IgA, IgM or IgG labeled with horseradish peroxidase (Sigma–Aldrich, St. Louis, MO, United States) diluted in BSA/PBS/T was added, followed by incubation at RT for 1 h. This was followed by repeated washing with PBS/T. Then, 100 μL TMB solution (Invitrogen Life Technologies, Carlsbad, CA, United States) was added, and the plates were reincubated for 30 min at RT. Finally, the reaction was stopped with 100 μL of 2 N sulfuric acid. Reactions were read at 450 nm in a SpectraMax M3 spectrophotometer (Molecular Devices, San Jose, CA, United States). The results were recorded as the average optical density (O.D.) of triplicate samples, and

the assay was repeated if the coefficient of variance was >10%. The sample index was calculated by dividing their O.D. per the established cut-off for each immunoglobulin. Indices above 1.0 were considered positive.

Statistical Analysis

All data were analyzed by GraphPad Prism v. 9.0 software (GraphPad Inc., La Jolla, CA, United States). Statistical variations were analyzed by the Mann–Whitney and Kruskal–Wallis tests, followed by Dunn's test. Spearman's correlation was used to compare the immunoglobulin levels of IgA, IgM, IgG anti-Mce1A and IgM anti-PGL-I. The ability of immunoglobulin levels to discriminate HD patients from controls (EC) was evaluated by receiver operating characteristic (ROC) curves. The chi-square test was used to assess associations among categorical variables and the presence of antibodies. Comparisons of the qPCR-RLEP positive test results were performed by the *t*-test. The level of statistical significance was set at $p < 0.05$. The Venn diagrams were generated using the online tool Draw Venn Diagram[1] to represent the overlap in the number of antibodies differentially determined by indirect ELISA in each of the comparison groups. Binomial logistic regression analysis was performed to assess the potential predictor of antibodies for the outcome of having an HD diagnosis with the jamovi project (2021). *jamovi* (Version 1.6) (Computer Software). Retrieved from https://www.jamovi.org.

Role of the Funding Source

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Study Population Characteristics

The study included 82 volunteers, grouped as new HD patients from Parnaíba (PAR-NC; $n = 26$; 31.7%), treated HD patients (CAR-TP) from the colony ($n = 19$; 23.2%), HHC ($n = 17$; 20.7%), and EC ($n = 20$; 24.4%). Of the 26 PAR-NC, 24 (92.3%) were multibacillary (MB). Thirteen (50%) patients were classified to have borderline borderline (BB) clinical forms of HD, 7 (27%) as lepromatous leprosy (LL), and 3 (11.6%) as borderline lepromatous (BL). Among the 19 CAR-TP patients, 10 (52.6%) received the MB scheme, and 5 (26.3%) received monotherapy with dapsone (DDS). A significant difference was observed among the ages of the volunteers from all four groups ($p < 0.0001$) and sex ($p = 0.03$) due to the inclusion of a special population with only elderly people from the colony (mean:58.6, $SD = 13$). Descriptive characteristics of the study population are summarized in **Table 1**. The frequency of positive PCR-RLEP tests in PAR-NC and CAR-TP was 57.7 and 57.9%, respectively, but the cycle threshold showed significant differences between these two groups ($p = 0.03$), demonstrating a decreased bacillary load after CAR-TP and/or the use of monotherapy (DDS) in the initial treatment.

Antibodies Against Mce1a Protein Are Biomarkers for the Diagnosis and Monitoring Treatment of Hansen's Disease Patients and Their Contacts

The antibody profiles against Mce1A protein and PGL-I levels in PAR-NC, CAR-TP, HHC, and EC are shown in **Figure 1**. IgA anti-Mce1A levels were significantly higher in the HHC [median: 2.5 (IQR:1.6–3.7), $p < 0.0001$], PAR-NC [median: 2.7 (IQR:1.6–4.8), $p < 0.0001$] and CAR-TP [median: 1.3 (IQR:0.7–1.9), $p = 0.0004$] groups as compared to the EC group [median: 0.4 (IQR: 0.3–0.6)], and the IgA indices were lower in CAR-TP as compared to PAR-NC ($p = 0.007$) (**Figure 1A**). IgM anti-Mce1A was increased in PAR-NC [median: 1.03 (IQR:0.6–1.8)] as compared to the EC group [median: 0.5 (IQR:0.4–0.9), $p = 0.006$] and CAR-TP [median: 0.3 (IQR:0.2–0.6), $p < 0.0001$], which showed negative test results for all individuals. HHC had high antibody levels [median: 0.8 (IQR: 0.6–1.04), $p = 0.0009$] compared to treated patients (CAR-TP), proving to be a potential marker of active disease (**Figure 1B**). In these three groups, HHC [median: 1.2 (IQR: 0.9–1.7), $p = 0.0003$], PAR-NC [median: 1.5 (IQR: 1.1–2.0), $p < 0.0001$] and CAR-TP [median: 1.2 (IQR: 1.04–1.9), $p < 0.0001$] IgG anti-Mce1A were higher than in EC [median: 0.4 (IQR: 0.3–0.5)] (**Figure 1C**). The PAR-NC group had moderately higher levels of IgM anti-PGL-I [median: 0.7 (IQR: 0.3–2.5)], compared to EC [median: 0.2 (IQR: 0.1–0.5), $p = 0.0007$], HHC [median: 0.4 (IQR: 0.1–0.8), $p = 0.03$] and CAR-TP [median: 0.3 (IQR: 0.2–0.5), $p = 0.009$] (**Figure 1D**).

Enzyme-Linked Immunosorbent Assay Performance of Anti-Mce1A Antibody Levels for Hansen's Disease Diagnosis

A panel comprising plasma samples from PAR-NC, CAR-TP and HHC was examined (**Table 2**). The detection of IgA was strongly correlated with the PAR-NC and HHC groups ($p < 0.0001$), as well as with CAR-TP, although it was weaker ($p = 0.0012$). The IgA performance showed an area under the curve (AUC) > 0.8, sensitivity and specificity between 52.6 and 100% for treated patients and 93.2 and 88.2% for untreated patients, respectively (**Figures 2A–C**). In the HHC group, the test was 82.3 and 100% sensitive and specific, respectively. Additionally, the pairwise comparison of ROC curves did not show a significant difference between the IgM and APGL-I values for CAR-TP and HHC but APGL-I performance showed sensitivity and specificity of 38.4 and 100%, respectively, for untreated HD patients ($p = 0.0002$, respectively) (**Table 2**). The ROC curve for the IgM anti-Mce1A test did not show a significant performance, with AUCs ranging from 0.6 to 0.64 for these two groups (**Figures 2E,F**). The best performance of the ROC curve for IgM anti-Mce1A (AUC = 0.83) was found in the group of patients at baseline, at the time of diagnosis (PAR-NC), showing 57.6 and 85% sensitivity and specificity ($p < 0.0001$), respectively (**Figure 2D**). ROC curve analysis revealed excellent performance for IgG in all three

[1] http://bioinformatics.psb.ugent.be/webtools/Venn/

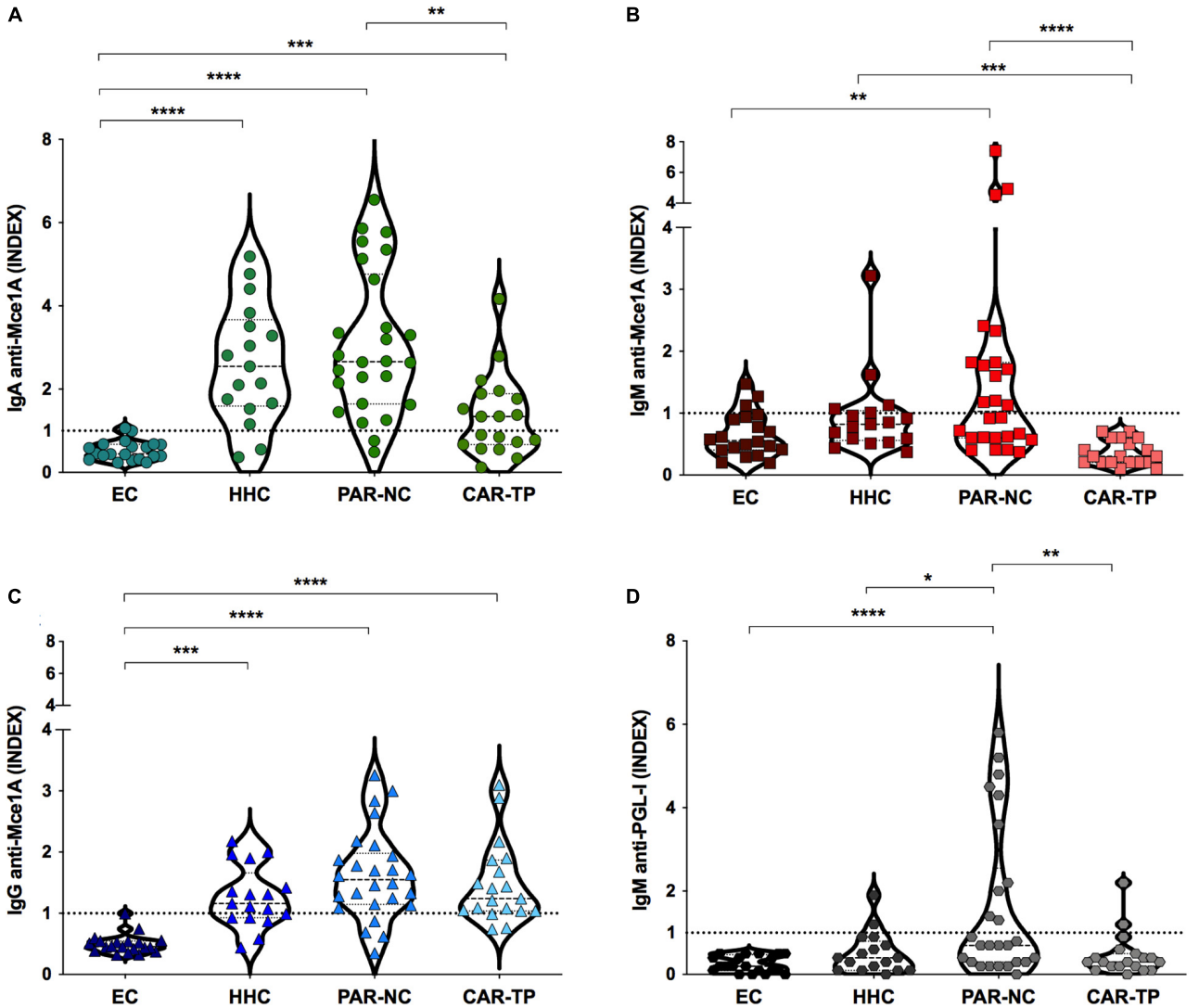

FIGURE 1 | Antibodies anti-Mce1a are biomarkers for the diagnosis and monitoring of HD. IgA **(A)**, IgM **(B),** and IgG **(C)** antibody indices against Mce1A protein and PGL-I **(D)** in different groups of plasma samples tested. Statistical significance was determined by the Kruskal–Wallis test followed by the Dunn test. Data are presented as the median and interquartile range (IQR); significance was considered at $p < 0.05$, $p < 0.01$, $p < 0.001$, or $p < 0.0001$ as represented by *, **, ***, and ****, respectively. EC endemic controls ($n = 20$); HHC household contacts of HD patients in Parnaíba ($n = 17$); PAR-NC new cases of HD in Parnaíba ($n = 26$); CAR-TP patients treated in Colony Carpina ($n = 19$). The respective index was calculated by dividing the optical density (O. D 450 nm) of each sample by the cut-off, and indices above 1.0 were considered positive represented by horizontal dotted line.

groups vs. EC ($p < 0.0001$), with an AUC ≥ 0.95, sensitivity ranging from 88.2 to 94.2% and 100% specificity for all groups (**Figures 2G–I**).

Seropositivity Pattern for New Serological Biomarkers in Hansen's Disease

The IgA titer was positive in 92.3% of PAR-NC patients, and 84% were positive for IgG, regardless of the clinical and operational classification of the evaluated cases. The Anti-Mce1A IgM titer was positive in 50% of HD patients, while the positive APGL-I titer was detected only in 38.5%. In contrast, treated patients had no detection of anti-Mce1A IgM antibodies, and only 11.1% were positive for APGL-I. IgA and IgG titers remained positive in 52.6 and 89.5% of cases, respectively. Among the HHC, IgA and IgG antibody titers were positive in 88.2 and 64.7% of patients, respectively. The IgM titer among HHC was positive in 29.4% [index median: 0.8 (IQR: 0.6–1.04)], 17.6% better than APGL-I [index median: 0.4 (IQR: 0.1–0.8)] (**Table 3**).

The antibody titers differed for the three study groups (PAR-NC, CAR-TP, and HHC), considering that newly diagnosed patients (PAR-NC) had a greater number of positive individuals for all biomarkers tested (IgA, IgM, IgG, and APGL-I) with a predominance of positive anti-Mce1A IgM titers. Treated patients (CAR-TP) had positive titers only for IgA and IgG

TABLE 2 | Comparison of receiver operating characteristic curve analysis for IgA, IgM, and total IgG against Mce1A protein and IgM anti-PGL-I in discriminating among new HD patients, treated HD patients and HHC vs. endemic controls.

Group	Antibody	AUC (95%CI)	*p*-value	Cut-off(O.D)	Sensitivity% (95%CI)	Specificity% (95%CI)	LR +
	IgA	0.95	<0.0001	0.203	93.2 (74.8–99.0)	88.2 (72.5–96.7)	7.8
	IgM	0.83	<0.0001	0.184	57.6 (36.9–76.6)	85.0 (62.1–96.7)	3.8
PAR-NC	IgG	0.97	<0.0001	0.302	88.4 (69.8–97.5)	100 (83.1–100)	–
	APGL-I	0.81	0.0002	0.295	38.4 (20.2–59.4)	100 (83.1–100)	–
	IgA	0.80	0.0012	0.203	52.6 (28.8–75.5)	100 (83.1–100)	–
	IgM	0.60	0.27	0.184	100 (82.3–100)	15 (3.2–37.8)	1.1
CAR-TP	IgG	0.99	<0.0001	0.302	94·7 (73.9–99.8)	100 (83.1–100)	–
	APGL-I	0.60	0.26	0.295	10.5 (1.3–33.1)	100 (83.1–100)	–
	IgA	0.92	<0.0001	0.203	82.3 (56.5–96.2)	100 (83.2–100)	–
	IgM	0.64	0.12	0.184	58.8 (32.9–81.5)	60 (36.0–80.8)	1.4
HHC	IgG	0.96	<0.0001	0.302	88.2 (63.5–98.5)	100 (83.1–100)	–
	APGL-I	0.67	0.06	0.295	11.7 (1.4–36.4)	100 (83.1–100)	–

AUC, area under the receiver operating characteristic curve; CI, confidence interval; O.D, optical density; LR+, positive likelihood ratio; PAR-NC, new cases of HD in Parnaíba; CAR-TP, patients treated in Colony Carpina; HHC household contacts of HD patients in Parnaíba.

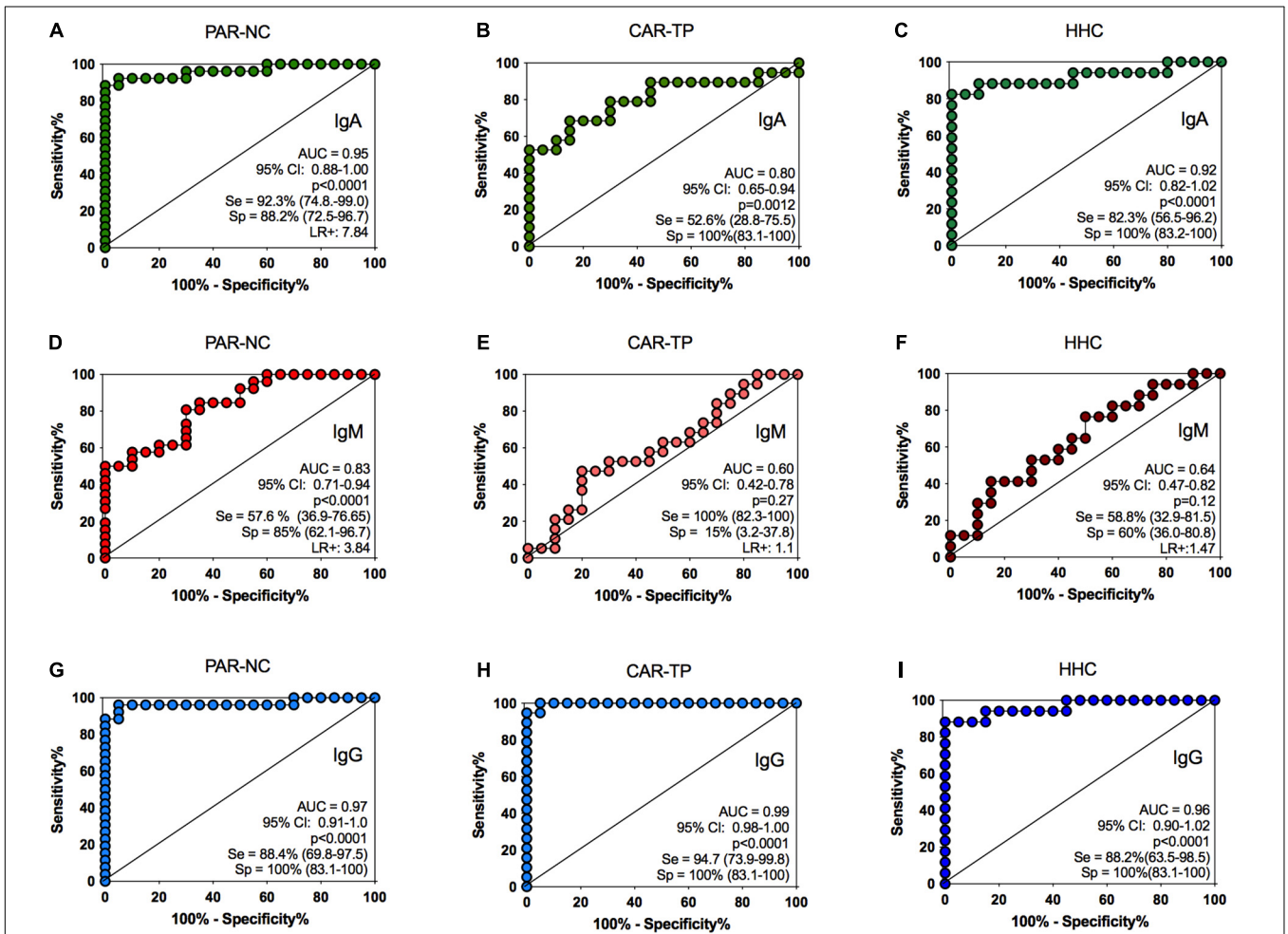

FIGURE 2 | ELISA performance of anti-Mce1A antibody levels. Receiver operating characteristic analysis for comparison of IgA anti-Mce1A **(A–C)**, IgM anti-Mce1A **(D–F)**, and IgG anti-Mce1A **(G–I)** between HD patients and HHC vs. endemic controls. PAR-NC new cases of HD in Parnaíba (*n* = 26); CAR-TP patients treated in Colony Carpina (*n* = 19); HHC household contacts of HD patients in Parnaíba (*n* = 17). AUC, area under the curve; CI, confidence interval; Se, sensitivity; Sp, specificity; LR+, positive likelihood ratio.

TABLE 3 | Positivity to antibodies against Mce1A protein and PGL-I in different groups of studies.

Groups	No of cases	IgA*n* (%)	x^2; *p*-value	IgM*n* (%)	x^2; *p*-value	IgG*n* (%)	x^2; *p*-value	APGL-I*n* (%)	x^2; *p*-value
PAR-NC	26	24 (92.3)	34.7;<0.0001	13 (50)	6.1; 0.01	22 (84.6)	32.4;<0.0001	10 (38.5)	9.8; 0.001
CAR-TP	19	10 (52.6)	10.9; 0.0009	0 (0)	3.0; 0.07	17 (89.5)	31.7;<0.0001	2 (11.1)	2.2; 0.1
HHC	17	15 (88.2)	25.9;<0.0001	5 (29.4)	1.1; 0.2	11 (64.7)	18.4<0.0001	2 (11.8)	2.4; 0.1
EC	20	1 (5.0)		3 (15)		0 (0)		0 (0)	

Chi-squared test between HD patients and HHC vs. EC.
PAR-NC, new cases of HD patients Parnaíba; CAR-TP, patients treated in Colony Carpina; HHC, household contacts of HD patients in Parnaíba; EC, endemic controls.

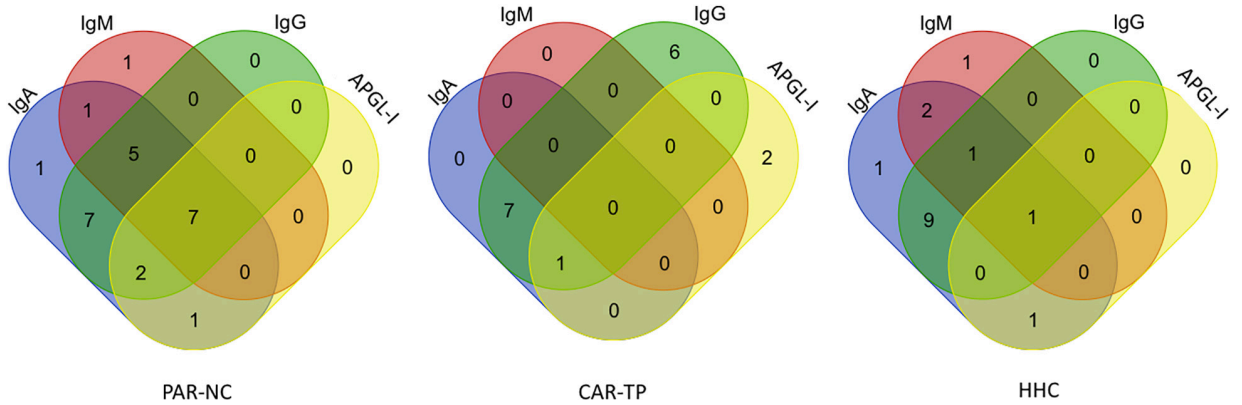

FIGURE 3 | Seropositivity pattern of antibodies in HD. The Venn diagrams represent the overlap in the number of positive IgA, IgM, IgG anti-Mce1A, and APGL-I antibodies in each of the comparison groups new cases of HD in Parnaíba (PAR-NC), treated HD patients in Colony Carpina (CAR-TP) and household contacts of HD patients in Parnaíba (HHC).

anti-Mce1A antibodies. APGL-I antibodies did not achieve a satisfactory response for detecting HD cases and infected HHC (**Figure 3**).

Correlation Between Immunoglobulins and Mce1A Protein

Correlation analyses were performed to assess the different levels of the serological markers tested. There was a strong positive correlation between the indices of anti-Mce1A IgA and IgG in the PAR-HD group ($r = 0.76$; $p < 0.0001$) (**Figure 4A**), CAR-TP ($r = 0.76$; $p = 0.0002$) (**Figure 4C**), and HHC ($r = 0.85$; $p < 0.0001$) (**Figure 4D**). Similarly, a moderate correlation was found between plasma anti-Mce1A IgG and IgM ($r = 0.40$; $p = 0.04$) in the new HD cases (**Figure 4B**).

Logistic Regression Analysis to Evaluate the Potential of Anti-Mce1A Antibodies as Predictors of the Diagnosis of Hansen's Disease

The logistic regression model demonstrated an association of IgM, IgA and PCR-RLEP with the clinical outcomes. ($X^2 = 45.8$; $p < 0.001$; R^2 MacFadden = 0.49; Accuracy = 0.88; Specificity = 0.927; Sensitivity = 0.80; AUC = 0.915), Patients with positive anti-Mce1A IgM titers had a 3.6 chance [OR = 3.6 (95% CI = 1.1–11.6); $p = 0.028$] and anti-Mce1A IgA titer had a 2.3 chance [OR = 2.3 (95% CI = 1.2–4.3); $p = 0.005$] of being diagnosed with HD compared to healthy volunteers. PCR-RLEP had a 16.0 chance of identifying HD [OR = 16.0 (95% CI = 2.8–89.2); $p = 0.002$]. The age [OR = 1.0 (95% CI = 0.9–1.0); $p = 0.7$) and sex [OR = 1.0 (95% CI = 0.2–4.4); $p = 0.9$] of the population did not affect the model. The positive anti-Mce1A IgG titer [OR = 0.3 (95% CI = 0.08–1.7); $p = 0.2$) was not associated with the outcome of the diagnosis for HD because this test was positive in the different groups of patients and contacts (**Figure 5A**). The second logistic regression model also showed the association of serological markers with the active disease ($X^2 = 40.5$; $p < 0.001$; R^2 MacFadden = 0.68; Accuracy = 0.90; Specificity = 0.94; Sensitivity = 0.88; AUC = 0.96) using treated patients (CAR-TP) and new cases (PAR-NC). The results are maintained in relation to the IgM [OR = 3.080 (95% CI = 1.9–4.95e + 6); $p = 0.03$;] and IgA [OR = 8.8 (95% CI = 1.2–66.0); $p = 0.03$] titers and clinical outcome with an increased association between these tests and the outcome of having HD. PCR-RLEP was not associated with the outcome in this model [OR = 1.7 (95% CI = 0.1–18.1); $p = 0.6$]. This result is due to the presence of positive PCR tests in the group of treated patients with high bacillary load at the time of baseline diagnosis and untreated new cases. The anti-Mce1A IgG titer was not associated with disease activity [OR = 0.04 (95% CI = 0.002–0.8); $p = 0.038$] because the tests were positive in new cases and treated patients (PAR-NC and CAR-TP) (**Figure 5B**). The APGL-I serology was removed from the analysis because it interfered with the performance of the logistic regression model, given the high number of negative results in common in the patient and contact groups evaluated.

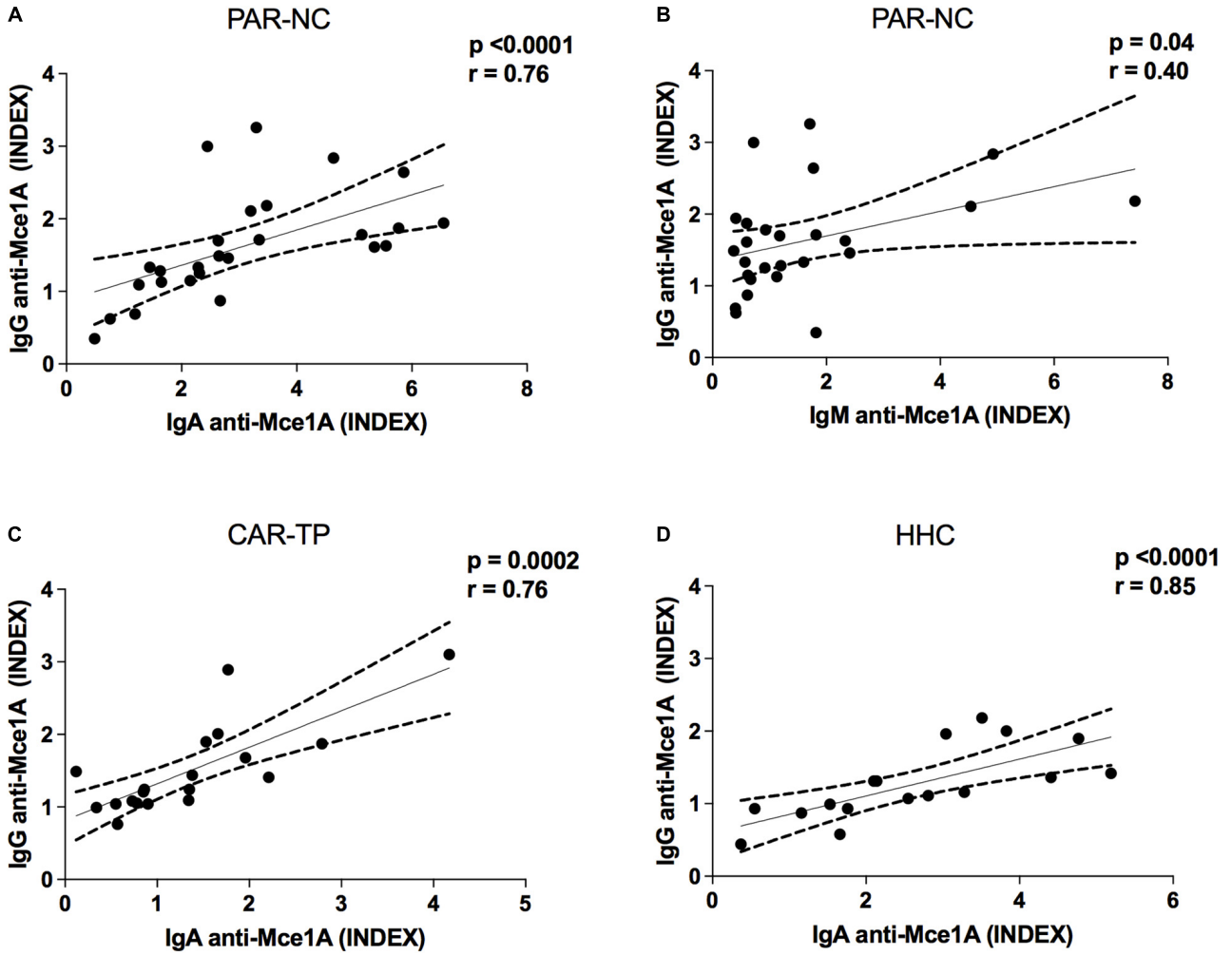

FIGURE 4 | Immunoglobulins correlation profile in HD. Spearman's correlation coefficient was used to compare immunoglobulin indices. PAR-NC new cases of HD in Parnaíba **(A,B)**; CAR-TP patients treated in Colony Carpina **(C)**; HHC household contacts of HD patients in Parnaíba **(D)**.

Age was not included in the second model due to the interference of the elderly population from the colony.

DISCUSSION

HD patients were compulsorily institutionalized in HD colonies before the establishment of specific treatment. Due to late diagnosis, insufficient treatment, or patients initially highly infected, colony cases of HD had a high bacillary load at diagnosis, who remained test positive by PCR in 57.9% of treated patients. Large numbers of dead *M. leprae* may persist for several years after the killing of all bacilli by effective MDT (20). The absence of diagnostic tests, especially subclinical infection, frequently leads to a delayed diagnosis, resulting in large numbers of undetected cases and not reaching the WHO target for the elimination of HD as a public health problem (21). Our anti-Mce1A serology results show they may serve as biomarkers capable of detecting cases of HD and HHCs who have not yet developed dermatoneurological classical signs and symptoms, and for monitoring treated patients.

The levels of IgA and IgG antibody titers against the Mce1A protein in the three groups we evaluated (contacts, new cases and treated patients) were significantly elevated but not among the healthy controls in endemic communities. IgM appeared to be a sensitive biomarker for identifying active disease, since no colony population treated with MDT had a positive titer. Positive IgM titer indicates a need for a robust clinical investigation of HHCs and individuals in endemic regions for HD.

The serological assays using PGL-I or LID antigens (NDO-BSA, NDO-LID, LID-1, and others) already reported present results in the literature with high seropositivity almost only in MB patients. However, anti-Mce1A antibodies demonstrate satisfactory seropositivity for the antibodies tested in both PB and MB patients and even in household contacts, as a complementary diagnostic tool capable of detecting potential cases early, as previously described by Lima et al. (15). IgG antibodies are characterized by prolonged exposure to the

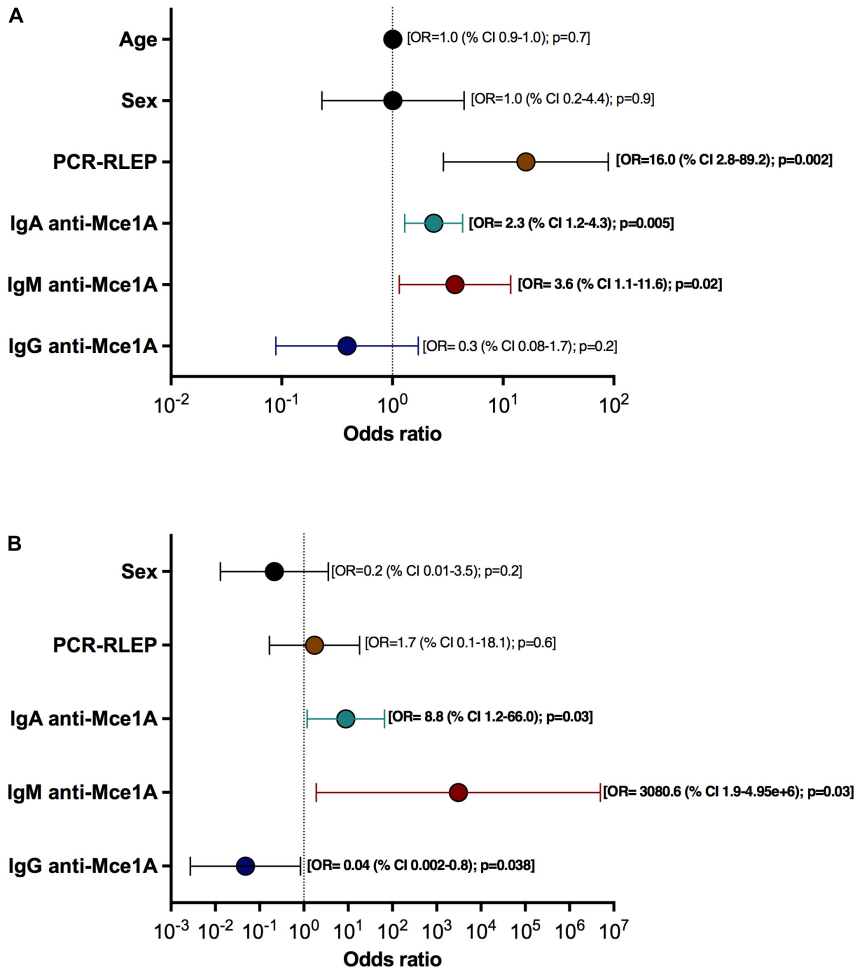

FIGURE 5 | Potential of anti-Mce1A antibodies as predictors of the diagnosis of HD. Binomial logistic regression analyses showing odds ratios (ORs) and 95% confidence intervals (CIs) for HD diagnosis with independent variables: age, sex, PCR-RLEP, and antibodies against Mce1A (IgA, IgM, and IgG) for all groups (PAR-NC, CAR-TP, HHC, and EC) **(A)** and between untreated patients (PAR-NC) and treated patients (CAR-TP) **(B)**.

bacillus (22), corroborating our results of anti-Mce1A IgG ELISA with high seropositivity in new cases and treated patients with MDT. Early detection of HD cases is an important goal for disease control and elimination. Thus, the screening of different classes of immunoglobulins increases the possibility of diagnosis, with anti-Mce1A IgA being a potential biomarker in the screening of contact with *M. leprae*. In line with the findings of our study, Silva et al. (23) reports that IgA antibodies play a role in protecting against mycobacteria in the nasal mucosa and a biomarker of contact with the bacillus (23). As also, the search for an ideal serology is also associated with a satisfactory marker of disease activity. Thus, our results suggest that anti-Mce1A IgM ELISA is the indicator marker of active disease, due to the absence of positivity in treated patients. Another alternative for the use of serological tests is community screening, being positive results indicative of potential community contact with the bacillus. The presence of positive serology among endemic controls (healthy individuals without dermatoneurological signs of HD) may be associated with increased exposure in hyperendemic regions, identified in our study in 5% of the IgA ELISA and 15% of the IgM ELISA. However, further studies need to be performed to clinically follow-up these individuals. Therefore, this means that all isotypes should always be measured. We believe that those individuals that are positive for IgM or two isotypes with high indices should be clinically followed every year.

The positive anti-Mce1A IgG antibody titers (89.5%) with high diagnostic accuracy (94.7% sensitivity and 100% specificity) and negative anti-Mce1A IgM antibody titers (0%) among treated HD patients in the colony indicate the potential of these serological markers to monitor treatment response.

This group's previous work in a population from another endemic region in the state of Bahia showed elevated IgA, IgM, and IgG titers in cases of paucibacillary and multibacillary HD. BCG vaccination and latent tuberculosis infection did not induce cross-reactive anti-Mce1A antibodies in HD patients (15). Despite the presence of Mce1A protein in the cell wall of *Mycobacterium bovis* BCG (24), no statistical difference was

observed in the anti-Mce1A ELISA response between vaccinated and non-vaccinated patients (11, 25) and prior BCG vaccination does not influence antibody levels against *M. tuberculosis* proteins (26). A linear immunodominant epitope KRRITPKD (residues 131 and 138 in Mce1A) is highly conserved in *M. tuberculosis*, which is a possible explanation for the difference in response between patients with tuberculosis and Hansen's disease, despite the homology between the mce1 gene (25). Although mce genes have been reported in many bacterial species, these genes exist as operons in mycobacteria only, hence regarded as important virulence attributes (24, 27).

The antibody response to PGL-I is the most widely evaluated biomarker for HD, and it has been shown extensively that the detection of α-PGL-I antibodies only is not sufficient to identify all HD patients, and PB cases generally lack an antibody response against PGL-I (21, 22). ELISAs targeting the PGL-I antigen showed lower sensitivity than the other antigens, but it did not affect the specificity, and a meta-analysis study showed a mean sensitivity of 59.1 (95% IC 50.6–67.1) and 91.7% (95% IC 83.9–94.9) specificity. Of all available serological tests in 78 studies, ELISA was predominantly studied, and its sensitivity varied widely from 0 to 100% and the specificity varied from 13 to 100% (28). The summary ROC plot using other antigens showed the sensitivity of PGL-I ELISA was 63.8% (95% CI 55.0–71.8), and the specificity was 91.0% (95% CI 86.9–93.9) (28). The sensitivity of quantitative polymerase chain reaction (qPCR) varied from 51 to 91%, and the specificity varied from 46 to 100%. The summary sensitivity of the RLEP test was not different from that of the other PCR targets, and the specificity was greater in studies that used RLEP as a conventional PCR target (28). These false-negative patients will not be treated, and if these patients are MB patients, then transmission could continue due to using tests with low diagnostic accuracy (29).

Summarizing and aggregating the results from our three previous published studies searching for HD involving prison male/female populations and from the community (n = 2,133 evaluated individuals) in São Paulo state, the authors found 112 new cases of HD, with new case detection rate (NCDR) 6.5%; macular mild lesion in 93.7%; nerve impairment on palpation in 91.9 and 67% defined as having grade 2 disability (67%). On the other hand, APGL-I titer was positive in only 31.3% of the general population, 30.3% of the non-HD group and 54.4% of HD patients, although it was officially considered high for the non-endemic state, highlighting the hidden presence and the diagnostic challenge of HD and the low sensitivity as diagnostic test for new cases and screening contacts (8, 30, 31).

Several tests have been developed to assess anti-PGL-I antibody, a known biomarker of *M. leprae* infection, including ELISA and lateral flow rapid tests that incorporate synthetic PGL-I (ND-O-BSA) or protein glycoconjugates, such as NDO-LID (27). However, anti-Mce1A serologic assay remedies the main gaps of the previous serologic test (PGL-I), as it demonstrates higher sensitivity, regardless of the clinical form or bacillary load, and increased seropositivity in paucibacillary cases of difficult clinical diagnosis.

Therefore, compared to the traditional clinical and other laboratory tests for HD, the anti-Mce1A serology results proved to be superior for the diagnosis of new cases of HD (including PB cases), monitoring treatment response, and identifying infected HHC of index cases.

Brazilian epidemiological indicators and the current global HD situation confirm the scenario of continued transmission and its maintenance as a public health problem that has not yet been resolved. The reality of the disease in Brazil reinforces the importance of developing new tools for the diagnosis and monitoring of HD, and studies that address the humoral immunological profile of HD patients and their contacts in addition to anti-PGL-I are rare, with results limited to multibacillary only and which is still being implemented in public health services in the country. Therefore, the development of HD diagnostic techniques in all clinical forms, both multi and paucibacillary and/or as a way of monitoring cases, in addition to their contacts and the expected search for an early diagnosis in its subclinical phase become goals to be sought to achieve the HD control goals recommended by the WHO and intended by the Ministry of Health.

In summary, in addition to understanding of the role of Mce1A in the pathogenesis of HD, it offers a highly useful target for immunological biomarker response for the implementation strategies of low-cost and easy-to-perform serological diagnostic platforms for HD. Such platforms will constitute an important technological advance for public health control of HD that can interrupt the chain of transmission of the disease, in addition to preventing deformities, disability and stigma associated with this ancient disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board for Human Research of the HCFMRP-USP (MH-Brazil Project—Protocol number 16620/2014). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FL and MF substantially contributed to manuscript conception and design, acquisition of data, and analysis and interpretation of data. MF, JS, RA, and EA contributed to the clinical care of patients and collected the samples. FL, VA, VB, and NP contributed to the development of experiments. FL and DT contributed to the statistical analysis and interpretation of the data. LR and SA conducted scientific guidance and advice. MF gave final approval of the final submitted version. All authors contributed to the article and approved the submitted version.

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The Armadillo as a Model for Leprosy Nerve Function Impairment: Preventative and Therapeutic Interventions

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Mycobacterium leprae infection of peripheral nerves and the subsequent nerve function impairment (NFI), especially in response to reactional episodes, are hallmarks of leprosy. Improved treatments for *M. leprae*-induced nerve injury are needed, as most if not all of the disability and stigma associated with leprosy arises from the direct or indirect effects of NFI. Nine-banded armadillos (*Dasypus novemcinctus*), like humans, exhibit the full clinical spectrum of leprosy and extensive involvement of the peripheral nerves. In this study, state-of-the-art technology was used to compare nerve function between uninfected and *M. leprae*-infected armadillos. Motor nerve conduction velocity (MNCV) and compound muscle action potential (cMAP), which measure changes in the rate of impulse conduction velocity and amplitude, revealed a progression of impairment that was directly correlated with the duration of *M. leprae* infection and enabled development of an objective nerve impairment scoring system. Ultrasonography accompanied by color Doppler imaging detected enlargement of the *M. leprae*-infected nerves and increased vascularity, possibly due to inflammation. Assessment of epidermal nerve fiber density (ENFD), which shows a length-dependent innervation in armadillos that is similar to humans, identified small fiber degeneration early after *M. leprae* infection. Staining for neuromuscular junction (NMJ) integrity, which is an indicator of signal transduction efficiency into skeletal muscle, discerned a markedly lower number and structural integrity of NMJ in *M. leprae*-infected armadillo footpads. These tools for assessing nerve injury were used to monitor the effects of intervention therapy. Two potential neuro-protective drugs, ethoxyquin (EQ) and 4-aminopyridine (4-AP), were tested for their ability to ameliorate peripheral nerve injury in *M. leprae*-infected armadillos. 4-AP treatment improved MNCV, cMAP, and EFND compared to untreated animals, while EQ had less effect. These results support the armadillo as a model for *M. leprae*-induced peripheral nerve injury that can provide insights toward the understanding of NFI progression and contribute to the preclinical investigation of the safety and efficacy of neuro-preventive and neuro-therapeutic interventions for leprosy.

Keywords: *Dasypus novemcinctus*, *Mycobacterium leprae*, armadillos, leprosy, neuropathies, nerve function impairment

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INTRODUCTION

Mycobacterium leprae and the closely related *Mycobacterium lepromatosis* infect the Schwann cells of the peripheral nerves. This infection can result in neuropathy that affects at least a third (1, 2) of the individuals diagnosed with leprosy. Since treatment of the infection often does not lead to resolution of the neuropathy, the number of affected patients is cumulative. Consequently, ∼2 million people currently suffer from leprosy-related nerve function impairment (NFI) (3).

Leprosy neuritis has been well described clinically and histologically for more than 100 years (4–6). In humans, the ulnar, radial, median, and peroneal nerves are primarily involved (7). Robinson et al. (8) reported that the lateral plantar branch of the tibial nerve is also significantly affected. Unfortunately, the mechanisms of nerve injury following *M. leprae* infection are difficult to study in humans as these nerves cannot be biopsied. Therefore, most investigations have been in human, mouse, and rat Schwann cell tissue culture models (9–11) and to a limited extent *in vivo* in mice (12). The mouse, however, may not be a suitable model to study *M. leprae*-induced neuropathy as it is neither naturally susceptible to *M. leprae* infection, nor does it develop any appreciable nerve involvement (7).

Nine-banded armadillos (*Dasypus novemcinctus*), as well as red squirrels (13) and chimpanzees (14), can become naturally infected with *M. leprae* (15–18). Armadillos develop an extensive nerve involvement that closely resembles the neuropathy seen in humans (19, 20). In response to *M. leprae* infection, the majority (>70%) of armadillos show lepromatous-type disease, but borderline and tuberculoid responses are also found (21, 22).

Standardized tests that measure nerve damage are well established for humans (23) and there has been some use of these tests to evaluate neuropathy in persons affected by leprosy (2, 24–28). Validation of such tests in *M. leprae*-infected armadillos would support its use as an animal model to study leprosy NFI. Therefore, we present here several methods to assess peripheral neuropathy in armadillos at early, mid and late stages of leprosy disease. These include motor nerve conduction tests (MNCT), such as motor nerve conduction velocity (MNCV) and compound motor action potential (cMAP), peripheral nerve ultrasonography with Doppler imaging, quantification of epidermal nerve fiber density (ENFD), neuropathic changes in muscle physiological cross sectional area (PCSA), and evaluation of neuromuscular junction (NMJ) number and integrity. Following extensive validation of these tools in both uninfected and *M. leprae*-infected armadillos, two potential neuroprotective drugs were tested in a pilot study to determine the suitability of these methods to evaluate therapeutic interventions to arrest or prevent leprosy neuropathy.

MATERIALS AND METHODS

Armadillos

Wild captured and captive born (genetically identical) nine-banded armadillos were used in this study. The female armadillo gives birth to four genetically identical siblings, which are hand raised and brought to the NHDP after they are weaned. The use of genetically identical siblings enables a higher statistical power for experimental interventions. All animals were conditioned to our facility for a period of 3–6 months (wild captured) or 12 months (captive born). During conditioning, the armadillos were housed in modified rabbit cages and received the following treatments: Penicillin (2 mL IM repeated at 5 days), Ivermectin (0.1 mL SC), Droncit (0.4 mL IM) and Meticorten (0.25 mL IM). At the end of the conditioning period, they were tested for the presence of anti-Phenolic glycolipid-1 (PGL-1) antibodies to determine the presence of pre-existing *M. leprae* infection. Armadillos with pre-existing infection were not used in this study. To determine their immune responsiveness to *M. leprae*, a Lepromin test (heat-killed nude mice footpad derived *M. leprae* strain Thai-53 at 1×10^8 bacilli in 0.1 mL total volume) was administered by intradermal injection to the abdominal skin. After 21 days, the skin test sites were biopsied using a 4 mm biopsy punch and examined histopathologically to score the reactivity of each animal on the Ridley-Jopling scale (29, 30). Only those animals classified as lepromatous (LL) were included in this study.

Infection of Armadillos With *Mycobacterium leprae*

Armadillos were experimentally infected via intravenous (IV) inoculation of a suspension of 1×10^9 viable *M. leprae* freshly harvested from nude mice footpads (31).

Anti-pGL-1 Antibody ELISA

PGL-1 antigen (BEI Resources) was used in immunoassays to detect anti-PGL-1 IgM circulating antibody levels in serum samples as previously described (32). Levels of OD > 0.700 (540 nm) were considered positive.

Light Microscopic and Electron Microscopic Studies

At the time of sacrifice the posterior tibial nerve was collected, fixed in 10% buffered formalin and processed for light microscopic studies, or fixed in 5% glutaraldehyde and processed for electron microscopic studies as previously described (33, 34).

Motor Nerve Conduction Test

Following anesthesia with 0.4 ml Detomidine and 0.6 ml Ketamine, the animals were placed in a wooden trough ventral side up inside a Faraday cage. Skin temperatures were taken on the medial aspect of each hind limb between the knee and hip to ensure that the MNCT tests were done at body temperatures of 31 ± 2.0°C, because high body temperatures (>35°C) can affect the speed of conduction velocity. An infrared thermometer was used to take the body temperature. We adapted a Cadwell Sierra Summit (Cadwell) portable electrophysiology unit for use with the armadillos. 6.5mm gold cup percutaneous electrodes were prepped with Ten20 conductive paste and used as active, reference, and ground electrodes. After the hind limbs were cleaned with alcohol, the reference electrode was placed at the sulcus of the third toe and the active electrode was placed on an intrinsic muscle between the third and fourth

metatarsal bones midshaft. The ground electrode was placed on the anterior/lateral aspect of the hind limb. A cortical disposable stimulator (Natus Neurology) was used to produce a motor stimulus. This stimulator has two 2.2 mm gold plated tips with 5 mm spacing. The negative pole of the stimulator was placed posterior to the medial ankle maleolus for the distal stimulation site. Percutaneous stimulation of the posterior tibial nerve was done near the ankle (distal), and near the knee (proximal) on the medial/posterior shaft of the tibia. Measurements were taken from the proximal stimulation site to the active electrode and from the distal stimulation site to the active electrode to measure the distance between the stimulation sites. Recordings were always done at a supramaximal stimulus. Motor nerve conduction velocity (MNCV) was calculated using the formula: MNCV (in m/s) = (Distance between Proximal and Distal stimulation sites)/(Proximal Latency – Distal Latency). Compound Motor Action Potential (cMAP) amplitude was measured from baseline to negative peak and was recorded in millivolts (mV). Room temperature was maintained between 24 and 26°C throughout the procedure.

Ultrasonography of the Peripheral Nerves

Posterior tibial nerves were examined via ultrasonography using a SmartUS transducer probe with a linear broadband frequency of 7.5–15 MHz connected to a Sierra Summit basic system (Cadwell). Color Doppler imaging was used to detect blood flow in the posterior tibial nerves. The colors show the speed and direction of blood flow through the vessel. Flow that travels away from the transducer (negative Doppler shift) is depicted in blue. Flow traveling toward the transducer (positive Doppler shift) is depicted in red. Lighter shades of each color denote higher velocities.

Neuropathic Changes in Muscle Physiological Cross Sectional Area

Following euthanasia, each hind limb was disarticulated at the hip joint and frozen until dissection. The dissection technique used was adapted from Brand et al. (35). The skin, superficial fascia, and fat were removed from the limbs and upper leg muscles were removed for better visualization. Blunt dissection was used to isolate the foot muscles from surrounding tissue. The tendon of insertion was severed distal to the muscle, clamped with a hemostat, and pulled laterally from the tendon of origin. Both tendons were traced into the muscle belly, lightly teasing away the connective tissue holding the muscle in its fusiform shape (**Figures 6A,B**). This allowed the fibers to align parallel to each other as they course from the tendon of origin to the tendon of insertion. Muscle fibers were measured with a Fowler precision dial caliper. The muscles were severed at their tendon of origin and weighed on an Ohaus scale. The fiber length (in centimeters) and mass (in grams) for each muscle was entered into the following equation to obtain the muscle PCSA: PCSA = Mass/1.02/Fiber length. The PCSA values for each muscle were analyzed along with the MNCT values recorded for the respective hind limb to determine any association.

Evaluation of Neuromuscular Junctions in the Footpad Muscles

Three uninfected and five *M. leprae*-infected armadillos were used to evaluate integrity and numbers of NMJ in the intrinsic muscles of the hind footpads. Following euthanasia, intrinsic muscles from both hind footpads were carefully dissected and collected in 4% paraformaldehyde at 4°C. After fixing in paraformaldehyde for 4 h, the muscles were incubated overnight in 30% sucrose solution, embedded in OCT (Thermo Fisher Scientific), and stored at -80°C. Thirty μm longitudinal sections were prepared and stained with FITC-labeled Bungarotoxin (Sigma-Aldrich) and PE-labeled anti-myosin antibody (Sigma-Aldrich). A 30 mm^2 area through the entire thickness of the section was analyzed for the number and integrity of NMJ. Images were captured on an Olympus BX-70 microscope and analyzed using Image Pro software (Dimension, Cellsen).

Quantification of Epidermal Nerve Fiber Density in the Armadillo Hind Limb

Skin biopsies were obtained from the armadillo hind limb using a 3 mm biopsy punch. The biopsy was fixed in Zamboni solution (Newcomer Supply) for 24 h, washed with 0.08 M Sorenson's phosphate buffer, fixed in cryoprotectant (0.2 M Sorenson's phosphate buffer and Glycerol), and stored at -20°C. The biopsies were sectioned with a sliding microtome into 50 μm thick, frozen, vertical, free-floating sections. Four sections were randomly selected and immunostained with rabbit anti-PGP 9.5, a pan axonal marker (Chemicon, Temecula, CA, United States dilution 1:10,000). ENFD were determined following established counting rules (36–38).

Evaluation of Effectiveness of Neuroprotective Drugs in Preventing and Improving NFI in *Mycobacterium leprae*-Infected Armadillos

Five sets (3 pairs and 2 triplets) of *M. leprae*-infected siblings were randomly allocated into 3 treatment groups as follows: 4-Aminopyridine (4-AP) (N = 4), Ethoxyquin (EQ) (N = 3) and untreated control (N = 5). 4-AP (0.9 mg/day, Sigma-Aldrich) and EQ (2 mg/day, Sigma-Aldrich) were administered orally with the food on a daily basis. The treatment started when the set of animals showed a decrease in cMAP (about 3–7 months post-inoculation) and ended after 60 days. MNCT was performed before, during, and at the end of treatment. At the end of treatment, a 3 mm punch biopsy was collected from the hind leg and fixed in Zamboni fixative. The biopsies were washed, fixed in cryoprotectant, and stored at -80°C for quantification of ENFD.

Statistical Analysis

Mann–Whitney Rank Sum test and Spearman's rank test was used to compare differences and correlation, respectively, between different groups using either GraphPad Instat software, version 3.10 (GraphPad Software, Inc.) or Sigma Plot version 12.0 (Systat Software, Inc.). $p < 0.05$ was considered significant.

RESULTS

A total of 154 armadillos were used in this study, including 36 uninfected and 118 that were experimentally infected with *M. leprae*. All the animals were classified as LL.

Localization of *Mycobacterium leprae* in the Posterior Tibial Nerve of Infected Armadillos

Armadillos, like humans, exhibit peripheral nerve involvement upon *M. leprae* infection. **Figure 1A** depicts a posterior tibial nerve section from a *M. leprae*-infected armadillo showing the presence of intraneural acid-fast bacilli. Transmission electron microscopy shows *M. leprae* located inside the unmyelinated (**Figure 1B**) and myelinated (**Figure 1C**) Schwann cells. The effects of this nerve invasion, however, are poorly understood in this model. Therefore, in this study we adapted and applied several non-invasive and invasive tests to evaluate the resultant neuropathy.

Determination of MNCV and cMAP Values in the Posterior Tibial Nerve of Uninfected Armadillos

Twenty-five uninfected armadillos were used to develop and standardize MNCT and obtain the normal values for MNCV and cMAP. Upon evaluation of the posterior tibial nerve of each hind limb, a mean MNCV of 62.09 ± 10.72 m/s (range = 88.24–43.75 m/s) was found, with no significant difference between the right and left hind limbs of an individual animal (**Figure 2**). The mean proximal amplitude was 1.53 ± 0.30 mV (range = 2.12–1.02 mV) and the mean distal amplitude was 1.55 ± 0.33 mV (range = 2.18–0.98 mV). Again, there were no large discrepancies between the right and left cMAP amplitudes of an individual animal. Based on this data we established that a MNCV of <40 m/s (mean - 2 SD) and a cMAP amplitude of <0.9mV (mean - 2 SD) should be considered abnormal for the posterior tibial nerves in armadillos.

Progression of Abnormal MNCV and cMAP in the Posterior Tibial Nerve of *Mycobacterium leprae*-Infected Armadillos and Development of a MNCT Scoring System

Mycobacterium leprae-infected armadillos began to exhibit a low cMAP (<0.9 mV) at an early stage of infection (∼4 months post-inoculation) (**Figure 3A**). As the animals progressed to later stages of leprosy disease they showed a further depressed cMAP (**Figure 3B**) and eventually complete block of conduction at the knee (**Figure 3C**). A reduction in cMAP (signifying axonal degeneration) was more common than slowing of MNCV (related to demyelination). Importantly, these results indicated that measurable motor nerve conduction deficit, especially reduced cMAP, could occur early in the course of infection in these animals.

Based on these results we developed the following simple qualitative MNCT scoring system to categorize the armadillos according to their MNCV and cMAP abnormal readings in the posterior tibial nerve. The animals with both normal MNCV and cMAP in both hind limbs were arbitrarily assigned a score of 5. Each abnormality, low MNCV or cMAP in each hind limb, was considered as 1-point, which was substracted from the normal score of 5 to obtain the MNCT for a particular animal as follows:

Score 5: animals having no abnormal MNCV or cMAP in either hind limbs.
Score 4: animals having only one abnormality, either slow MNCV (<40 m/s) or low cMAP (< 0.9mV) in only one hind limb.
Score 3: animals having any combination of two abnormal readings in the MNCV and/or cMAP.
Score 2: animals having any combination of three abnormal readings in the MNCV and/or cMAP.
Score 1: animals having abnormal MNCV and cMAP in both right and left hind limbs.

Therefore, an animal showing both abnormal MNCV and cMAP in both hind limbs will have a score of 5–4 = 1, the lowest possible in this system.

Correlation of MNCT With Disease Progression and Anti-PGL-1 Antibody Levels

A set of 20 *M. leprae*-infected armadillos were individually evaluated for NFI throughout their leprosy disease progression using the MNCT scoring system standardized above. **Figure 4A** shows the MNCT scores of individual animals at pre-inoculation and at 4, 9, 12, and 24 months post-inoculation. At time 0 (uninfected) all the animals show a score of 5 with no abnormalities in the MNCV or cMAP in either hind limb. The scores began to decrease as early as 4 months post-infection with the majority of animals showing low scores at the late stages of leprosy disease (12 and 24 months post-infection).

We have previously shown that antibody levels for the *M. leprae*-specific PGL-1 correlate with bacterial load in armadillos (39–41). In order to determine the correlation between anti-PGL-1 antibody levels and impaired nerve conduction, we stratified 71 *M. leprae*-infected armadillos at different stages of leprosy disease into two groups according to serum anti-PGL-1 IgM antibody levels. As shown in **Figure 4B**, sera with OD 540nm values between 0.700 and 0.900 were considered as a low (+) anti-PGL-1 titer group (30/71) while sera with OD values of >0.900 were considered as a high (++) anti-PGL-1 titer group (41/71). Overall, 80.3% (57/71) of infected armadillos developed measurable motor nerve conduction abnormality in at least one of their posterior tibial nerves (MNCT score < 5). Within the low anti-PGL-1 group, 73.3% (22/30) showed impaired nerve conduction in at least one hind limb while in the high anti-PGL-1 group, 85.4% (35/41) showed impaired nerve conduction (**Figure 4B**). Although no significant difference (p = 0.3297) was found in impaired nerve conduction between low and high PGL-1

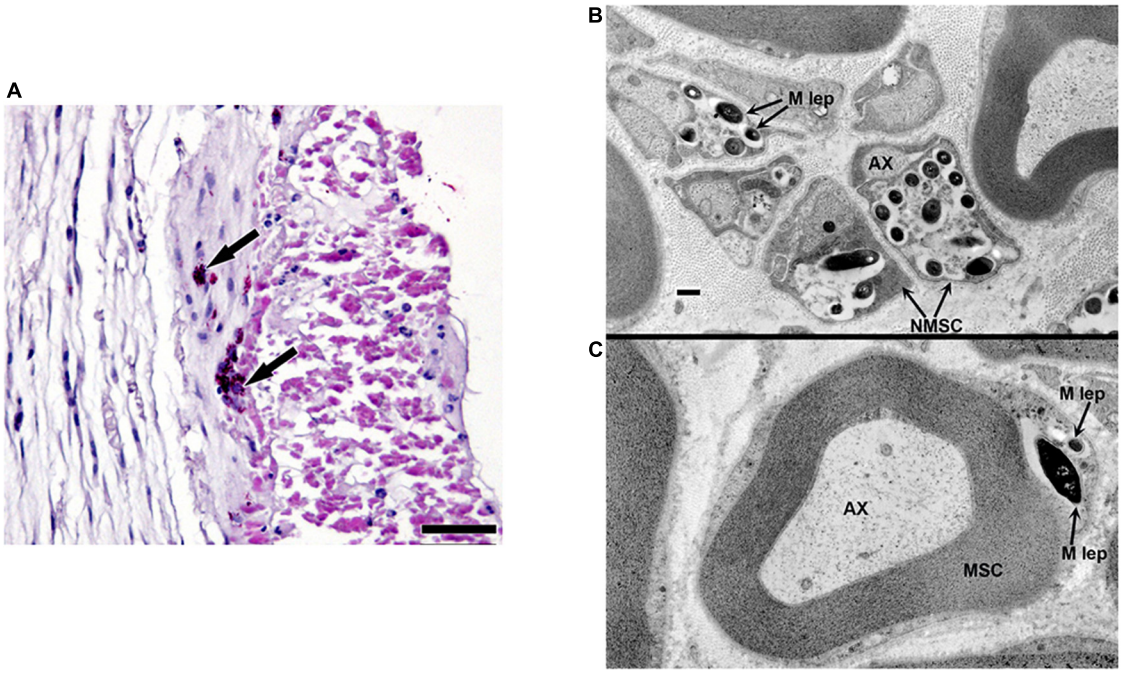

FIGURE 1 | Localization of *M. leprae* in the posterior tibial nerve of infected armadillos. **(A)** Posterior tibial nerve showing clumps of intraneural *M. leprae* (arrows); modified Fite/Faraco stain. Electron micrographs showing *M. leprae* (M. lep) within unmyelinated **(B)** and myelinated **(C)** Schwann cell (SC) located by the axon (AX) in the post-tibial nerve of an armadillo at 24 months post-experimental infection.

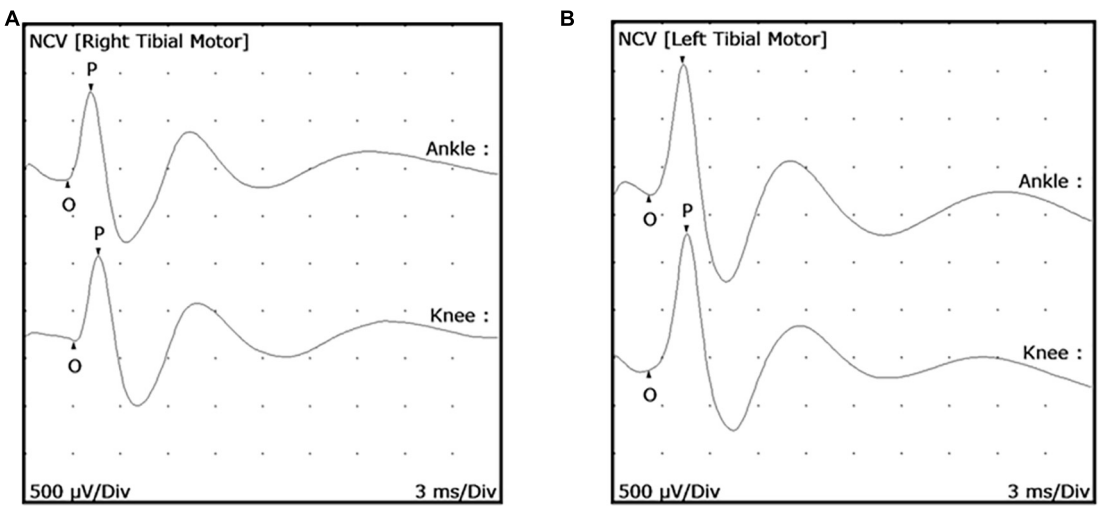

FIGURE 2 | Posterior tibial nerve conduction wave forms in uninfected armadillos. Motor Nerve Conduction Tests (MNCT) were performed on the posterior tibial nerve of uninfected armadillos (*N* = 25) using a Cadwell Sierra Summit portable electrophysiology unit. Representative wave forms are shown of a right posterior tibial **(A)** and left posterior tibial **(B)** nerve. Measurements were taken from the proximal stimulation site to the active electrode and distal stimulation site to the active electrode to measure the distance between stimulation sites. Compound muscle action potential (cMAP) was measured from onset (O) to the peak (P) in both ankle and knee in response to supra-maximal electrical stimulation.

groups, conduction abnormality among *M. leprae*-infected armadillos generally tends to increase with rising anti-PGL-1 antibody levels. In the low anti-PGL-1 group, 36.6% (11/30) of the animals had a score of 3 or below while in the high anti-PGL-1 group 61.0% (25/41) of the animals had a score of 3 or below. In addition, nearly all of the animals that developed conduction deficit also eventually exhibited signs of clinical neuropathy in their footpads. Decreased cMAP correlated well with the number of wounds and heavy calluses ($r = -0.72$, $p = 0.02$) on the plantar surface of the foot (data not shown).

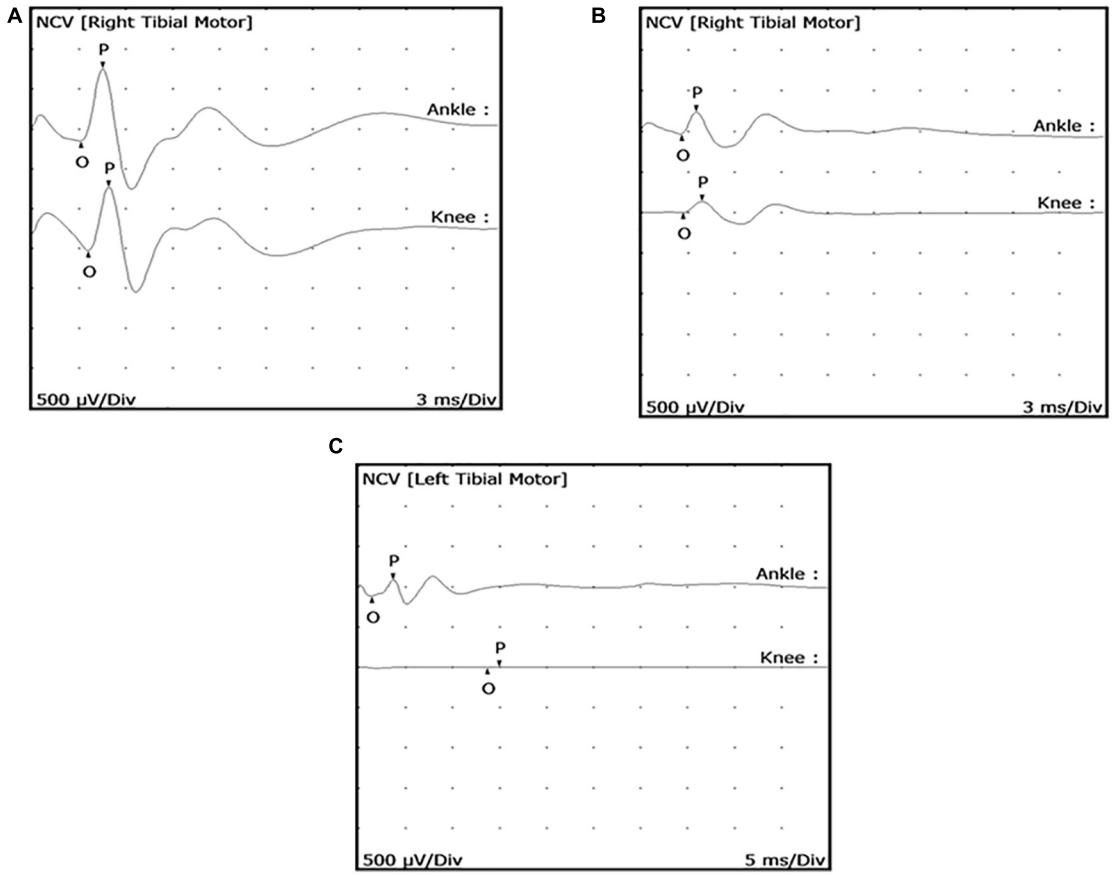

FIGURE 3 | Posterior tibial nerve conduction wave forms showing reduced compound muscle action potential (cMAP) in *M. leprae*-infected armadillos. MNCT were performed on the posterior tibial nerves of *M. leprae*-infected armadillos ($N = 71$). Representative motor nerve conduction wave forms depict early **(A)** (4 months infection) and mid **(B)** (9 months infection) stage leprosy disease and show abnormal wave forms with declining degrees of cMAPs (<0.9 mV). Armadillos progressing to late stage leprosy disease (12–24 months post-inoculation), exhibit conduction block at the knee **(C)**.

The occurrence of hypertrophic nails and nail avulsion also tend to increase along with decreasing cMAP and increased anti-PGL-1 antibodies.

Ultrasonography of the Posterior Tibial Nerves

Figure 5 shows ultrasonography of the posterior tibial nerve in transverse and longitudinal axes of uninfected (A and B) and *M. leprae*-infected (C and D) armadillos at an early stage (4 months post-inoculation) of leprosy disease. The posterior tibial nerve of the *M. leprae*-infected armadillo was larger when measured at the transverse axis (0.096 cm^2; P: 13.1 mm) compared to the uninfected armadillo (0.037 cm^2; P: 8.3 mm). Nerve enlargement can also been seen when examined at the longitudinal axis. **Figure 5E** shows the ultrasonography of the posterior tibial nerve of an *M. leprae*-infected armadillo at a late stage of leprosy disease. Increased blood flow, indicating possible inflammation, was seen using color Doppler imaging in an armadillo at 23 months post-*M. leprae* inoculation. Interestingly, this animal had a high level of anti-PGL-1 IgM antibody (2.104 at OD 540 nm). No increased blood flow (5F) was seen in an armadillo at a mid-stage disease (9 months post-inoculation) that was negative for anti-PGL-1 IgM antibody (0.225 at OD 540 nm).

Neuropathic Changes in Muscle Physiological Cross Sectional Area

We examined the PCSA of footpad muscles of 18 armadillos, of which 10 were infected with *M. leprae* and 8 were uninfected (**Figures 6A,B**). The PCSA values of the small lateral flexor, lateral lumbrical (**Figure 6C**), and both insertions (medial and lateral) of the medial lumbrical muscles (**Figure 6D**) of the *M. leprae*-infected armadillos averaged 20% lower than that of the uninfected animals ($p < 0.02$). Furthermore, there were statistical differences ($p = 0.008$) between MNCT scores between the groups.

Integrity of the Neuromuscular Junction in the Footpad Muscles

Intrinsic muscle from the hind footpads of 3 uninfected and 5 *M. leprae*-infected late-stage armadillos were used to quantify the numbers and fragmentation of post-synaptic NMJ. A significant reduction ($p = 0.0012$) in the number of NMJs was observed

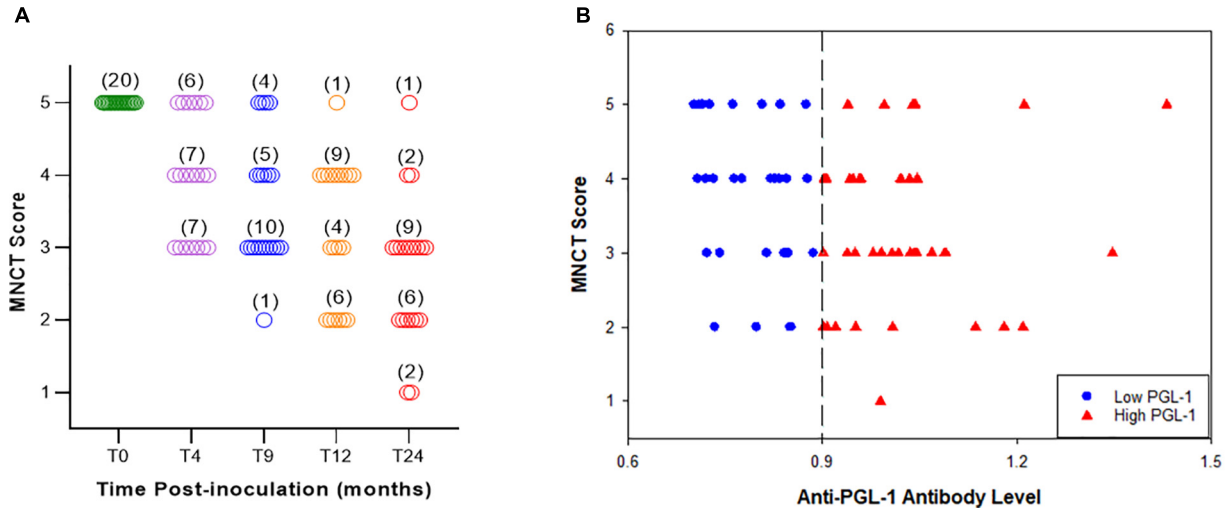

FIGURE 4 | MNCT scores decrease with duration of infection and correlate with anti-PGL1 antibody titers. A longitudinal study **(A)** demonstrated negative correlation between the MNCT score and duration of *M. leprae* infection, indicating progressive nerve injury (*N* = 20). **(B)** Phenolic glycolipid I (PGL-1) antigen was used in an enzyme immunoassay to detect anti-PGL1 IgM circulating antibody levels in armadillo serum samples. Armadillos with high (>0.900 OD 540 nm) anti-PGL1 antibody titers have lower MNCT scores, while those with low (0.700–0.900 OD 540 nm) anti-PGL-1 antibody titers have higher MNCT scores (*N* = 71).

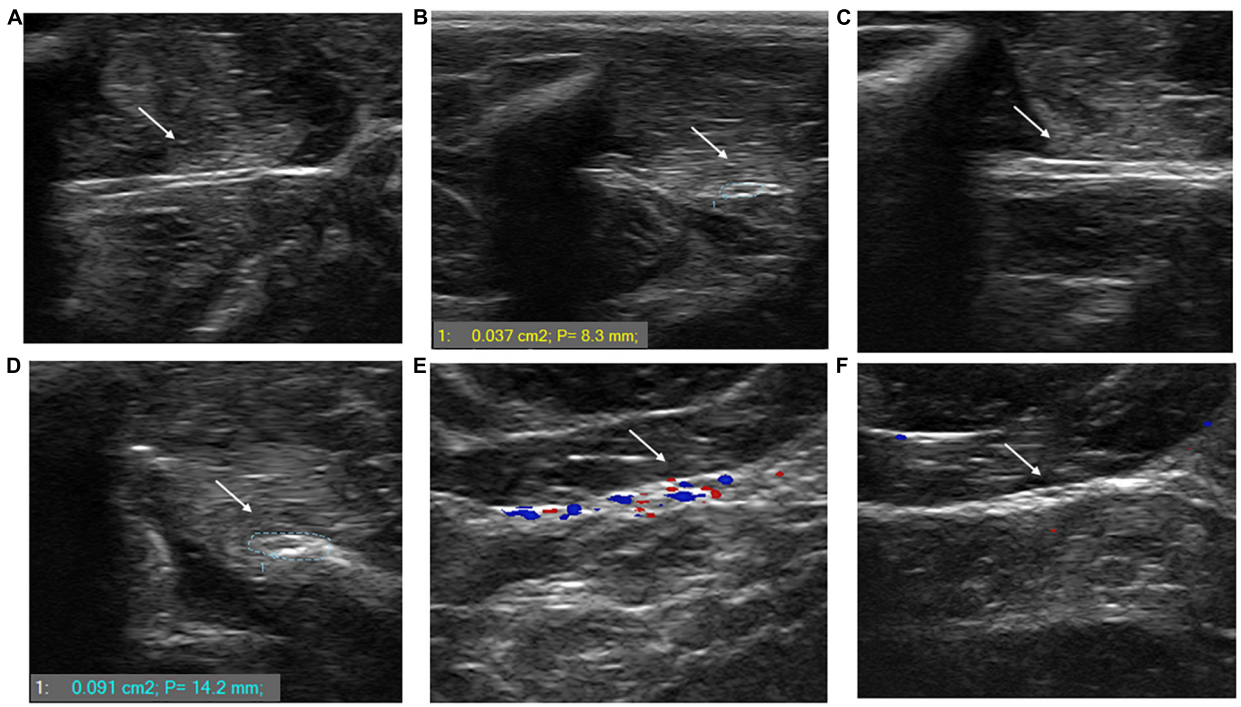

FIGURE 5 | *M. leprae* infection causes nerve enlargement in armadillo peripheral nerves. Ultrasonography of the posterior tibial nerve was performed using a SmartUS transducer probe with a linear broadband frequency of 1.5–15 MHz connected to a Sierra Summit basic system. The posterior tibial nerve is shown along its longitudinal **(A)** and transverse **(B)** axes in an uninfected armadillo and a *M. leprae*-infected armadillo [**(C,D)**, respectively]. The nerve diameter of the *M. leprae*-infected armadillo is 0.096 cm^2 indicating nerve enlargement compared to the nerve diameter of the uninfected armadillo (0.037 cm^2). Ultrasound with color Doppler imaging in *M. leprae*-infected armadillos showed increased blood flow in animals at late stage leprosy disease (23 months post-infection) that were positive for anti-PGL-1 antibodies **(E)** compared to armadillos that were negative for anti-PGL-1 antibodies at 9 months post-infection **(F)**. The intense colors indicate lower velocity of the blood flow traveling away and toward the transducer (*N* = 4).

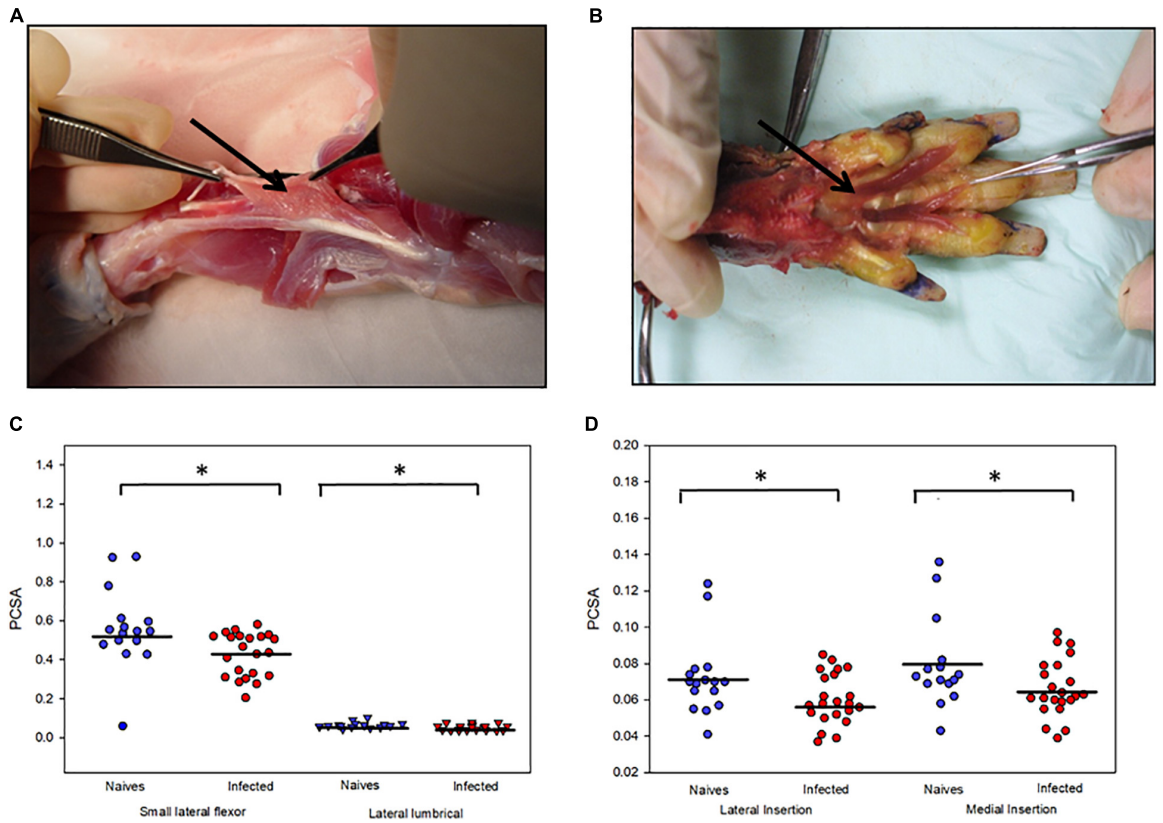

FIGURE 6 | *M. leprae* infection causes atrophy of armadillo footpad muscles. Photographs of the dissection of a representative animal showing **(A)** the small lateral flexor muscle of the footpad and how the fibers run between the tendons and **(B)** the lumbrical muscles of the right hind limb that were released from their tendon of insertion. **(C)** Atrophy of small lateral flexor and lateral lumbrical muscles in *M. leprae*-infected armadillos compared to uninfected armadillos. **(D)** Atrophy of medial and lateral insertions of the medial lumbrical muscle in *M. leprae*-infected armadillos ($N = 10$) compared to uninfected armadillos ($N = 8$). $^*p < 0.05$.

in this muscle in *M. leprae*-infected armadillos (**Figure 7A**). Furthermore, ∼50% of the NMJs in the infected armadillos (**Figure 7B**) were fragmented when compared to those in uninfected animals.

Evaluation of Effect of Neuroprotective Drugs in Preventing and Improving Nerve Function Impairment in *Mycobacterium leprae*-Infected Armadillos

Motor nerve conduction studies together with ENFD for sensory nerves were used to evaluate the ability of two neuroprotective drugs, 4-AP and EQ, to improve nerve function impairment in *M. leprae*-infected armadillos. Electrophysiological studies indicated that armadillos treated with 4-AP and EQ showed a significantly higher ($p = 0.001$ and $p = 0.012$, respectively) cMAP than the untreated controls at 1 month post-treatment (**Figure 8a**). No significant differences were seen in the cMAP between EQ and 4-AP treated animals ($p = 0.105$). At the end of the treatment all the untreated (N = 4) and EQ (N = 3) treated animals had a MNCT score of 3 while the 4-AP treated animals (N = 3) yielded MNCT scores of 3, 4 and 5. Similarly, armadillos treated with 4-AP showed higher ENFD than untreated controls and EQ treated armadillos; however, the differences in this limited number of animals did not reach significance (**Figure 8b**). **Figure 8c** depicts a skin section from an *M. leprae*-infected armadillo stained with PGP.9.5, showing fewer branching of the epithelial nerve fibers compared to a 4-AP-treated armadillo (**Figure 8d**). EQ-treated armadillo also shows fewer branching than 4-AP-treated armadillo (**Figure 8e**).

DISCUSSION

The lack of an effective animal model to study *M. leprae*-induced nerve injury has greatly hampered understanding of the mechanisms involved. Nine-banded armadillos are naturally susceptible to *M. leprae* infection (16, 17) and show the full histopathological spectrum of leprosy. More importantly, armadillos develop extensive peripheral nerve involvement due to *M. leprae* infection that closely resembles neuropathy in humans (19, 42). Previous histopathological studies performed in the armadillo posterior tibial nerve suggested that the localization of *M. leprae* in the peripheral nerve proceeds from outside in as opposed to an ascending-type of infection. Similar to human LL cases, infected armadillo nerves demonstrate a heavy

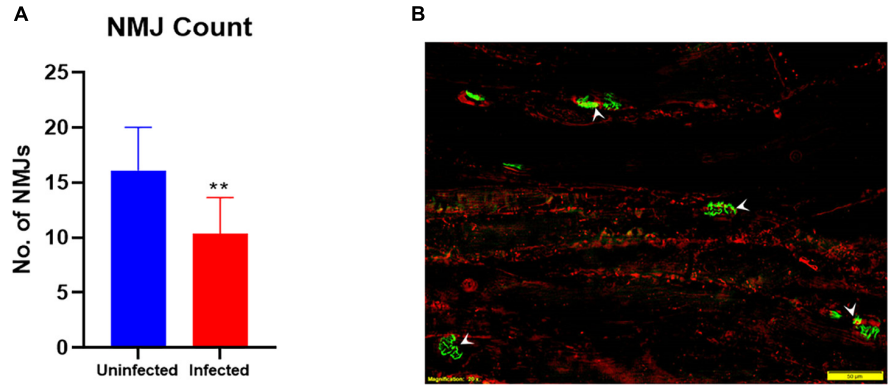

FIGURE 7 | *M. leprae* infection causes degeneration of neuromuscular junctions (NMJ's) in the armadillo footpad muscles. The number of NMJs **(A)** is reduced (**p = 0.0012) in *M. leprae*-infected armadillos (terminal stage) as measured in 30μm-thick, 30^2 mm muscle sections. Fluorescent micrograph **(B)** of footpad intrinsic muscle in an *M. leprae*-infected armadillo showing NMJs (green, arrowheads) stained with FITC-α-bungarotoxin and muscle fibers (red) stained with PE-anti-myosin. (N = 8).

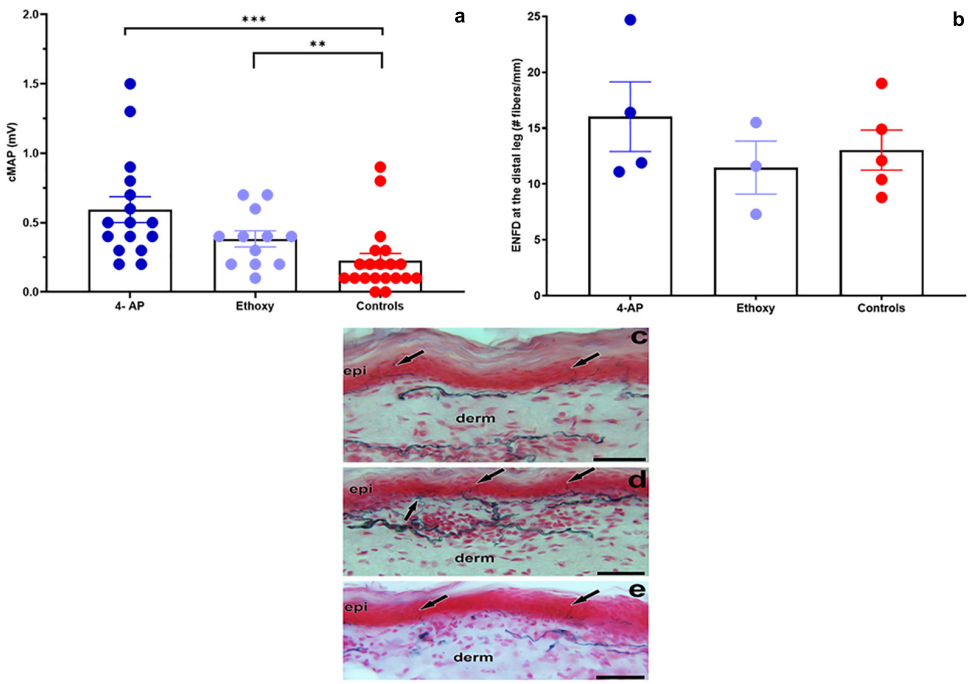

FIGURE 8 | Evaluation of the effect of neuroprotective drugs on NFI in *M. leprae* infected armadillos. **(a)** Armadillos treated with 4-Aminopyridine- (4-AP) (N = 4) and Ethoxyquin (EQ) (N = 3) showed a significantly higher (***p = 0.001 and **p = 0.012, respectively) cMAP (mV) than the untreated controls (N = 5) at 1 month post-treatment. **(b)** Epithelial nerve fiber density (ENFD, #fibers/mm) in 4-AP- and EQ-treated and untreated armadillos. **(c)** Skin section from an untreated armadillo stained with PGP9.5 showing epidermal nerve fibers (arrows) terminating at the epidermis (epi). **(d)** Skin section from a 4-AP-treated armadillo showing more branching of the epidermal nerve fibers (arrows) and that the dermal nerve bundles are dense at the papillary dermis (derm) in comparison to the untreated armadillo. **(e)** Skin section from an EQ treated armadillo showing single epidermal nerve fibers (arrows) entering the epidermis. Scale bars = 50 μm.

infiltration of *M. leprae*-loaded nucleated cells in many fascicles of nerve trunks and invading demyelinating Schwann cells (5, 42, 43). However, due to the exotic nature of this animal model, no standardized methods are available to detect and accurately measure the degree of neuropathy or nerve damage in armadillos. Standardized tools to study the progression of neuropathy as well as to evaluate any therapeutic interventions are essential to advancing the armadillo as a model for NFI. The purpose of this study was to establish multiple non-invasive tests, like MNCT, ENFD and ultrasonography, which could be performed on the same animal to follow disease progression in the peripheral nerves. We also evaluated muscular atrophy and NMJ integrity in the footpad muscles as end-point assessments for the degree of neuro-muscular damage at the

terminal stage of the disease. Together these tests form a robust set of tools to evaluate peripheral neuropathy in *M. leprae*-infected armadillos.

Neuropathies in humans are diagnosed using peripheral motor and sensory nerve conduction studies that evaluate the integrity of nerve-muscle and the somatosensory circuit (23). Motor nerve conduction studies are commonly performed by electrically stimulating (percutaneous) a peripheral motor or mixed nerve and recording the electrical response from a suitable muscle innervated by the same nerve. Analysis of the conduction velocity (MNCV), negative peak amplitude (cMAP), and the shape of the wave form following electrical stimulation reveal the degree of demyelination and axonal loss in the studied segment of the peripheral motor or mixed nerve. Demyelination results in slowing of conduction velocity measured in meters/second (m/s). In some cases, demyelination can also cause dispersed cMAP. In contrast, axonal loss leads to a decrease in the cMAP measured in millivolts (mV) (27). Since percutaneous nerve conduction studies are non-invasive, they are convenient ways to study onset and progress of peripheral neuropathy over time in the same subject.

Although the hard carapace and thick skin limit the number of nerves that may be examined in armadillos, we have developed and validated MNCT techniques and an MNCT scoring system that can be used to assess motor nerve conduction in both hind limbs by examining the posterior tibial nerve that lies beneath the skin surface between the ankle and knee and innervates the small lumbrical and flexor muscles of each foot. Similar to that seen in humans, leprosy is asymmetrical and manifests randomly in different nerves in these animals. Moreover, like in humans there is a high probability that the posterior tibial nerve will become involved, and that probability increases as the disease progresses (20, 42). We have shown in previous electron microscopy studies that posterior tibial nerves of armadillos progressing at 28 months post infection exhibited *M. leprae* within both myelinated and unmyelinated axons and Schwann cells. The axons showed edema with demyelination and axonal degenerative changes (20, 43).

Peripheral conduction deficit among *M. leprae*-infected armadillos began early in the course of their disease and progressed over time. Decreased cMAP amplitude (<0.9mV), which indicates axonal loss, was the most common impairment. *M. leprae*-infected armadillos rarely showed dispersed cMAP, which also indicates axon loss, but abnormal nerve conduction velocity (MNCV < 40 m/s) was observed. Some studies have reported demyelination as the main cause of nerve dysfunction in leprosy disease (44–46). Another study, however, found that axonal injuries occurred at a higher frequency than myelin injuries in the posterior tibial nerve (8). Hence, the sequence of events leading to nerve injury may vary among different nerves or an individual's position in the clinical spectrum. 70% (14/20) of experimentally infected armadillos developed demonstrable conduction deficit in at least one of their posterior tibial nerves within 4 months of infection, and its onset generally coincided with detectable anti-PGL-1 IgM antibodies. Eventually, nearly all *M. leprae*-infected armadillos will exhibit neuropathy as their disease disseminates. As the disease progressed, both right and left posterior tibial nerves were compromised showing delayed MNCV, which is a consequence of demyelination of the nerve fibers, and in some cases total conduction block. Armadillos in very late stages of experimental leprosy may develop lesions in their footpads that can make MNCT difficult to perform or can produce inaccurate results. Extending our electrophysiological techniques to include sensory nerve and F-wave responses will likely increase the sensitivity of detecting peripheral neuropathy and will likely reveal even greater involvement of nerves at these early stages of infection. Nevertheless, our data clearly shows that in armadillos there is a significant association between the onset of the host immune response (anti-PGL-1 antibodies) and conduction abnormalities in the peripheral nerves of the lower extremities which progresses over time to manifest classical lesions in the foot.

Peripheral nerves are often enlarged in leprosy patients and ultrasonography is currently used to determine nerve enlargement (28, 47, 48). It provides an objective measure of the nerve dimensions in addition to revealing structural changes over a longer length of the nerve (49). Enlarged nerves of patients undergoing leprosy reactions show increased endoneurial and epineurial vascularity that can be measured by ultrasonography with color or power Doppler (24, 28). We also used ultrasonography of the posterior tibial nerve to show that armadillos, like humans, show enlargement of peripheral nerves due to *M. leprae* infection. Although leprosy reactions have not yet been determined in armadillos, increased vascularity, most likely due to inflammation, could be demonstrated using color Doppler (49). Unlike MNCT, ultrasonography can be done on multiple peripheral nerves of upper and lower extremities of the animal, thereby increasing the probability of detecting neuropathy in focal and asymmetric disease progression.

Monitoring warm/cold detection or grip dynometry are currently among the most effective early indicators of nerve injury in human leprosy, and these tests often show changes well before conduction abnormality is apparent (2, 35). Unfortunately, armadillos cannot grip objects and they are generally unresponsive to common temperature stimuli. However, Brand showed that the PCSA (cross sectional area/mass) of muscles in the leprotic hand could be used as a surrogate measure of grip strength and index muscle atrophy (35). We examined the PCSA of footpad muscles of *M. leprae*-infected and uninfected armadillos and found that the infected armadillos showed significant muscle atrophy indicated by lower fiber density compared to the uninfected armadillos. Additionally, we compared neuromuscular junction (NMJ) integrity and numbers in footpad intrinsic muscles, between end stage *M. leprae*-infected and uninfected armadillos and found significant loss of NMJs in infected armadillos. Further studies are required to understand the mechanisms of motor NMJ innervations and decay dynamics in *M. leprae*-infected armadillos and to evaluate reconnection of the NMJ's, which can give important insights into possible therapeutic interventions to arrest or reverse loss of neuro-muscular function in leprosy.

Motor nerve conduction and sensory tests together with ultrasonography are suitable for evaluation of peripheral nerve function. Since these are non-invasive tests they can be used to

follow disease progression in experimentally infected armadillos and consequently to evaluate efficacies of therapeutic and immune interventions in preventing or reversing peripheral nerve damage. Within sensory tests, quantification of ENFD is a test that is used to diagnose small fiber neuropathies including leprosy induced neuropathies (50). In small fiber sensory neuropathies associated with diabetes, HIV and idiopathic small fiber neuropathies, a decrease in epidermal density in the distal leg has been demonstrated (51–53). We have previously reported that armadillos show a length dependent innervation similar to humans (42, 50); therefore, this test can be used to diagnose small fiber neuropathies associated with *M. leprae*-infection in armadillos. Using MNCT, it was demonstrated that Lepvax, a vaccine against leprosy, showed some nerve protection in *M. leprae*-infected armadillos, indicated by higher cMAP recordings and restoration of axonal size of the tibial nerve fibers in vaccinated armadillos compared to the unvaccinated (43).

In the current study, we evaluated two neuroprotective drugs that have been used successfully in cancer models to ameliorate neuropathy following chemotherapy (54) and in acute peripheral nerve injury models promoting remyelination (55). EQ, a quinolone-based antioxidant, has been shown to prevent axonal loss in peripheral nerves of mice with chemotherapy-induced neurotoxicity. EQ provided a dose-dependent neuroprotection indicated by higher intraepidermal nerve fiber density and sensory nerve potential amplitude, and lower thermal hypoalgesia compared to the untreated controls (54). A second drug, 4-AP, has been used in Lambert-Eaton myasthenic syndrome and multiple sclerosis and acts by blocking potassium channels, prolonging action potentials, and increasing neurotransmitter release at the neuromuscular junction. It was also shown to be effective in promoting remyelination and axon regeneration in acute peripheral nerve injuries. In clinical trials, administration of 4-AP improved multiple neurological signs of multiple sclerosis such as vision, muscle strength and coordination (56, 57). 4-AP treatment in acute peripheral injury enhanced both the speed and extent of restoration of normal NCV and caused regenerative increases in axonal area, myelin thickness, and levels of the myelin-specific protein (55). Both EQ and 4-AP were effective in preventing demyelination and axon loss, and treatment resulted in higher epidermal nerve fiber density (ENFD) indicating higher sensory integrity in treated animals (54, 55). We used motor nerve conduction studies together with sensory tests (ENFD) to assess the effect of these two neuroprotective drugs (4-AP and EQ) in preventing and or improving nerve function impairment in *M. leprae*-infected armadillos in a limited pilot study. After one month of treatment, 4-AP improved motor nerve function in armadillos that were progressing at an early stage of leprosy disease, indicating that this drug merits further evaluation in this model. This pilot study shows that the armadillo model and the various nerve structure and function assessment methods validated in this study have the required sensitivity to evaluate efficacy of therapeutic interventions against peripheral neuropathy in leprosy.

In summary, multiple non-invasive and invasive methods to evaluate *M. leprae*-induced peripheral NFI in nine-banded armadillos were validated, and a quantitative nerve deficit scoring system was developed. The data clearly indicate that the armadillo lower extremity becomes heavily involved and exhibits many of the same abnormalities as seen in humans. This study further suggests that, like humans, armadillos suffer an insidious silent neuropathy that commences early in the infection, and serological screening can be useful to stage the disease status for nerve injury studies. These techniques detected pathological events at early stages of disease and will benefit future studies to elucidate mechanisms involved in early leprosy neuropathy. Moreover, the armadillo model and these tests to evaluate peripheral neuropathy will support the screening of new preventive interventions for leprosy control such as leprosy specific vaccines and chemotherapies to either prevent, arrest or reverse nerve damage.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the NHDP Institutional Animal Care and Use Committee (Assurance #D16-000019 [A3031-01]) and was performed in accordance with the United States Department of Agriculture and Plant Health Inspection Service.

AUTHOR CONTRIBUTIONS

MP and RL validated MNCT test and ultrasonography of peripheral nerves and analyzed the data. GE counted and analyzed the EFND data. JF was instrumental in validating the MNCT test. SW validated the ultrasonography in peripheral nerves in armadillos. LA and RT conceived the study and secured funding. MP, RL, and LA wrote the manuscript. All authors provided critical reviews and approved the submitted version.

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CASE REPORT
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Case Report: A Case Series of Immunobiological Therapy (Anti-TNF-α) for Patients With Erythema Nodosum Leprosum

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Patients with leprosy may experience a chronic and severe type II leprosy reaction (ENL) erythema nodosum leprosum that may not respond to thalidomide and systemic immunosuppressants or may even cause serious adverse events. We here present four patients in whom anti-TNF-α therapy was used with successful results and compare our findings with other published cases. Four patients with chronic and severe ENL who did not respond to, at least, thalidomide and steroids (high doses) were followed up at two reference centers in Brazil. A thorough laboratory investigation was performed to exclude tuberculosis and other diseases before the start of immunobiological medication. Three patients were started on etanercept, and one patient was started on adalimumab. Of all patients, three developed severe adverse events resulting from the use of classical immunosuppressants for ENL (cataracts, deep vein thrombosis, diabetes, and osteoporosis). In all cases, a reduction in the number of ENL and, at least half of the immunosuppressant dose between 6 months and 2 years, were observed. Long-term follow-up of one patient revealed a dramatic reduction in hospital admissions due to ENL, from 12 instances in 1 year (before biologic therapy) to none (after biologic therapy), along with an improvement in condyloma acuminatum. In addition, no direct adverse events were observed with biologics. Treatment with anti-TNF-α therapy may be used as an alternative in patients with chronic and severe ENL who do not respond to traditional treatment (e.g., thalidomide, steroids, and other immunosuppressants). This treatment can help reduce the frequency of ENL, the immunosuppressive burden, and the number of hospital admissions.

Keywords: leprosy, dermatology, immunosuppression therapy, autoimmunity, leprosy reaction

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INTRODUCTION

Leprosy is a disabling disorder that mainly affects the skin and the peripheral nervous system. This disorder may be complicated by immune-mediated inflammatory reactions, such as reversal reaction (type I reaction) and erythema nodosum leprosum (ENL, type II reaction), as well as by peripheral nerve damage (1, 2).

Erythema nodosum leprosum is a clinical feature of multiple systemic symptoms, such as fever, crops of tender erythematous nodules, neuritis, arthritis, orchitis, lymphadenitis, and iritis (1, 2). It is considered an emergency event (3), which may affect 5–10% of patients with borderline leprosy and up to 50% of those with lepromatous leprosy (LL) (4). Given its magnitude and social significance, including the possibility of social stigma and sequelae for the patients and their families, leprosy is still one of the most serious public health problems in Brazil (5).

Among the most common treatments prescribed for ENL are thalidomide, prednisone/prednisolone (systemic steroids), and clofazimine (3). However, using high doses of steroids for prolonged periods of time may lead to well-known side effects, such as diabetes, cataracts, osteoporosis, and obesity (2, 6). Similarly, thalidomide has highly teratogenic effects and must be used with extreme caution, especially for women at childbearing age (2, 3). Both thalidomide and systemic steroids are associated with a risk for deep vein thrombosis and pulmonary embolism, and they even have a synergic risk effect if used in combination (3). Clofazimine is associated with gastrointestinal effects and skin pigmentation and may pose a cosmetic issue (7). Furthermore, systemic immunosuppressants, such as methotrexate, azathioprine, and cyclophosphamide, may increase the risk of infection and hepatic and renal damage (2, 8).

Although thalidomide is the first-line treatment for ENL in many countries, combining it with at least one additional agent, such as pentoxifylline or an immunosuppressant (3, 8), may be necessary for refractory cases (9). Multibacillary patients may experience ENL for many months (3, 8). Therefore, not only can ENL expose its patients to different sequelae and neurological impairments, but also the therapies targeting it may pose a high risk of side effects (7).

Several alternative therapies have been proposed for ENL, such as vaccination with *Mycobacterium indicus pranii* (10), apremilast (an oral phosphodiesterase IV inhibitor), cyclosporine, and tenidap (an anti-inflammatory drug) (7), as well as anti-TNF-α therapy (1, 2, 6, 11, 12). We here present four patients with severe, recalcitrant, and chronic ENL who did not respond to multiple therapeutic regimens and were treated with anti-TNF-α therapy. We compared these cases to other similar cases and discussed the rationale behind the use of anti-TNF-α therapy.

CASE DESCRIPTION

All four patients presented with anesthetic skin patches and/or thickened nerves and acid-fast bacilli on slit skin smears and/or a histopathology compatible with leprosy or ENL (9). Leprosy was classified according to the Ridley–Jopling system using clinical, histological, and bacteriological indices (13). A case definition of ENL was considered if a patient presented with crops of tender cutaneous or subcutaneous erythematous nodules, with or without systemic symptoms, or a suggestive histopathology (9).

The nature of ENL was defined as acute for a single episode lasting less than 24 weeks, recurrent for a patient experiencing a second episode of ENL 28 days or more after stopping the treatment for ENL, and chronic if over 24 weeks or more a patient required ENL treatment either continuously or 27 days or less after the last treatment period (14). In terms of severity, ENL can be classified according to the clinical manifestations as mild if the patient presented with less than 10 nodules per affected body, moderate if the patient presented with 10–20 nodules, and severe if the patient presented with more than 20 nodules (15). This study was approved by the institutional review boards of the original centers (CAAE: 93279318.9.0000.5558), and written consent was obtained from each individual.

Table 1 shows the clinical data of the four patients, along with another five similar published cases. As can be observed, the ratio between the males and females was equal (1:1), with a median age of 44.2 [range: 29–59] years for patients with ENL. The patients were originally from two hospitals at the center of Brazil: Hospital de Doenças Tropicais (Goiás) and Hospital Universitário de Brasília (Distrito Federal). All four patients were diagnosed with LL, according to the Ridley–Jopling classification (13). At the time of diagnosis, they presented with a bacteriological index (BI) of five or more on at least four body sites, and their ENL began when they were started on multidrug therapy (MDT). One of the patients was treated twice with MDT. ENL was classified as severe and chronic with common morphological features compatible with multiple (>50) tender, erythematous papules and nodules, followed by systemic symptoms, such as fever, malaise, arthralgia, joint swelling, and peripheral edema with neurological features of neuritis. No reversal reaction was observed in any of the patients. Thalidomide and high doses of systemic steroids were used in all patients. Methotrexate was used in two patients (Patients 1 and 3). However, in Patient 1, the fourth drug was pentoxifylline. These drugs were used for at least 1 year. Patient 1 was started on high doses of thalidomide, systemic steroids, methotrexate, and pentoxifylline for 4 years. Three patients developed severe adverse events because of the ENL treatment (Patients 1, 2, and 4), including cataracts, deep vein thrombosis (Patient 4), diabetes, obesity, and osteoporosis.

Before anti-TNF-α therapy was started, a complete investigation was performed to rule out latent foci of tuberculosis (Mantoux test and chest X-ray), hepatitis B and C, and HIV. Etanercept was the preferred anti-TNF-α therapy in three patients, with one patient undergoing therapy using adalimumab. Improvements were observed as early as 3 days (**Figure 1**) to 1 month, and the duration of anti-TNF-α therapy varied from 6 months to 2 years.

The outcomes considered to judge an improvement as a result of anti-TNF-α therapy were as follows: a reduction in the number of ENL cases, a reduction in the rate of hospitalization, and a reduction in the dosage of common treatments for ENL (thalidomide, steroids, methotrexate, pentoxifylline, or clofazimine). All patients exhibited a reduction in ENL, which particularly manifested as a reduction in the number of ENL, in weeks, in Patient 1 (**Figure 1**). Interestingly, in the year before the anti-TNF-α therapy, Patient 1 was hospitalized 12 times for ENL. However, in the year after therapy, she was hospitalized only once for neuritis. She presented with genital condyloma acuminatum, which was resistant to different treatment modalities. However, after methotrexate was stopped

TABLE 1 | Demographic and clinical data of leprosy patients with severe, chronic and refractory ENL ($n = 4$ patients), and cases already published ($n = 5$ patients) (2006–2021).

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5 Faber et al. (2006)**	**Patient 6 Ramien et al. 2011**	**Patient 7 Chowdhry et al. 2016**	**Patient 8 Santos et al. 2017**	**Patient 9 Cogen et al. (2020)**
Sex	F	M	F	M	F	F	M	M	M
Age in years (at diagnosis)	29	32	57	59	52	33	49	40	28
Country	Brazil	Brazil	Brazil	Brazil	Argentina	Philipines	India	Brazil	Samoa
Leprosy classification	LL	LL	LL	LL	BL	LL	LL	LL	LL
BI at leprosy diagnosis	5	5.25	6	5.5	5	2–4 (at 6 sites)	6	NI	5
Temporal relationship of reactional status and MDT	6 months after MDT	During MDT treatment	During MDT treatment	During MDT treatment	1.5 years after MDT	Mild ENL was present prior to MDT	2 months after MDT	Since the diagnosis of leprosy	During MDT treatment
Number of MDT treatment	2	1	1	1	1	2-year treatment	NI	1	MDT and change after to clofazimine + levofloxacin + clarithromycin
ENL type	Chronic	Chronic	Chronic	Chronic	Chronic	Chronic	Chronic	Chronic	Chronic
ENL severity	Severe	Severe	Severe	Severe	NI	NI	Severe	NI	NI
Morphologic features of ENL	Erythematous nodules, necrotic, and ulcerated lesions	Erythematous nodules, necrotic, and ulcerated lesions	Painful erythematous nodules and plaques	Painful erythematous nodules and plaques	Painful erythematous nodules and plaques	Erythematous papules and nodules	Erythematous tender papules, nodules and necrotic ulcers	Nodules	Painful and ulcerated cutaneous nodules
Symptoms related to ENL	Fever, malaise, arthalgia, joint swelling, peripheral oedema	Fever, malaise, arthalgia, joint swelling, peripheral oedema	Fever, malaise, arthalgia, joint swelling, peripheral oedema	Fever, malaise, arthalgia, joint swelling, peripheral oedema	Tender axillary lymph node	Fever, malaise	High grade fever, myalgia, lymphadenopathy and epididymo-orchitis	NI	Polyarthitis, acral oedema, and uveitis
Neurologic impairment	Neuritis, neuropathy	Diffuse neuritis	Diffuse neuritis	Diffuse neuritis	Thickened and tender ulnar nerve	Neuropathy	Severe neuritis	NI	Peripheral neuritis
Reversal reaction	No	No	No	No	NI	NI	NI	NI	NI
Skin biopsy	No	Consistent with ENL	Consistent with ENL	Consistent with ENL	NI	NI	Consistent with ENL	NI	NI
Number of ENL at the time of anti-TNF	50	>50	>50	>50	NI	NI	NI	NI	NI
Therapy for ENL prior to anti-TNF-α therapy	Thalidomide 400 mg/day, prednisone 1.5 mg/kg/day, methotrexate 15 mg/week, pentoxifylline 1,200 mg/day	Thalidomide 200 mg/day, prednisone 1 mg/kg/day	Thalidomide 400 mg/day, prednisone 1 mg/kg/day, methotrexate 20 mg/week	Thalidomide 200 mg/day, prednisone 1 mg/kg/day	Thalidomide 300 mg/day, prednisolone 40 mg/day, pentoxifyline 1,200 mg/day	Thalidomide 100 mg/day, prednisone 80 mg/day, clofazimine	Thalidomide 300 mg/day, prednisolone up to 80 mg/day, clofazimine 300 mg/day, clarithromycin 1 g/day, ofloxacin 400 mg/day, pentoxifylline 1,200 mg/day, azathioprine 300 mg/day	Thalidomide 300 mg/day and Prednisone up to 40 mg/day	Thalidomide 300 mg/day, prednisone up to 120 mg/day, clofazimine 150 mg/day, cyclophosphamide 200 mg/day, prednisolone eye drops

(Continued)

TABLE 1 | Continued

Time between using ENL treatment and anti- TNF-α therapy	4 years	1 year	2 years	1 year	NI	6 years	6 months	4 years	NI
Adverse events related to classical ENL treatment	Osteoporosis, cataracts, giant condyloma acuminatum, insulin resistance, 12 hospitalizations	Obesity, diabetes	None	Cataracts, arterial thrombosis	NI	Cushingoid features, hypertension, osteoporosis, toe fractures, recurrent soft-tissue infections, deep-vein trhombosis	Oral candidiasis, abdominal pain, diarrhea, worsening of his diabetes, hypertriglyceridemia and aggravation of peripheral neuropathy with thalidomide	NI	3 hospitalizations
Anti-TNF-α therapy	Etanercept 50 mg/week SC	Adalimumab 40 mg/biweekly SC	Etanercept 50 mg/week SC	Etanercept 50 mg/week SC	Infliximab 5 mg/kg	Etanercept 50 mg/week	Etanercept 50 mg/week for 16 weeks and then biweekly for 16 weeks	Etanercept 50 mg/week	Infliximab 5 mg/kg in 7 infusions —weeks 0, 2, 7, 16, 78, and 88, 99
Time for symptoms to subside after anti-TNF- α therapy	3 days	1 week	1 month	1 month	Hours	6 weeks	48 h in the first dose	48 h experiencing recurrence after 7 days	Immediate
Duration of anti-TNF-α therapy	1.5 years	6 months	1 year	2 years	Infusion in weeks 0.2 and 6	2 years and remain asymptomatic 2.5 years after anti-TNF-α stop	32 weeks	11 months	7 infusions when flares
ENL treatment after anti-TNF -α therapy	Thalidomide 100 mg/day and prednisone 0.5 mg/kg/day (1 year after anti-TNF therapy)	Thalidomide 100 mg/day and prednisone 0.5 mg/kg/day	Thalidomide 100 mg/day and prednisone 20 mg/day	Prednisone 0.5 mg/kg/day	No treatment after 1 year	After year, just thalidomide 100 mg/day	Prednisolone discontinuation after 12 weeks	Prednisone 10 mg/week	Discontinuation of prednisone after 4 years
Adverse events related to anti-TNF-α therapy	No	Sepsis (during the concomitant use of adalimumab and prednisone 1 mg/Kg/day)	No	No	NI	NI	NI	NI	7th infusion: hypotension, oedema, and throat tightening

F, female; M, male; LL, lepromatous leprosy; BI, baciloscopic index; MDT, multidrug therapy; ENL, erythema nodosum leprosum; SC, subcutaneous; TNF, tumoral necrosis factor; NI, not informed.

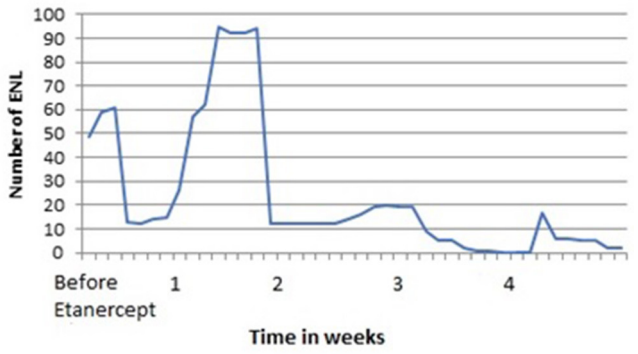

FIGURE 1 | Behavior of the reduction of ENL after etanercept therapy in Patient 1.

and the dose of systemic steroids was decreased, she improved without a specific treatment for human papillomavirus. Overall, a reduction of at least half in thalidomide and systemic steroids was observed in all four patients on anti-TNF-α therapy over a duration of 6 months−2 years.

DISCUSSION

Leprosy is a severe, neglected chronic disease. Besides the physical disabilities resulting from the neurological sequelae of this disease, type II reactional states are considered a public health problem in Brazil (12).

Our cases did not demonstrate a gender-related difference for ENL. In fact, several studies have highlighted that age and gender are not risk factors for ENL (6). Rather, the risk factors for developing ENL include a LL type and a high bacillary index. The relative risk of developing ENL is 3.6 with an LL spectrum and 8.6 with a bacillary index of 6, which is the case for patients with refractory, chronic, and severe ENL (16). Although the onset of ENL has been found to be highest during the first year of MDT (17), some studies claim a higher incidence in the second and third years after MDT is started (7). In three patients, MDT was changed (Patient 9) or repeated (Patients 1 and 6). Although the World Health Organization has estimated the rate of relapse after MDT as 0.77% for multibacillary patients 9 years after treatment (18), ENL may still pose a significant challenge to differentiate from a recurrence of leprosy, especially after many years of MDT (3).

Neurological impairments are common in patients with ENL (8, 19). Recently, Andrade et al. (19) described a link between demyelination and acute neuritis during leprosy. However, it remains unknown whether demyelination is a consequence of the inflammatory process of neuritis or whether it is directly induced by *Mycobacterium leprae*. Persistent demyelination is associated with axonal damage, which progressively compromises large fibers, leading to motor impairment. Damage to the myelin sheath may be a consequence of the inflammatory process resulting either from humoral immunity or from the release of immune mediators. TNF acts on Schwann cells (SCs) by stimulating the production of IL-6 and IL-8, contributing both directly and indirectly to neuroinflammation (20). Moreover, *M. leprae* stimulate the secretion of IL-23 in SCs, a cytokine that is believed to be involved in demyelinating processes. Thus, focal demyelination may potentially become a prime target for therapeutic interventions aimed at improving nerve function during leprosy (19). One of the contraindications for the use of anti-TNF-α therapy is demyelinating disorders (21). Since demyelination is regarded as a new discovery in leprosy (19), carefully evaluating patients who undergo this treatment to control their ENL is important. Importantly, none of our cases showed any neurological impairments caused by anti-TNF-α therapy.

We compared all cases with already published ones and found that most of the patients who were started on anti-TNF-α therapy were using at least two medications at high doses for 6 months−6 years (**Table 1**). Although thalidomide is regarded as the drug of choice for treating ENL, using it at high doses for prolonged periods of time may cause nerve injury and be a confounding factor with the neurological effects of the disease itself (1). Prednisone is added to the treatment regimen when the reactions are difficult to control or in the presence of complications, especially neuritis (22). The data outlined in **Table 1** show that prolonged classical treatment is associated with increased side effects (Patients 1, 7, and 8) (1, 12). In addition, the synergic association between systemic steroids and high doses of thalidomide is considered a risk factor for deep vein thrombosis (Patients 4 and 6) and pulmonary thromboembolism (6). Hence, precociously using anti-TNF-α therapy may prevent prolonged exposure to immunosuppressant agents and their side effects (2).

Etanercept was the most used drug for ENL, followed by infliximab and adalimumab. Infliximab is a human-murine chimeric monoclonal antibody against TNF-α, and etanercept is a dimeric fusion protein of the extracellular portion of the p75 TNF receptor coupled to IgG1. Both agents can effectively reduce the levels of TNF-α (7). Although the underlying immunological mechanism of ENL remains unclear, it is important to highlight the role of cytokines. High levels of TNF-α and IL-6 are consistently found in patients with severe disease (23). Moreover, the overexpression of TNF-α and the fact that thalidomide is an important anti-TNF-α agent allowed Farber et al. (11) to treat a patient with an uncontrolled type II reaction using classic systemic therapy with infliximab. After this study, four more cases were described, but only one patient continued to use infliximab (2). Etanercept was chosen instead of infliximab because of the increased risk of reactivation of latent tuberculosis and granulomatous diseases (24). The disadvantage of infliximab is its mode of administration (endovenous), which requires a hospital setting and may lead to adverse events, such as hypotension, edema, and throat tightness (2). Only one study has focused on adalimumab in the context of the reversal reaction, but not in that of the type II reaction. Compared to the administration of prednisolone for 20 weeks, the efficacies of both drugs against skin lesions in the context of the reversal reaction were similar. Interestingly, adalimumab is superior to prednisolone in terms of improving the nerve function and sensory and motor loss (25).

Unlike psoriasis, which is also treated using anti-TNF-α therapy, reactive leprosy episodes seem to exhibit a rapid response. The improvement observed in ENL seems to be immediate or within hours with infliximab (Patients 5 and 9) (2, 11), whereas etanercept may take days to a month (Patients 1, 2, 3, 4, 6, 7, and 8) (1, 6, 12). However, since only nine patients underwent anti-TNF-α therapy for ENL, we cannot guarantee the superiority of a specific anti-TNF-α agent. Nevertheless, in psoriasis, another skin disease treated by this class of drugs, infliximab (26) and adalimumab (27) have a faster response and are more efficacious than etanercept, in achieving a clear or almost clear stage.

The dosage of anti-TNF-α therapy used for treating ENL is considered another challenge. Should biologics be administered only during flares or perhaps as a prophylactic therapy? In Patients 5, 7, and 9, the drug was administered at a custom dose, preferably during flares. Since ENL flares decrease over time, specific drugs for ENL treatment are expected to be tapered down (7). However, as systemic steroids are the most detrimental drugs for such patients, it is advisable to stop such drugs in advance and to continue anti-TNF-α therapy along with thalidomide. It is also worth noting that the gaps between the doses of anti-TNF-α therapy may lead to the formation of neutralizing antibodies and, hence, the loss of efficacy (28). Therefore, maintaining a uniform and specific dose is considered a favorable choice.

Overall, no side effects with anti-TNF-α therapy were observed, except in Patient 2, who had sepsis but has also been on systemic steroids for a year. During the early 2000s, when anti-TNF-α therapy was released, the side effects of this type of therapy were strongly debated. In addition, the well-established connection between the inhibition of TNF-α and reactivation of tuberculosis may similarly hold true for leprosy. The rapidity with which patients develop signs of leprosy after receiving the biological agent suggests possible recrudescence of latent leprosy or initial misdiagnosis. Therefore, using anti-TNF-α therapy for patients with leprosy before complete treatment with MDT may reduce the efficacy of antimicrobial agents or promote infection by *M. leprae*. Although anti-TNF-α therapy is contraindicated in the case of lupus and cardiac congestive failure (stages III–IV) (29), given their target specificity, anti-TNF agents are considered safer than a wide immunological blockade provided by steroids and other immunosuppressants (30, 31).

The indications of anti-TNF-α agents are increasing in many diseases (21). Despite their high cost, more affordable biosimilars are reaching the market (2). It is also worth highlighting that the indication of biologic therapy for ENL may be limited because ENL occurs in ∼50% of LL cases (2). Moreover, flares are auto-limited, especially within the first 3 years after MDT, and only 26.8% of patients with ENL require at least one additional agent (16).

Our study has some limitations. For example, it lacks prospective controls (as in all case reports), it lacks laboratory tests aimed at verifying the behavior of anti-inflammatory markers, and it lacks more well-defined and uniform outcome measures for ENL. Hence, some questions remain unanswered: Who is the best patient with ENL for biologic therapy? Is it safe to use biologic therapy along with MDT or is it necessary to wait until treatment is completed? For how long should systemic agents be used until classical treatment of ENL is considered a failure? What is the best anti-TNF-α candidate to treat ENL? What is the best strategy, during flares or as a prophylactic? How does anti-TNF-α therapy affect nerve damage?

In our series, patients who presented with recalcitrant, chronic, and severe ENL were classified as LL patients with a BI higher than five, who had their first episode of ENL within the first year of MDT along with neurological impairments. All patients who received biologic therapy did not respond to high-dose systemic steroids or thalidomide. Anti-TNF-α therapy demonstrates rapid improvements and allows the reduction of ENL, the frequency of hospitalizations, and the doses used of classical drugs. Clinical trials on this population are, therefore, important to determine the role of biologicals in leprosy and neurological impairments and their reactive states.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comite de Etica do Hospital de Doenças Tropicais - HDT GO and by the Comitê de Ética da Faculdade de Medicina da Universidade de Brasília. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

AM: formal analysis, investigation, and resources. CG, PK, and MI: supervision, validation, visualization, writing—original draft, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Downmodulation of Regulatory T Cells Producing TGF-β Participates in Pathogenesis of Leprosy Reactions

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Leprosy reactions are an acute and systemic manifestation, which occurs suddenly, can be severe and lead leprosy patients to disability. Reactional episodes are observed among half of the multibacillary patients, mainly in borderline lepromatous and lepromatous forms. They may begin at any time during multidrug therapy, and even before the treatment. Physical disabilities, which are the source of extreme suffering and pain for patients, occur in progression of the cellular immune response associated with a reaction and are still poorly understood. Thus, this work aimed to phenotypically and functionally characterize $CD4^+$ and $CD8^+$ Treg cells *ex vivo* and in response to *Mycobacterium leprae* (ML). We studied 52 individuals, including 18 newly diagnosed and untreated multibacillary leprosy patients, 19 reactional multibacillary patients (Type I or Type II episodes) and 15 healthy volunteers, included as controls, all residents of the city of Rio de Janeiro. The functional activity and frequencies of these cells were evaluated through multiparametric flow cytometry. In addition, the production of cytokines in supernatant from peripheral blood mononuclear cell cultures was also investigated against ML by enzyme-linked immunosorbent assay. Our results showed a decrease in $CD4^+TGF\text{-}\beta^+$ Treg and $CD8^+$ $TGF\text{-}\beta^+$ Treg in leprosy multibacillary patients during both types of reactional episodes. Alterations in the cytokine profile was also observed in Type II reactions, along with upregulation of IL-17 and IL-6 in supernatant. Thus, our study suggests that downregulation of Treg cells is related with both classes of reactional episodes, improving our understanding of immune hyporesponsiveness in multibacillary patients and hyperresponsiveness in both reactions.

Keywords: leprosy, reactions, T lymphocytes, Treg, cytokines

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INTRODUCTION

Leprosy is a chronic systemic infectious disease that affected 202,256 new individuals worldwide in 2019 (1). Brazil occupies the second place in newly diagnosed cases, totaling 27,863 patients (1). The etiological agent, *Mycobacterium leprae*, mainly infects macrophages, Schwann cells and the vascular endothelium (2). The disease causes lesions in skin and peripheral nerves, promoting loss of sensitivity, deformities in the limbs/face and even blindness (3). Leprosy presentation differs according to the host immune response to the pathogen, which leads to a clinical spectrum of polar

clinical forms, which varies from the tuberculoid (TT) to lepromatous (LL) pole, including the intermediate forms called borderline (BT, BB and BL) and the indeterminate form (I) (4, 5).

Leprosy reactions are the main cause of morbidity and permanent damage to peripheral nerves. About 50% of leprosy patients develop this clinical condition, which is an acute inflammatory response manifested with local and systemic involvement (6, 7). These episodes can manifest at any time throughout the course of the disease, may affect patients of all clinical forms (6), and are classified into Type 1 (T1R) and Type 2 (T2R) reactions (8). It is not yet clear whether changes in immune response patterns are associated with the pathogenesis of reactions.

Borderline patients are the most affected by T1R, which develop gradually and may have a natural course of several weeks. Clinically, in this reaction, there is an increase in the inflammatory process in existing lesions, as well as the appearance of new skin lesions and painful neuritis (9). The T2R consists of a systemic episode of acute inflammation that occurs primarily in LL and in BL patients. This reaction could occur before, during and after multidrug therapy (MDT). In these episodes, patients develop an acute systemic inflammatory response, have deep and painful subcutaneous nodules, often present high fever, oedema and cyanosis of the extremities, weight loss, general malaise, and damage to nerves, skin, eyes and testicles (3, 10). Several studies aim to investigate immunological mechanisms of susceptibility and identify biomarkers, which can explain, predict and control T1R and T2R reactions (11–15). Activation of T helper 1 (Th1) cells and production of proinflammatory cytokines, such as IFN-γ and TNF, are described by several studies to explain the pathogenesis of T1R and T2R (16–18). Although multibacillary patients are hyporesponsive to *M. leprae,* during onset of reactions they develop a sudden exacerbated immune response against the pathogen (15). One of the most accepted hypotheses regarding the triggering of reactions is a change in the regulatory T cell profile (19).

Regulatory T lymphocytes (Treg) present the CD25$^+$FOXP3$^+$ profile (20), a cell subset which is important for immune system effector suppression mechanisms and is fundamental in the control of autoimmunity in peripheral organs (22). It has been shown that some microorganisms evade elimination promoted by the immune response through the modulation of regulatory T cell mechanisms (23). Treg can suppress Th1 and Th17 cells through TGF-β and IL-10 production and constitutive molecules, such as CTLA-4 (21, 24).

However, the exact role of Treg during reaction episodes is poorly studied and still very controversial. Azevedo and coworkers observed reduced Treg markers in skin lesion samples of T1R and T2R. This data demonstrates that an abrupt reduction in the frequency of Treg cells can contribute to the trigger of exacerbated inflammatory responses during leprosy reactions (25). Similar results have also been described in other diseases, such as atopic dermatitis (26), active tuberculosis (27), and sepsis (28).

Palermo and coworkers were pioneers in the study of Treg in leprosy. They suggested that these cells play a central role in the *M. leprae*-host interaction. The authors demonstrated a decreased frequency of CD4$^+$ Treg in peripheral blood and cutaneous lesions of lepromatous in comparison to tuberculoid patients (29). Others also revelated a higher CD4$^+$ Treg in LL patients in comparison to BT/TT (30, 31). Analyzing, CD3$^+$CD4$^+$ T cells in blood, Saini and collaborators saw lower mean fluorescence intensity (MFI) of FOXP3 and TGF-β cells in T1R and T2R compared to BT and LL patients, respectively (32). In addition, depletion of CD4$^+$ Treg participates in T2R pathogenesis and prednisolone treatment upregulates this cell profile in blood leucocytes (33).

Most studies on Treg in leprosy reactions focus on the CD4$^+$ subset, and information concerning CD8$^+$ cells still very scarce. Previous studies in leprosy, related a higher frequency of CD8$^+$ Treg in blood of LL/BL patients in comparison to BT/TT (19, 30). Negera et al. (33) did not observe a difference in CD8$^+$ Treg frequency between LL patients with or without T2R, and the same cell profile appears to be depleted during prednisolone treatment but did not increase after treatment (33).

We hypothesized that at some point in the clinical course of leprosy *per se*, Treg downregulation occurs, and this change may trigger reactions in the host. Therefore, the aim of our study was to investigate the participation of CD4$^+$ and CD8$^+$ Treg in the genesis of both Type 1 and Type 2 reactions through peripheral blood mononuclear cells (PBMC) by flow cytometry. In this context, our results show the involvement of TGF-β producing CD4$^+$ and CD8$^+$ Treg in maintenance of the hyporesponsive response in multibacillary leprosy patients favoring *Mycobacterium leprae* survival.

MATERIALS AND METHODS

Ethical Considerations

The study was approved by the Institutional Ethics Committee of the Oswaldo Cruz Foundation/FIOCRUZ (protocol number 518/09). All participants were informed, and written consent was obtained prior to specimen collection. For the sake of privacy and wellbeing of the studied individuals, we refrained from disclosing their identity.

Studied Population

Thirty-seven leprosy patients were selected from both sexes, defined according to clinical profile, and distributed into newly diagnosed and untreated lepromatous (LL) and borderline lepromatous (BL) patients, while T1R and T2R patient samples were collected after the diagnosis of any reactional episode and before specific treatment for the reactions. All individuals were followed up at the Leprosy Unit/Fiocruz. The diagnosis of disease and reactional episodes was based on clinical signs and symptoms presented by the patients, confirmed by the histopathological analysis of skin lesion fragments, according to Ridley and Jopling criteria (4) and the treatment of all patients followed the rules defined by the WHO (34). During the study, any multibacillary patients developed reactional episodes.

Furthermore, 15 healthy donors were included in the study. All individuals lived in Brazil, a leprosy endemic area. Patients with a history of BCG vaccination within the last 3 months,

patients under 15, co-infections (HIV, TB, Hepatitis B and C), autoimmune or allergic diseases, diabetes, hypertension and pregnancy were excluded from the study.

Isolation of Peripheral Blood Mononuclear Cells (PBMC) and *in vitro* Assay

About 40 mL of blood were collected under endotoxin-free conditions from heparinized venous blood of all individuals, and PBMC was isolated by Ficoll-Hypaque (GE Healthcare AB, Uppsala, Sweden) centrifugation. After three washes with sterile phosphate-buffered saline (PBS; Gibco Invitrogen Co., USA) the pellet of cells was resuspended in 5 mL AIM-V medium (Thermo Fisher Scientific, USA). Cell viability was determined by 0.4% sterile Trypan Blue solution (Sigma Aldrich, MO, USA) and ranged from 94 to 98%. PBMCs were adjusted to 1×10^6 (for cytometric analysis) and transferred to U-bottomed 96-well polyethylene plates (Nunc, USA) with 200 μL per well for *ex vivo* and *in vitro* assay evaluation. For *in vitro* assay, cells were added into wells with specific antigen or mitogen, namely: *M leprae* antigen (20 μg/mL) irradiated and sonicated, from armadillo (provided by NIH/ NIAID N01 Al-25469 contract with Colorado State University, CO, USA) and as mitogen Phytohemagglutinin (Sigma, USA-1 μg/mL). In all wells, including unstimulated cultures, 1μg/mL anti-CD28 and anti-CD49d was added (Biolegend, USA). PBMC were maintained for 72 h in AIM-V medium at 37°C with 5% CO_2. After 68 h of culture, intracellular protein transport blocker (BD GolgiPlugTM, USA−10 μg/mL) was added in order to evaluate the production of cytokines and/or molecules inside the cells. Cells were then stained with specific monoclonal antibodies and analyzed by multiparametric flow cytometry. Response kinetics to *M. leprae* and PHA were previously performed in healthy volunteers (HVs), reaching a peak in cultures at 72 h.

Flow Cytometry Analysis of Cell Surface and Intracellular Molecules

Following incubation, cells were centrifuged for 5 min at 1,500 rpm at 4°C, at which time supernatants were collected and stored in a −80°C freezer. PBMC were washed twice with PBS (Gibco Invitrogen Co., USA) at 1,500 rpm for 5 min at 4°C. Cells were resuspended in PBS containing 0.02% ethylenediaminetetraacetic acid (EDTA; Sigma), and incubated for 15 min at room temperature, followed by a further wash (1,500 rpm for 5 min at 4°C). PBMCs were stained at a 1:5 ratio with the Live/Dead Kit (Invitrogen, USA) according to manufacturer's instructions. Human TruStain FcXTM—Fc Receptor Blocking Solution (5 μL, Biolegend, USA) was add in 100 μL of PBS/FACS and cells were incubated for 10 min at 4°C. After this step, PBMCs were labeled with different specific monoclonal antibodies (anti-CD4 FITC, anti-CD8 Pe-Cy7, anti-CD25 Alexa 700—Biolegend, USA) and incubated with appropriate isotype controls, used to define the phenotypic subset profiles, for 30 min at 4°C (Biolegend, USA). PBMCs were then resuspended in 1% paraformaldehyde (PA; Sigma) and incubated for 30 min at 4°C. After this step, cells were resuspended in 1% Foxp3 Fix/Perm (Biolegend, USA) and incubated for 20 min at 4°C. PBMCs were subjected to two washes, the first with PBS/FACS and the second with Foxp3 Perm Buffer (1%, Biolegend, USA) and centrifuged at 1,500 rpm for 5 min at 4°C each. Perm Buffer solution (150 μL) was added to PBMCs, which were incubated at room temperature for 15 min and protected from light. Subsequently, PBMCs were stained with monoclonal antibodies for intracellular proteins (anti-FOXP3 Pe, anti-IL-10 APC and anti-TGF-β PerCp—Biolegend, USA) and their respective isotype controls (Biolegend, USA) for 30 min at 4°C. Paraformaldehyde 1% (PA; Sigma) was added and cells were evaluated on the CytoFLEX flow cytometer (Beckamn Coulter, USA). A total of 100,000 events in the strategic lymphocyte region were observed, followed by analysis in FlowJo version 3.0 software (Tree Star Inc., USA) (**Figures 1A–E,G,H**).

Cytokine Evaluation by Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed according to the manufacturer's instructions for each kit. Cytokine profile was measured in duplicate using culture supernatants for analysis of IFN-γ, IL-17, IL-23, IL-6, and IL-10 (eBioscience, USA). Supernatants were incubated in 96-well plates (Nunc, Rochester, NY, USA) for 12 h at 4°C with diluted capture antibody for each cytokine. After the sensibilization step, supernatants were washed 3 times with PBS 0.05% Tween 20 after which blocking solution was added to samples that were incubated for 1 h at room temperature. Subsequently, diluted supernatant/standard were added, and plates were incubated overnight at 4°C. Supernatants were washed (three times), diluted detection antibody was added and maintained for 1 h. Avidin-HRP complex (ThermoFisher Scientific, Massachusetts, EUA) was incubated for 30 min at room temperature in the dark and the supernatants were washed (four times). After adding TMB solution (ThermoFisher Scientific, Massachusetts, EUA), the plate was incubated for ∼15 min and stop solution was added (2N H_2SO_4). Test was performed using SpectraMax 190 (Molecular Devices—USA/Canada) at 450 nm using SoftMax Version 5.3 software (Molecular Devices-US/Canada).

Statistical Analysis

Before assays, data was tested for normality (Shapiro-Wilk normality test), and non-normally distributed results were analyzed by non-parametric tests. Results were expressed as mean and standard deviation. Significant differences between groups were determined by the non-parametric One Way Anova and Mann Whitney test. GraphPrism version 6.0 (Graph Prism, USA) was used for statistical and graphical analysis. The values with $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$ were considered significant.

RESULTS

Clinical and Demographic Characteristics of Volunteers

The participants evaluated in this study were defined according to the clinical presentation of leprosy and monitored at the Leprosy Unit (LAHAN-IOC-Fiocruz). The collection of biological

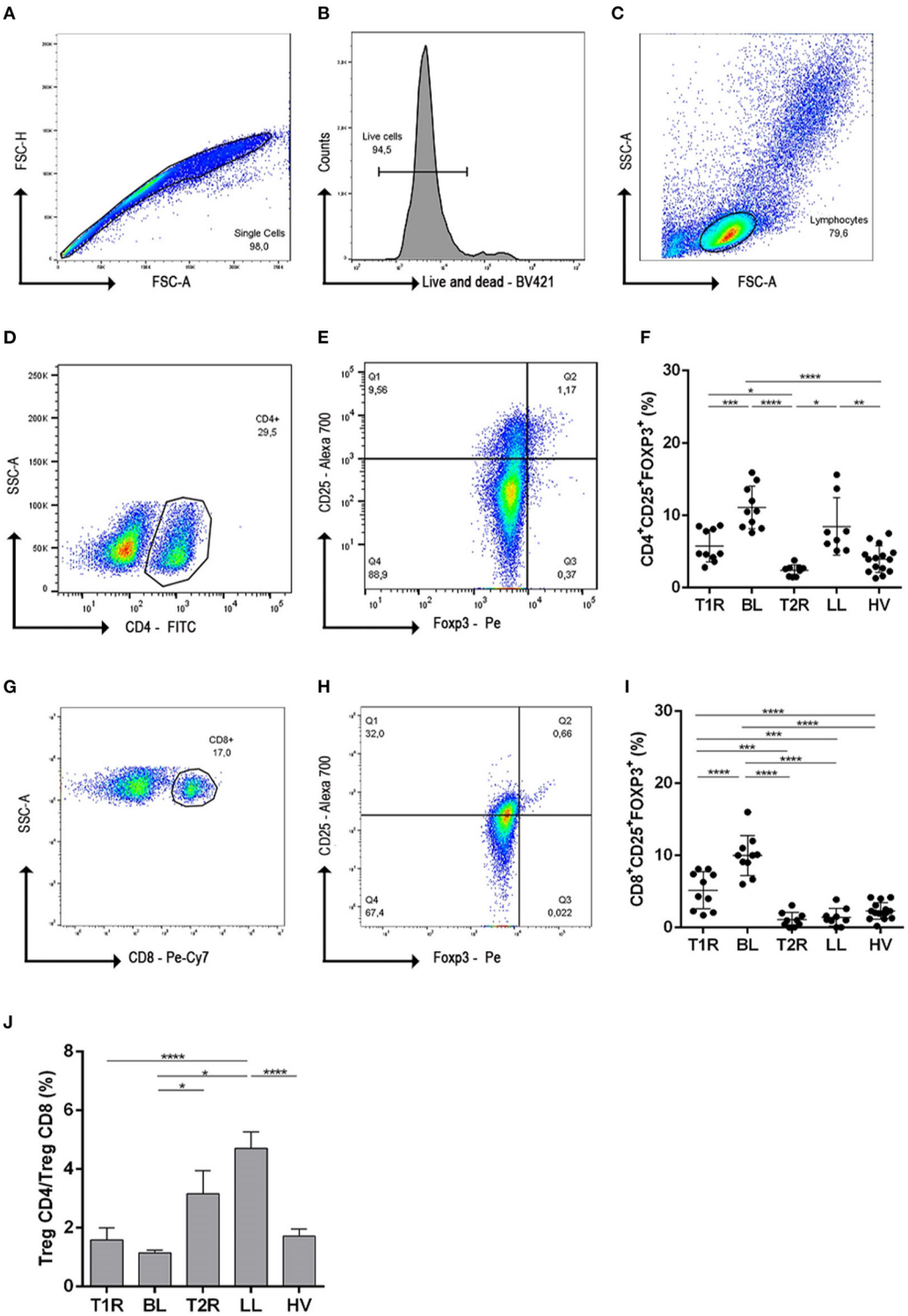

FIGURE 1 | Gating strategies and frequency evaluation of regulatory T cells subsets. To exclude cell doublets and debris from data, cells were gated on singlet regions through dot plot FSC-H vs FSC-A **(A)**; Next analysis was initiated from singlets and gated in live and dead histogram, for viability evaluation **(B)**; lymphocyte population were determined from viable cells in SSC-A vs FSC-A dot-plot **(C)**; the dot-plot from lymphocyte cells was used for CD4^{+} T cell determination **(D)**; CD25 vs Foxp3 gated on CD4^{+} was used to define CD4^{+} T reg cells **(E)**. Graphical representation of CD4+CD25+Foxp3+ frequencies in different clinical forms of leprosy **(F)**. From lymphocyte dot-plot, CD8^{+} cells were gated **(G)** and CD25 vs Foxp3 were defined **(H)** graphical representing of CD8+CD25+Foxp3+ cells **(I)**; Ratio determination of CD4+CD25+Foxp3/CD8+CD25+Foxp3+ frequencies in PBMC **(J)**. In **(F,I)** each symbol represents an individual studied according to the group; horizontal lines represent the mean. In **(J)** each bar represents the mean and standard deviation of the groups and the culture condition. One-way Anova was used for statistical analysis where, *$p \leq 0.05$, **p *leq* 0.01, ***$p \leq 0.001$ and ****$p \leq 0.0001$. T1R, Type 1 Reaction; BL, borderline lepromatous; T2R, Type 2 Reaction; LL, polar lepromatous patients; HV, healthy volunteers.

samples occurs after confirmation of the diagnosis of disease and/or reaction episode, immediately before starting MDT or use of anti-reaction drugs (Predinisone and/or Thalidomide). Disease diagnosis was performed by specialized physicians from the Leprosy Unit based on the clinical signs and symptoms presented by patients and confirmed by pathologists through histopathological skin lesion analysis obtained from skin biopsy.

The mean age observed in all groups were similarly around 47 years and the majority of participants were male, accounting for 72%. Bacilloscopic index (BI) mean was 3.9 ± 0.6 in BL patients and 3.5 ± 1.0 in T1R patients (**Table 1**). Also, the BI mean was 4.6 ± 1.0 in LL patients and 3.4 ± 2.0 in T2R patients. The bacteriological lesion index (BLI) was also analyzed revealing a decreased index in both reactional groups when compared to multibacillary patients (T1R/BL $p = 0.0449$; T2R/LL $p = 0.0011$). Disability grade (DG) was 1 in most reactional patients, while in non-reactional patients the DG was 0. All leprosy patients presented a negative lepromin test. Multibacillary patients presented reactions at different times after starting MDT, 7 ± 1.5 months for T1R and 17.6 ± 12.5 months for T2R. Most individuals had the reaction after the end of the therapeutic scheme.

We conducted a retrospective analysis in order to analyze the evolution of patients regarding the onset of reactions. One-year after blood collection, medical records were analyzed, and during this time about 39% of multibacillary patients (22% BL and 17% LL) presented one type of reactional episode during MDT.

Downmodulation of Regulatory CD4$^+$ and CD8$^+$ Subpopulations in PBMC of Reactional Leprosy Patients

Anti-CD28/CD49d were used in all the culture of mononuclear cells as costimulatory molecules and to test whether Tregs influence development of leprosy reactions, we analyzed CD4$^+$ and CD8$^+$ Treg cells, defined as CD25$^+$Foxp3$^+$, in multibacillary patients, T1R, T2R and HV. Frequencies of CD4$^+$ Treg subpopulations were significantly higher in BL patients when compared to T1R ($p = 0.0001$), T2R ($p < 0.0001$) and HV ($p < 0.0001$; **Figure 1F**). Data also demonstrated a lower frequency of CD4$^+$ Treg in T2R in comparison to LL ($p = 0.0020$) and T1R ($p = 0.0348$). Similar cell frequency was observed in T1R, T2R and HV individuals. Thus, in reactional episodes, patients return to a normal CD4$^+$ Treg frequency.

In regards to CD8$^+$ Tregs, this cell profile decreased in BL patients during T1R genesis ($p < 0.0001$). This difference did not occur between LL and T2R individuals. This result indicates that T2R genesis is not CD8$^+$ Treg dependent. However, the imbalance of this cell profile appears to be linked to T1R genesis in BL patients. Analyzing both reactional groups, T1R presents higher CD8$^+$ Treg than T2R ($p = 0.0002$; **Figure 1I**).

CD4$^+$/CD8$^+$ Treg ratio was also evaluated and was shown to be <2 in T1R, BL and HV groups (**Figure 1C**), representing twice the amount of CD4$^+$ Treg than CD8$^+$. However, the CD4$^+$/CD8$^+$ Treg ratio was significantly higher in LL patients when compared to BL ($p < 0.0001$; **Figure 1J**). This high ratio is due to the lower frequency of CD8$^+$ Treg than CD4$^+$ Treg in LL

TABLE 1 | Clinical and sociodemographic characterization of study participants.

Study population	No. of individuals	Age, years (Mean ± SD)	Sex (% male)	BI[a] (Mean ± SD)	DG[b] (%)			LST[c]	Time to diagnosis (months)	Time to reaction onset[d]
					0	I	II			
BL/T1R	10	55.5 ± 13.2	70	3.5 ± 1.0	30	60	10	NEG	–	7 ± 1.5
BL	10	41.7 ± 22.0	81	3.9 ± 0.6	50	50	–	NEG	43	–
LL/T2R	9	47.8 ± 17.1	75	3.4 ± 2.0	41.7	50	8.3	NEG	–	17.6 ± 12.5
LL	8	28.1 ± 10.4	62	4.6 ± 1.0	87.5	12.5	–	NEG	17	–
HV*	15	33.5 ± 11.9	60	–	–	–	–	–	–	–

[a] *BI, bacteriological index.*
[b] *DG, disability grade.*
[c] *LST, lepromin skin test [NEG = negative (<5.0 mm) and POS = positive (>5.0 mm)].*
[d] *Time to onset of reaction: Measure assessed through MDT time currently recognized by WHO (6 or 12 doses).*
* *HV, Healthy volunteers. The results were expressed as mean and standard deviation.*

and T2R groups. We then went on to investigate the function of these Treg subsets in patients and healthy controls.

Regulatory T Cells Producing IL-10 Do Not Participate in Reactional Episode Pathogenesis

Given that reaction episodes in multibacillary patients are characterized by an exacerbated proinflammatory response, according to our hypothesis, this change in immune response depends on downmodulation of $CD4^+$ and $CD8^+$ Treg. Thus, the functional profile of Tregs producing IL-10 and TGF-β was evaluated, because these cytokines stimulate an anti-inflammatory environment, impairing Th1 and Th17 responses.

Peripheral frequency of IL-10 producing Treg cells was determined to be in $CD4^+CD25^+Foxp3^+$ or $CD8^+CD25^+Foxp3^+$ region of analysis (**Figure 2A**). $CD4^+CD25^+Foxp3^+IL\text{-}10^+$ did not present significant difference between reactional forms and non-reactional BL/LL patients. The same data was also observed for $CD8^+$ IL-10+ Treg.

In T1R patients, the frequency of IL-10 producing $CD4^+$ and $CD8^+$ Treg was higher than in patients with T2R ($p = 0.0006/p < 0.0001$; **Figure 2B**). An elevated frequency of IL-10 producing $CD4^+$ and $CD8^+$ Treg was also observed in BL patients in comparison to LL ones ($p < 0.0001/p < 0.0001$).

Ratio analysis of $CD4^+IL\text{-}10^+/CD8^+IL\text{-}10^+$ Tregs was higher in HV when compared to T1R ($p = 0.0109$) and BL ($p = 0.0158$; **Figure 2C**). This data shows elevated CD8+IL-10+ Treg cells in T1R and BL compared to normal individuals. But between both reactional and non-reactional states, all groups had a rate below 1 (**Figure 2D**). This similarity shows a non-participation of $CD4^+IL\text{-}10^+$ Treg and $CD8^+IL\text{-}10^+$ Treg in genesis of reactional episodes.

Downmodulation of $CD4^+TGF\text{-}\beta^+$ and $CD8^+TGF\text{-}\beta^+$ Cells Contribute to Pathogenesis of Leprosy Reactional Episodes

Treg induce an anti-inflammatory milieu through direct contact with the target cell and immunomodulatory action through TGF-β production (**Figure 3A**). This cytokine is pleiotropic and participates in uncounted infectious diseases. Therefore, we evaluated the cell production of this molecule by flow cytometry.

Patients with T1R had a lower frequency of $CD4^+TGF\text{-}\beta^+$ Treg compared to the non-reactional BL group ($p = 0.0132$). In the T2R group, TGF-β producing CD4+ Treg was significantly lower among LL patients ($p < 0.0001$; **Figure 3B**). This finding revealed the participation of this cell profile in the pathogenesis of multibacillary leprosy reactions. This indicates that *Mycobacterium leprae* infection affects systemic $CD4^+TGF\text{-}\beta^+$ Treg frequency and downmodulation of this cell profile is related to genesis of reactions. Comparing reactional groups, T1R had a higher frequency of $CD4^+TGF\text{-}\beta^+$ Treg than T2R ($p = 0.0087$). Multibacillary patients also had a different TGF cell frequency and LL presented a higher $CD4^+TGF\text{-}\beta^+$ Treg than BL patients ($p = 0.0215$). Healthy volunteers had a significantly lower frequency of $CD4^+TGF\text{-}\beta^+$ Treg subsets in comparison to some groups (T1R, $p = 0.0013$; BL, $p < 0.0001$ and LL, $p < 0.0001$).

Evaluation of $CD8^+TGF\text{-}\beta^+$ Treg was also different between T1R and BL ($p = 0.0132$) as well as between T2R and LL ($p = 0.0006$; **Figure 3C**). Both reactional groups had a lower $CD8^+TGF\text{-}\beta^+$ Treg frequency than multibacillary leprosy, respectively. Comparing multibacillary patients, LL had a higher $CD8^+TGF\text{-}\beta^+$ Treg frequency than BL ($p = 0.0215$). On the other hand, when reactional episodes were compared, T2R presented higher frequency of $CD8^+TGF\text{-}\beta^+$ cells than T1R ($p < 0.0001$). Cell production of TGF-β also was lowest in HV in comparison to T2R ($p < 0.0001$), LL ($p < 0.0001$) and BL ($p < 0.0001$).

The ratio of $CD4^+TGF\text{-}\beta^+/CD8^+TGF\text{-}\beta^+$ Treg was higher in T1R when compared with T2R ($p < 0.0001$), BL ($p = 0.0015$), LL ($p < 0.0001$) and HV ($p = 0.0007$; **Figure 3D**), with a ratio close to or below 1. The same was observed in BL compared to T2R ($p = 0.0024$) and LL ($p = 0.0369$). The ML and PHA stimuli showed high production of TGF-β and IL-10 in all groups studied, however, no differences between them were found.

Data indicate that *Mycobacterium leprae* induces an increase of $CD4^+TGF\text{-}\beta^+$ and $CD8^+TGF\text{-}\beta^+$ Treg in multibacillary patients. This hyporesponsive milieu suffers a rupture, causing $CD4^+TGF\text{-}\beta^+$ and $CD8^+TGF\text{-}\beta^+$ Treg decrease. This new environment in addition to high proinflammatory cytokines, could trigger the genesis of both types of reactional episodes.

Proinflammatory Cytokines Participate in T2R Episodes

To analyze the culture environment, we compared levels of proinflammatory and anti-inflammatory cytokines in PBMC supernatant. Supernatant was collected after PBMC were cultured for 72 h with or without irradiated and sonicated *M. leprae* (NIH/NIAID N01 Al-25469), phytohemagglutinin (Sigma, USA), CD28, CD49d (Biolegend, EUA) and incubated at 37°C in humidified 5% CO_2 air.

As shown in **Table 2**, there was an IL-17 increase in T2R compared to non-reactional LL patients, with ($p = 0.0173$) or without *M. leprae* stimuli ($p = 0.0177$). We also observed a difference in IL-17 levels between T2R and T1R ($p = 0.0333$). IL-6 also appears to play an important role in leprosy reactions. In T2R this cytokine is increased when compared to LL patients with ($p = 0.0056$) or without *M. leprae* ($p = 0.0025$). IL-10 and IL-23 did not change significantly between reactional patients and other groups. In BL patients these cytokines may not be involved in T1R genesis.

DISCUSSION

Mechanisms involved in immunopathogenesis of reactions are not entirely understood. Our data revealed that reactions appear to be related to downmodulation of $CD4^+TGF\text{-}\beta^+$ and $CD8^+TGF\text{-}\beta^+$ Treg cells in multibacillary patients, associated to imbalance of proinflammatory cytokines. These findings bring new Treg perspectives to the pathogenesis of leprosy reactions,

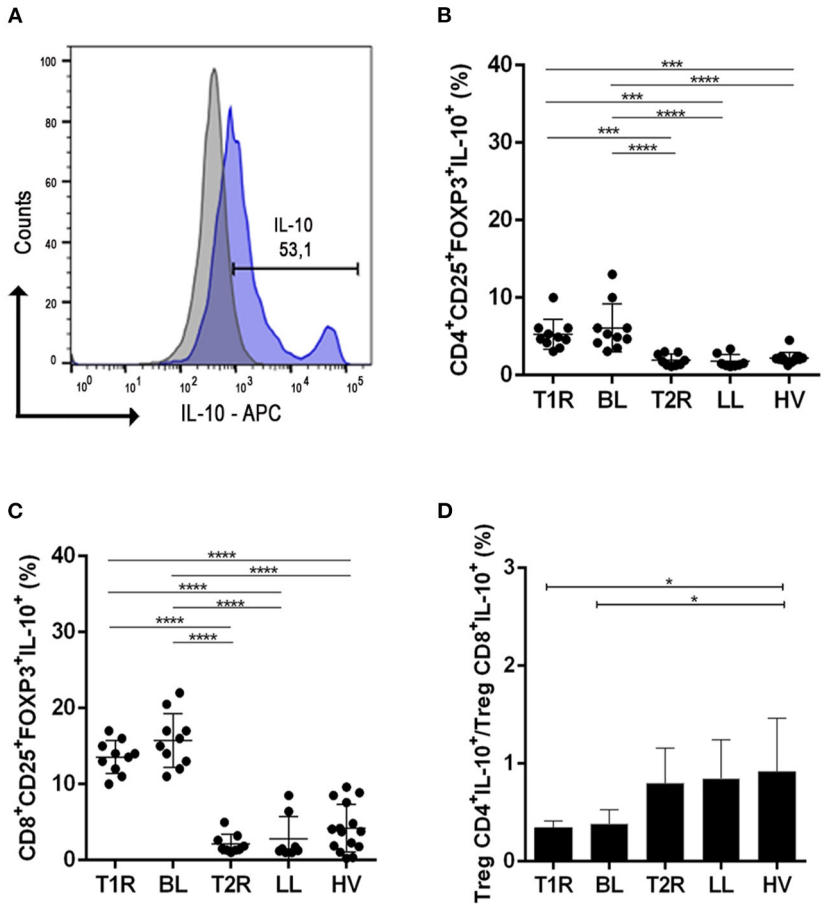

FIGURE 2 | Analysis of regulatory T cells producing IL-10. Evaluation of CD4^{+} and CD8^{+} Treg frequency in blood samples in vitro with no stimuli. Representative histogram from strategy analysis, cells were gated in the regulatory T cell phenotype **(A)**; Frequency of CD4+CD25+Foxp3+IL-10+ **(B)**; CD8+CD25+Foxp3+IL-10+ **(C)**; Ratio of CD4+CD25+Foxp3+IL-10+/CD8+CD25+Foxp3+IL-10 **(D)**. Each symbol represents an individual studied according to the group; the horizontal lines represent the median. One-way Anova was used for statistical analysis where, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$. T1R, Type 1 Reaction; BL, borderline lepromatous; T2R, Type 2 Reaction; LL, polar lepromatous patients; HV, healthy volunteers.

especially regarding the role of CD8^{+} Treg, a cell subset that has been poorly studied, but which has an important role in the clinical course of leprosy.

Most patients involved in this study were male. A similar proportion is observed in the world (1) and Brazilian epidemiological data among multibacillary patients (35). This is expected because disease impairment is related to lifestyle, work, and higher levels of testosterone found in this gender (36).

Regarding the genesis of reactional episodes, the high BI and number of lesions in LL patients are directly related to risk of initiating T2R (6). In our study, all lepromatous patients presented more than 20 widespread lesions over the body and a BI higher than 4.

Of note, anti-CD-28 and anti-CD49d antibodies were added in all the conditions of culture. The use of such molecules triggers the engagement of co-stimulatory receptors leads to recruitment of specific binding partners, such as adaptor molecules, kinases, and phosphatases, *via* recognition of a specific motif. Consequently, each co-stimulatory receptor transduces a unique pattern of signaling pathways (37, 38).

According to Sakaguchi et al. (20) and Corthay (21), regulatory T cells are identified as CD4^{+} or CD8^{+} subsets expressing CD25^{+}FOXP3^{+}. Therefore, characterization of this subpopulation started after duplet exclusion, viable cell determination and delimitation of lymphocyte area, followed by establishment of CD4^{+} and CD8^{+} gates. After analysis, our data revealed that negative modulation of TGF-β producing CD4^{+} and CD8^{+} Treg in multibacillary patients is related to reactional episodes. Similar results also showed the participation of CD4^{+} Treg in PBMC of T2R patients (32, 33) and in skin lesions (39).

Although our group also analyzed CTLA-4 frequency in regulatory T cells, reactional patients showed no differences when compared to multibacillary individuals. Viera e collaborators also observed that CTLA-4 does not participate as a suppressor molecule in multibacillary leprosy patients. Similar conclusions were obtained by Li and colleagues in patients with multidrug-resistant tuberculosis (27).

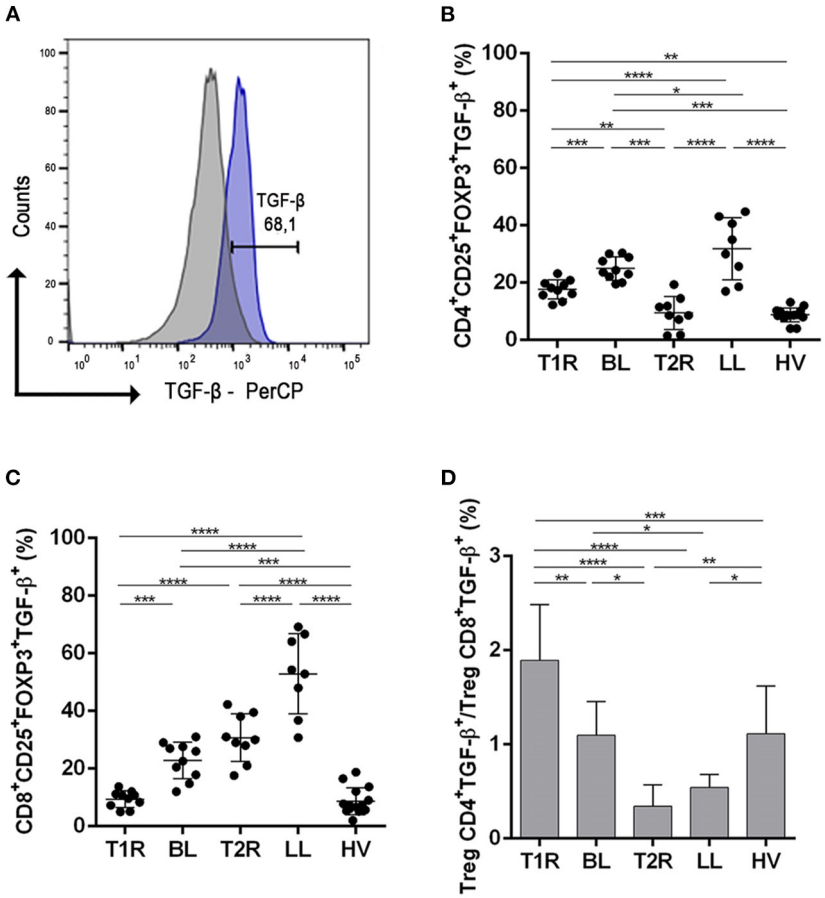

FIGURE 3 | Analysis of regulatory T cells producing TGF-β. Evaluation of CD4+ and CD8+ Treg frequency in blood samples in vitro with no stimuli. Representative histogram from strategy analysis, cells were gated in regulatory T cell phenotype **(A)**; Frequency of CD4+CD25+Foxp3+TGF-β+ **(B)**; CD8+CD25+Foxp3+TGF-β+ **(C)**; Ratio of CD4+CD25+Foxp3+TGF-β+/CD8+CD25+Foxp3+TGF-β+ **(D)**. Each symbol represents an individual studied according to the group; the horizontal lines represent the median. One-way Anova was used for statistical analysis where, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$. T1R, Type 1 Reaction; BL, borderline lepromatous; T2R, Type 2 Reaction; LL, polar lepromatous patients; HV, healthy volunteers.

As in our current work, Negera and co-works showed no reduction in $CD8^+$ Treg frequency in T2R, however, our study revealed that downregulation of $CD8^+TGF\text{-}\beta^+$ Treg participates in the onset of T1R and T2R (33). Thus, according to our hypothesis, the genesis of reactions is dependent on depletion of TGF-β producing $CD4^+$ and $CD8^+$ Treg. TGF-β is a cytokine capable of inhibiting macrophage activation and effector T lymphocyte action, reducing the secretion of proinflammatory cytokines, such as IFN-γ and TNF, in addition to favoring the Th17 profile shift (40, 41). Several researchers have shown TGF-β to induce apoptosis in different cells such tumors (42), embryonic cells (43), hepatocytes during alcohol abuse (44) and T lymphocytes infected by the bacillus Calmette-Guérin (BCG) vaccine provoking decreased protection against tuberculosis (45). It is possible that Treg cells may undergo apoptosis during the pathogenesis of leprosy reactions due to TGF-β.

In patients with active tuberculosis (TB), suppression of the proinflammatory response was observed before treatment. These patients had increased apoptosis of T lymphocytes in PBMC and cells in the pleural space, compared to healthy individuals (46). Also, in a coculture of PBMC from TB patients, high apoptosis of $CD4^+$ T lymphocytes was revealed when compared to controls. Complementary assays show an increase in FasL on the surface of $CD4^+$ T cells/m-RNA and BCL-2 reduction. Under the above conditions, TGF neutralization led to CD4 T lymphocyte survival (47). Quaresma et al. (48) demonstrated the relationship between TGF-β and caspase-3 in skin lesions of lepromatous patients. Thus, our data indicates that exacerbated production of TGF-β may be related to Treg apoptosis during onset of reactional episodes.

Higher frequency of TGF-β producing cells in multibacillary patients over time, could thus trigger apoptosis of Treg in an autocrine or paracrine manner, favoring performance of cells and cytokines with a proinflammatory profile and inducing reactions. Furthermore, apoptosis of these TGF-β producing Treg cells possibly appears to be linked to an increased frequency of a T lymphocyte proinflammatory environment that is widely recognized in the pathogenesis of these reaction episodes.

TABLE 2 | Cytokine production by PBMC, after 72 h cultures against *M. leprae* stimulus.

Cytokynes (pg/mL)		T1R	BL	*p* value	T2R	LL	*p*-value
		Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
IL-17	UNS	223.2 ± 41.3	176.4 ± 42.3	–	560.1 ± 299.3	207.5 ± 85.2	*
	ML	341.1 ± 80.8	196.1 ± 77.9	–	763.7 ± 316.8	295.4 ± 89.6	*
IL-23	UNS	137.7 ± 11.7	138.1 ± 13.1	–	207.9 ± 85.4	203.6 ± 81.1	–
	ML	129.8 ± 4.7	140.3 ± 9.7	–	191.7 ± 73.6	225.5 ± 74.3	–
IL-6	UNS	28.3 ± 7.7	20.3 ± 4.6	–	30.1 ± 1.8	27.1 ± 1.3	**
	ML	20.5 ± 8.7	19.7 ± 5.3	–	29.9 ± 1.1	25.9 ± 2.3	**
IFN-γ	UNS	130.8 ± 46.7	89.6 ± 14.8	–	153.6 ± 36.5	139.9 ± 33.7	–
	ML	142.5 ± 24.7	107.5 ± 34	–	147.2 ± 27.3	139.7 ± 40.9	–
IL-10	UNS	176.6 ± 10.9	157.8 ± 10.5	–	94.1 ± 39.4	137.7 ± 49.3	–
	ML	165.3 ± 30.2	160.3 ± 11.1	–	69.7 ± 24.5	88.6 ± 39.8	–

*The results were expressed as mean and standard deviation. The Mann-Whitney was used for statistical analysis where, *p≤ 0.05, **p≤ 0.01. T1R, Type 1 reaction; BL, borderline lepromatous; T2R, Type 2 reaction; LL, polar lepromatous patients; SD, standard deviation; UNS, unstimulated; ML, M. leprae stimulus. It was analyzed BL 7, T1R 3, LL 10 and T2R 10 individuals.*

Previous work by our group had already shown a high frequency of TBX21 as well as an increase in IFN-γ producing $CD4^+$ T cells in the genesis of T1R in borderline lepromatous patients (17). On the other hand, in T2R we found an increased in $CD8^+$ TNF producing cells (18). Thus, after apoptosis of TGF-β^+ Treg an increase in $CD4^+$IFN-γ^+ and $CD8^+TNF^+$ cells could occur, triggering T1R and T2R, respectively.

In addition, the present work also revealed the participation of IL-17 and IL-6 in the genesis of T2R in lepromatous patients. According to Saini and colleagues, the high *in vitro* production of IL-6 occurs in monocytes and granulocytes in T2R (32), and after treatment with prednisone there is a significant reduction of this cytokine (49). IL-6 and IL-17 are closely related to TGF-β. In synergy with IL-6, TGF-β stimulates the polarization of $CD4^+$ T lymphocytes to the Th17 profile, a subpopulation with proinflammatory action through the production of IL-17, IL-22, and IL-23 (50, 51). Increase in IL-17 producing T lymphocytes had already been observed in the pathogenesis of T2R in Ethiopian multibacillary patients (33). Negera also observed a reduction of the Th17 subpopulation after starting treatment with prednisone. According to our data, after Treg apoptosis there is an increase in IL-17, possibly triggering a proinflammatory environment in T2R. Moreover, pathogenesis of T2R appears to be independent of IL-23 presence, since this cytokine was not altered in the patients studied. This same data was also observed through RT-PCR analysis of skin lesions (25).

IFN-γ positive modulation may participate in some way in both Type 1 and 2 reactions, along with the negative modulation of IL-10 in T2R. These data appear to show a breakdown in the balance between an anti-inflammatory environment, maintained by Treg in multibacillary patients, and the beginning of reactions marked by a proinflammatory profile, with the involvement of the Th17 shift and IL17 production. This phenomenon can occur in T1R patients, but other mechanisms and mediators can participate in triggering the reaction.

Interleukin-10 producing $CD4^+$ and $CD8^+$ Treg and the presence of IL-10 in culture supernatants does not appear to be crucial for the maintenance of the hyporesponsive state observed in multibacillary patients under study conditions. Saini and collaborators observed $CD4^+$TGF-β^+ Treg in multibacillary patients (52). This frequency was also observed in multibacillary patients when compared with paucibacillary ones (32). Saini data corroborate our findings and suggest that IL-10 producing $CD4^+$ and $CD8^+$ Treg may not be crucial for maintaining the *Mycobacterium leprae* response in multibacillary patients.

Future experimental studies could demonstrate the mechanism proposed in this work such as adding neutralizing antibodies for TGF-β in PBMC cultures of multibacillary and reactional patients, as well as apoptosis assay analysis. In conclusion, our study showed the participation of TGF-β producing $CD4^+$ and $CD8^+$ Treg in the maintenance of a hyporesponsive profile in multibacillary leprosy patients. Thus, apoptosis of Treg subpopulations could be related to overproduction of TGF-β, promoting an imbalance of this cell profile and leading to genesis of T1R and T2R episodes. Finally, these conditions are influenced by higher synthesis of IL-17 and IL-6 in T2R but not in T1R pathogenesis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Oswaldo Cruz Foundation/FIOCRUZ (protocol number 518/09). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DE: conceptualization, funding acquisition, review, and editing the text. KG, PL, LN, JL, and MP: performed the experiments. IA: analyzed data of flow cytometric experiments. KG, PL, and DE: analyzed the data and writing the manuscript. All authors contributed to the article and approved the submitted version.

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Clinical, epidemiological, and laboratory prognostic factors in patients with leprosy reactions: A 10-year retrospective cohort study

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Introduction: Leprosy reactions, the main cause of neural damage, can occur up to 7 years after starting multidrug therapy. We aimed to approach the prognostic factors that may influence the leprosy reactions over the follow-up time.

Methods: Retrospective cohort study, encompassing 10 years of data collection, composed of 390 patients, divided into 201 affected by reactions and 189 reaction-free individuals. Epidemiological, clinical, and laboratory variables were approached as prognostic factors associated with leprosy reactions. The association among variables was analyzed by a binomial test and survival curves were compared by the Kaplan-Meier and Cox proportional-hazards regression.

Results: 51.5% (201/390) of patients were affected by leprosy reactions. These immunological events were associated with lepromatous leprosy (16.2%; 63/390; $p < 0.0001$) and multibacillary group (43%; 169/390; $p < 0.0001$). This study showed that survival curves for the prognostic factor anti-PGL-I, comparing positive and negative cases at diagnosis, differed in relation to the follow-up time (Log Rank: $p = 0.0760$; Breslow: $p = 0.0090$; Tarone-Ware: $p = 0.0110$). The median survival times (time at which 50% of patients were affected by leprosy reactions) were 5 and 9 months for those reactional cases with negative (26/51) and positive serology (75/150), respectively. The time-dependent covariates in the cox proportional-hazards regression showed anti-PGL-I as the main prognostic factor to predict leprosy reactions (hazard ratio=1.91; $p = 0.0110$) throughout the follow-up time.

Conclusions: Finally, these findings demonstrated that anti-PGL-I serology at diagnosis is the most important prognostic factor for leprosy reactions after starting multidrug therapy, thus enabling prediction of this immunological event.

KEYWORDS

antigens, leprosy, leprosy reaction, phenolic-glycolipid-1, survival analysis

Introduction

Leprosy reactions, classified as Type 1 or Type 2, occur before, during and after Multi-Drug Therapy (MDT), and may be triggered by different co-infections and/or antigens of *Mycobacterium leprae* (*M. leprae*) especially phenolic-glycolipid-1 (PGL-I) (1, 2).

Regarding to the time for leprosy reaction occurrence, a study reported that 9.5% of patient had late type 1 reaction up to 7 years after starting MDT (3).

The type 1 reaction (T1R), common between borderline tuberculoid (BT), borderline borderline (BB), and borderline lepromatous (BL), might be subdivided into upgrading and downgrading reaction (4).

A current study showed 27% of patients being affected by T1R with 63% ranging from moderate to severe cases (5). Furthermore, a study reported that 60% of patients developed T1R with 90% of cases presenting inflamed plaques as the main sign (6). The T1R presents cutaneous manifestations such as erythema, infiltration into the skin and edema in preexisting lesions, as well as arising of new skin lesions. As to the presence of neurological signs and symptoms, it is possible to highlight neural thickening (edema), pain in the peripheral nerve, sensory-motor changes with loss of muscle strength and consequent evolution to physical disabilities (7).

The type 2 reaction (T2R), whose main presentation is erythema nodosum leprosum (ENL), is systemic and associated with the formation of immune complexes (IC) in the blood such as found in serum samples, and are deposited inside tissues, especially skin, kidneys and joints, reported as extravascular complements, therefore being a type III hypersensitivity reaction (8–10). This type of reaction is considered an immunological complication for the clinical forms BL and lepromatous leprosy (LL) (8). The T2R affected 44% of BL and 71% of LL according to a study involving a period of 12 years of data collection. Furthermore, independent on clinical form, it was reported 26.8% of T2R in a referral center (11, 12). The systemic signs and symptoms that are commonly present in this type of reaction include malaise, loss of weight and injury to internal organs, which in turn may cause peripheral neuropathy, orchiepididymitis, glomerulonephritis, myositis, arthralgia, iridocyclitis, hepatomegaly, and ganglion infarction (13). Hematological and biochemical changes may be present in T2R as leukocytosis, neutrophilia, thrombocytosis, increased acute-phase proteins such as C-reactive protein, alkaline phosphatase, transaminases, fibrinogen, and elevated immunoglobulins of the IgG and IgM classes (14).

Studies analyzing prognostic factors in relation to the outcome of leprosy reactions are scarce. However, it is possible to find some research studies limited to risk factors associated with leprosy reactions.

Therefore, we aim to approach, by means of comparison among survival curves, the prognostic factors that may be associated with leprosy reactions across 10 years of follow-up.

Materials and methods

Sample, place, and study design

Retrospective cohort study, involving a sample of 390 patients, divided into 201 affected by leprosy reactions and 189 reaction-free individuals, whose follow-up period ranged from 2006 to 2015. The secondary data were collected in the National Reference Center in Sanitary Dermatology and Leprosy at Federal University of Uberlândia, Brazil, from 2014 to 2016.

Inclusion and exclusion criteria

The inclusion criteria were: leprosy patients affected by leprosy reactions type 1 and T2R; reaction-free patients, diagnosed by leprologists according to the clinical, histological and immunological criteria of Ridley and Jopling (15).

The exclusion criteria were: individuals with other chronic infectious diseases; patients affected by acute infections; relapses cases and/or patients with resistance to anti-leprosy drugs.

Criteria for definition of leprosy reactions

The leprosy reactions (T1R and T2R) were identified and classified by the expert leprosy physician who evaluated the patient during the clinical episodes. The diagnosis was based on clinical and immunological criteria.

Follow-up time

The follow-up time varied from time zero (t_0) to time of event/outcome; the data collection encompassed a period of 10 years, as reported previously. In this present study, time zero (t_0) was considered the date of the first dose of MDT to treat leprosy. On the other hand, the time-to-event/outcome was the first day of clinical manifestation of signs and symptoms associated with leprosy reactions. Each one of the patients have been followed for a total-person time of 7 years, by means of medical records, in order to registering the first leprosy reaction after starting the MDT.

Clinical and epidemiological variables

The main clinical and epidemiological variables used in the study were: clinical form of the disease, type of leprosy reaction, operational classification (OC), period of leprosy reaction presentation, sex, age group, skin phenotype, and disability degree.

Laboratory variables

The laboratory variables evaluated in this investigation were IgM antibodies to the PGL-I serology and dermal smear bacillary index.

Regarding anti-PGL-I serology, the cutoff point was equal to index 1. Thus, values below this point were negative and those above were positive. Indeterminate anti-PGL-I ELISA index values (equal to 1) were repeated. As to the bacillary index (BI) of dermal smear, the results equal to 0 were considered negative. On the contrary, BI values above 0 were classified as positive.

Anti PGL-I serology

The enzyme-linked immunosorbent assay (ELISA) was performed on all patients, against the native PGL-I molecule purified from the *M. leprae* cell wall, according to a methodology previously described in the literature (16).

Bacillary index of dermal smear

The mean of the dermal smear bacilloscopic index was obtained after collection of 7 standardized sites, such as: ear lobes, elbows, knees and main skin lesion. The BI, proposed by Ridley in 1962, is based on a logarithmic scale from 0 to 6, ranging from the absence of bacilli to the presence of more than 1,000 bacilli in each field examined (17).

Ethical approval

This study was approved by the Research Ethics Committee at the Federal University of Uberlândia – Brazil under registration number 28931320.9.0000.5152. The written informed consent was not needed given that this research was to be carried out by means of secondary data.

Statistical analysis

The binomial test was employed to compare the reaction and reaction-free groups regarding the proportions found in the epidemiological and clinical variables. Relative risk (RR) was used to assess the likelihood of the leprosy reactions in those individuals with the presence of factors assessed in this study. The comparison among survival curves was carried out by means of the Kaplan Meier test. The time-dependent covariates in the cox proportional-hazards regression was performed to ascertain the factors that influenced the outcome, leprosy reaction, in a multivariate model. The IBM Statistical Package for Social Sciences (SPSS) for Windows, version 22 (IBM Corp., Armonk, N.Y., USA) was used for all statistical analyses with a 5% significance level.

Results

Epidemiologic and clinical data

The sample was composed of 390 patients, 189 (48.5%) individuals were reaction-free and 201 (51.5%) affected by leprosy reactions. Among the reactive group, T1R predominated with 61.2% (123/201), while 38.8% (78/201) were T2R (Table 1). There was difference between the proportions of clinical form LL in the reactional individuals (31.3%; 63/201) when compared with the same clinical form in the reaction-free group (5.3%; 10/189) ($p < 0.0001$) (Table 1). All clinical and epidemiological variables are shown in Table 1.

Relative risk for the development of leprosy reactions

According to Table 2, the risk for leprosy reactions in those individuals with anti-PGL-I positive serology, at diagnosis, was 2.65 times more likely than in those with negative results ($p < 0.0001$; CI: 2.07–3.40). Table 2 highlights that the risk for leprosy reactions in individuals with positive dermal-smear BI at the diagnosis, was 2.56 times more likely than in those with negative results for the same test ($p < 0.0001$; CI: 2.05–3.20).

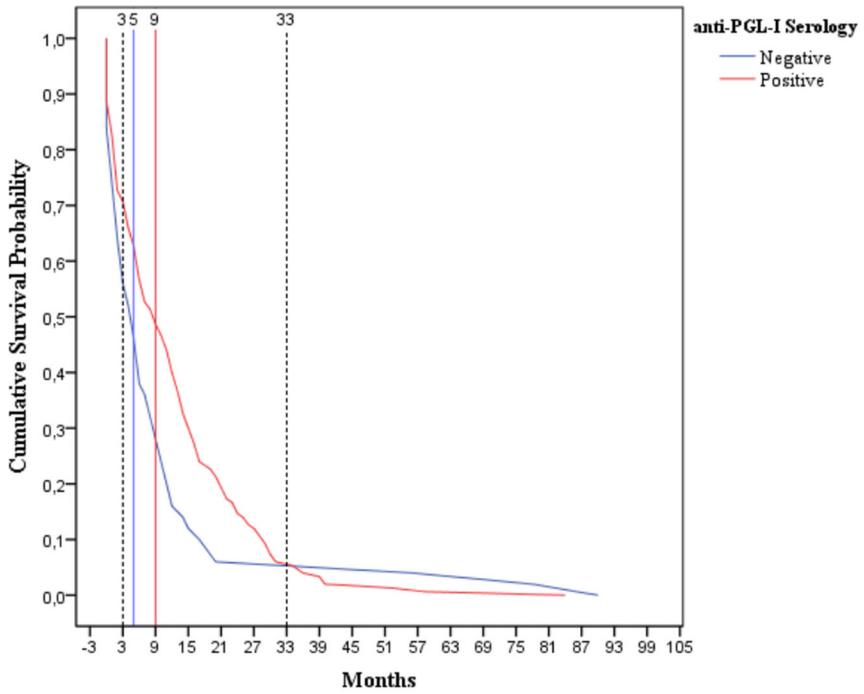

FIGURE 1
Survival curve (Kaplan-Meier) of 201 leprosy reaction individuals during the follow-up of 10 years according to the anti-PGL-I serology at diagnosis. The comparison between 2 cumulative survival probability curves that presented significant difference along all the time of follow-up (Log Rank, $p = 0.076$; Breslow, $p = 0.009$; Tarone-Ware, $p = 0.011$). Lines over all follow-up time: the blue line represents negative cases to anti-PGL1 serology ($n = 51$) and the red line the positive cases ($n = 150$).

Survival curves, prognostic factors and time-to-event for leprosy reactions

The time-to-event, that is, from t_0 to the first leprosy reaction (event/outcome), was determined by comparing survival curves assessing several prognostic factors that directly influenced the primary outcome, leprosy reaction. Figure 1 displays the comparison between two survival curves in those reactional individuals that were seronegative ($n = 51$) and seropositive ($n = 150$) for anti-PGL-I serology at diagnosis. It was observed that, within the first 3 months, 30% (45/150) of seropositive cases were affected by leprosy reactions, whereas 45% (23/51) seronegative ones presented this event within the same interval. The median survival times (times at which 50% of patients were affected by the event/leprosy reactions), were 5 and 9 months for those reactional cases with negative (26/51) and positive serology (75/150), respectively (Figure 1). Thus, reactional cases who presented negative serology had poor prognosis, due to the first reaction having occurred earlier after t_0 when compared to seropositive cases. We emphasized that 33 months after the t_0, the trend between prognostic factors changed, because the cases seronegative for anti PGL-I had better prognosis than seropositive patients. This finding may be confirmed by noting that after the 33rd month, the curve of seropositive individuals was under that of seronegative ones, indicating a higher leprosy reaction rate in seropositive patients after this period (Figure 1). Furthermore, the survival curves, as shown in Figure 1, were statistically different throughout the follow-up time cited in this study (Log Rank, $p = 0.076$; Breslow, $p = 0.009$; Tarone-Ware, $p = 0.011$).

As displayed in Figure 2, when was analyzed the BI of the dermal smear at diagnosis as a prognostic factor for leprosy reaction development, 50% (26/32) of those classified as negative at diagnosis presented reaction within 6 months after t_0, whereas half of positive cases (85/169) had this same outcome at 7 months (Log Rank, $p = 0.058$; Breslow, $p = 0.024$; Tarone-Ware, $p = 0.020$).

With respect to sex, age group, degree of physical disability, clinical form and skin color, these factors did not show differences between the survival curves for the leprosy-reaction prognosis across the follow-up time (Supplementary Figures).

Multivariate analysis of main prognostic factors

In Figure 3, a set of epidemiological (sex and age group), clinical (clinical form and type of leprosy reaction) and

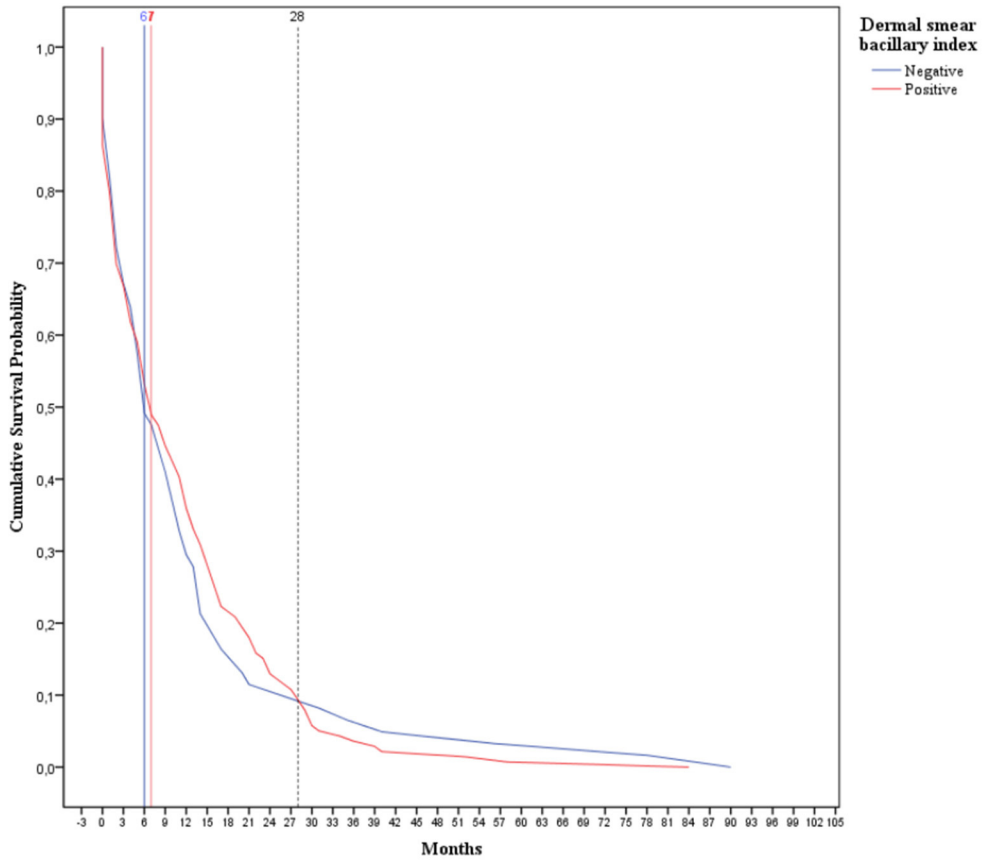

FIGURE 2
Survival curve (Kaplan-Meier) of 201 leprosy reaction individuals during the follow-up of 10 years according to dermal smear bacillary index at diagnosis divided into negative and positive. The comparison between 2 cumulative survival probability curves that presented significant difference along all the time of follow-up (Log Rank, $p = 0.058$; Breslow, $p = 0.024$; Tarone-Ware, $p = 0.020$).

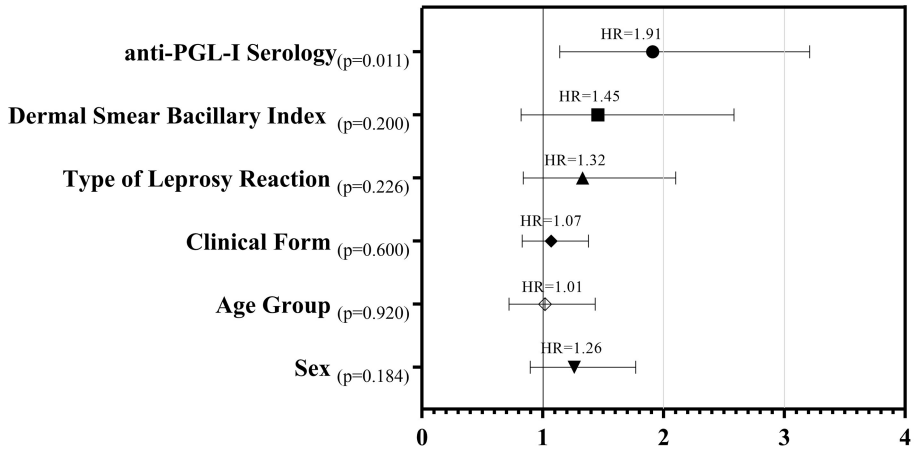

FIGURE 3
Forest plot of estimated *Hazard Ratios, p-values* and *confidence intervals* from epidemiologic, clinical and laboratory variables as prognostic factors for leprosy reactions - Cox Regression with time-dependent covariate analysis.

TABLE 1 Comparison among proportions of Epidemiologic and clinical data from leprosy reaction and reaction-free groups by means of Binomial test.

		Leprosy Reaction		Reaction-free		Total		*p
		n	%	*n*	%	*n*	%	
Clinical form	I	0	0.0	4	2.1	4	1.0	
	T	8	4.0	49	25.9	57	14.6	<0.0001
	BT	58	28.9	98	51.9	156	40.0	<0.0001
	BB	37	18.4	12	6.3	49	12.6	0.0003
	BL	35	17.4	16	8.5	51	13.1	0.0088
	LL	63	31.3	10	5.3	73	18.7	<0.0001
		201		189				
Type of leprosy reaction	type 1	123	61.2					
	type 2	78	38.8					
Operational classification	PB	32	15.9	126	66.7	158	40.5	<0.0001
	MB	169	84.1	63	33.3	232	59.5	
Disabiity degree	0	102	50.7	142	75.1	244	62.6	<0.0001
	1	63	31.3	32	16.9	95	24.4	0.0009
	2	36	17.9	15	7.9	51	13.1	0.0035
Sex	Male	63	31.3	92	48.7	155	39.7	0.0005
	Female	138	68.7	97	51.3	235	60.3	
Skin phenotype								
	White	108	53.7	92	48.7	200	51.3	0.3183
	Brown	61	30.3	60	31.7	121	31.0	0.7655
	Black	16	8.0	18	9.5	34	8.7	0.5843
	Not declared	16	8.0	19	10.1	35	9.0	0.4699
Age group								
	0–19	3	1.5	13	6.9	16	4.1	0.0074
	20–39	45	22.4	43	22.8	88	22.6	0.9316
	40–59	105	52.2	87	46.0	192	49.2	0.2204
	≥60	48	23.9	46	24.3	94	24.1	0.9158

BB, borderline-borderline; BL, borderline-lepromatous; BT, borderline-tuberculoid; I, indeterminate; LL, lepromatous-lepromatous; MB, multibacillary; PB, paucibacillary; T, tuberculoid; T1R, type 1 reaction; ENL, erythema nodosum.
*Binomial test.

TABLE 2 Laboratory risk factors for leprosy reactions.

		Leprosy Reaction		Reaction-free		Total		Relative Risk (RR)		
		n	%	*n*	%	*n*	%	RR	*p*	Confidence interval (CI)
anti-PGL-I serology	Positive	150	74.6	55	29.1	205	52.6	2.65	<0.0001	2.07–3.40
	Negative	51	25.4	134	70.9	185	47.4			
Dermal smear bacillary index	Positive	139	69.2	43	22.8	182	46.7	2.56	<0.0001	2.05–3.20
	Negative	62	30.8	146	77.2	208	53.3			

laboratory variables (anti-PGL-I serology and dermal smear Bacillary Index) were analyzed in a multivariate model, by means of the Cox Regression with time-dependent covariate analysis, in order to assess the more relevant prognostic factor. It was noted that anti-PGL-I serology was the principal prognostic factor with potential to predict the outcome, leprosy reaction, over the follow-up time with precision (*Hazard Ratio*: 1.91; $p = 0.011$) in a model with different factors.

Summarized interpretation

By means of survival curves and Cox Regression with time-dependent covariate analysis, this study showed that the negative anti-PGLI at diagnosis may predict up to 33 months, an early reaction in this group (50% of leprosy reactions occurred until 5 months) with a proportional risk of nearly 2-fold (hazard ratio of 1.91) when compared with positive cases (50% of reactional cases occurred up to 9 months). On the contrary, after 33 months there is a poor prognostic for seropositive cases.

Discussion

Among leprosy reaction cases, the borderline group, BT, BB and BL, have predominated, being considered by several studies as a risk factor for T1R (4, 5). The proportion of the reactional LL clinical form was higher than LL reaction-free in agreement with previous studies (13, 18). The low proportion of the reactional T form was expected, due to the fact that these cases may be subpolar tuberculoid (TTs), rare and immunologically unstable, being able to migrate on the clinical spectrum of disease toward borderline forms (19). The MB operational classification was associated with high bacillary load, which results in risk of leprosy reaction occurrence as reported in a past study (8). The disability degree 2, associated with leprosy reactions, corroborated a recently study that indicated a dependent relationship between these two variables (20). The association between female sex and leprosy reaction was cited in another study as a risk factor, especially for T1R (21). The low prevalence of reactional individuals that belong to the 0–19 age group may be related to the operational classification and clinical form of them, since they were treated in early stage of disease (22). Moreover, the efficient immune response in this group, since these individuals have regular production of B and T cells from bone marrow and the thymus (23). On the other hand, elderly individuals present an increased number of regulatory T lymphocytes (Treg), which may cause excessive suppression of immune responses; furthermore, degenerative disease associated with polypharmacy may favor immunologic abnormalities in the elderly (24).

The positivity of anti-PGL-I serology was mentioned in this study as a risk factor for leprosy reactions. Thus, this result may contribute as a risk factor for leprosy reaction when compared with those seronegative. This important finding is supported by a study that reported a high positivity proportion of this antigen in reactional individuals (25). Our results from dermal-smear BI indicated high risk for manifesting leprosy reaction when the results to this test were positive at diagnosis, as found in other studies that showed that positive BI raises the chance of developing leprosy reactions as compared with negative cases (7, 26).

The prognostic was poor among seronegative patients, due to half of seronegative individuals presented leprosy reaction up to 5 months, while those seropositive developed the event at 9 months after MDT (Figure 1). In this current research, the highest proportion of patients with negative anti-PGL-I serology at diagnosis, among reactional cases, belong to T and BT clinical forms (data not shown). These clinical forms exhibited T1R, cell-mediated immunity, with macrophage activation under the influence of cytokines such as tumor necrosis factor-α (TNF-α), interferon gamma (IFN-γ), IL-2 and Lymphotoxin-alpha (LT-α) (24). We hypothesized that early occurrence of this type of reaction in this seronegative to PGL-I IgM is associated with MDT action on *M. leprae* that after bacillus fragmentation releases antigens into the bloodstream activating Th1 response, predominant in these individuals (27). The humoral immune response prevailed in those cases with positive anti-PGL-I IgM serology, such as BL and LL, characterized by production of the cytokines L-4, IL-5 and IL-10, manifesting, therefore, T2R, which provokes an increase in circulating levels of TNF-α and IL-10 in some of them. It is important to highlight that IL-10 cytokine may favor bacillus survival and delay an efficient response against this mycobacterium (28, 29).

We emphasized in this present study that, 33 months after t_0, there was a change in the prognostic-factor profile related to anti-PGL-I serology, evidencing poor prognosis for those seropositive, which may indicate a persistent of bacillary load in cases with higher dermal-smear BI. In relation to dermal-smear BI, a change in the prognostic factor was also observed 28 months after the t_0. This previous finding might be related to the persistence of bacillary load, which is as common in MB as in BL and LL, which consequently, will present a time-dependent bacillary clearance (30). The bacillary clearance also depends on the immune competence of these clinical forms, given that, as reported previously, a longer duration was necessary to eliminate the bacilli from tissues in those with T2R when compared to those without T2R (30).

Leprosy patients classified as T and BT have developed reactions in less time when compared with BB and BL (Supplementary Table 1). This difference among clinical forms regarding the time-to-event may be associated with effective immune response against *M. leprae* in those individuals with low bacillary load, an immunological event, according to other authors, that occurs within 6 months (31). Half of LL individuals developed a leprosy reaction within 6 months after t_0, corroborating a previous study that reported more than 70% of LL being affected by this reaction in the first 6 months after starting MDT treatment (32). The higher percentage of LL affected by T2R in the first 6 months after t_0 is in accordance with risk factors associated

with this clinical form, since BI > 4, and hypothetically related to activation of immune complexes and release of TNF-α by macrophages in these individuals with high BI (33).

The idea about the presence of immune complexes during T2R/ENL episodes may be reinforced by other research that assessed genic expression in peripheral blood mononuclear cell (PBMC) from T2R/ENL patients, demonstrating the high expression of components from the classical complement pathway, such as C1qA (34).

With respect to anti-PGL-I translational application, we recommend the use of this marker as prognostic factor in order to screening patients according to clinical forms and median time for the first leprosy reaction as shown in this research. This serology test may be suitable for creating an assistance flowchart involving esthesiometry, electroneuromyography, and medical assessment in a short period of time among evaluations to prevent nerve damage. The use of steroid such as prednisolone 20 mg/day during the first 4 months after MDT was pointed out in another research for leprosy reaction prevention. However, this strategy is controversial and more studies should be performed (35). The use of steroid associated with positivity of PGL-I after treatment with the goal to prevent leprosy reactions should be avoided, since the bacillary load of dermal-smear positive falls 1 log per year what may indicate the persistence of positivity of anti-PGL-I titers after treatment for multibacillary forms (36). Even though we did not focus on data after MDT, the positive anti PGL-I showed to be, in another study of our group, a predictive factor for peripheral nerve impairment demonstrated by electroneuromyography evidencing 4-fold chance of nerve damage for positive households contact as compared with seronegatives (17).

Conclusion

Finally, this study showed that the anti-PGL-I should be considered the main prognostic factor for leprosy reactions prediction after MDT and pointed out a median time of 5 and 9 months for this event in seronegatives and seropositives, respectively. These data may facilitate the monitoring and follow-up of these patients in order to prevent potential peripheral neural damage.

The principal limitations of this study are related to the difficulty of testing cytokines and lipoarabinomannan (LAM) as prognostic factors for leprosy reactions in a large sample of patients, due to the high cost of these laboratory supplies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Research Ethics Committee at the Federal University of Uberlândia. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

DA, IG, LG, and DS designed the study. ML, LC, MC, EM, and NC collected data on reporting. The lab protocol was standardized and performed by ML, LC, MC, EM, and NC. DA performed the data analysis. DA and DS wrote the manuscript. Critical review was performed by IG and LG. IG and LG directed the research. All authors read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.841030/full#supplementary-material

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Bacilloscopy and polymerase chain reaction of slit-skin smears and anti-phenolic glycolipid-I serology for Hansen's disease diagnosis

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The bacilloscopy of the slit-skin smear (SSS) is the exclusive laboratory test associated with dermato-neurological evaluation for Hansen's disease (HD) diagnosis; however, it is negative in the majority of PB or primary neural forms. Thus, a PCR technique involving different sequences and target genes has been performed with an aim to increase the sensitivity and specificity of *M. leprae* identification, especially in patients with low bacillary loads. Additionally, serological assays based on antibody response reflect infection levels and indicate that this could be a simpler, less invasive technique for estimating *M. leprae* exposure. Serological tests and PCR have been shown to be more sensitive and accurate than the SSS. Our study aimed to measure accuracy and performance among the SSS and PCR of dermal scrapings stored on filter paper and APGL-I serology for diagnosis in HD. A cross-sectional study analyzing the medical records (n = 345) of an HD outpatient-dermatology clinic from 2014 to 2021 was conducted. Accuracy performance parameters, correlation, and concordance were used to assess the value among the SSS, PCR, and APGL-I exams in HD. The SSS presented 24.5% sensitivity, 100% specificity, 37.4% accuracy, and the lowest negative predictive value (21.5%). The PCR assay had 41, 100, and 51% sensitivity, specificity, and accuracy, respectively. PCR and APGL-I serology increased the detection of HD cases by 16 and 20.6%, respectively. PCR was positive in 51.3% of patients when the SSS was negative. The SSS obtained moderate concordance with PCR [k-value: 0.43 (CI: 0.33–0.55)] and APGL-I [k-value:

0.41 (CI: 0.31–0.53)]. A moderate positive correlation was found between the APGL-I index and the bacillary index (r = 0.53; $P < 0.0001$). Thus, the use of the SSS is a low sensitivity and accuracy method due to its low performance in HD detection. The use of PCR and serological tests allows for a more sensitive and accurate diagnosis of patients.

KEYWORDS

Hansen's disease, bacilloscopy, slit-skin smear, PCR, serology, diagnosis

Introduction

HD is a treatable infectious disease with a chronic evolution, and its etiologic agent is the slow-growing organism *Mycobacterium leprae* complex, which includes *M. leprae* and *M. lepromatosis*. The bacteria compromise mainly the skin and peripheral nerves and can leave serious sequelae when there is no diagnosis or early therapeutic intervention (1). There were more than 127,000 new cases detected globally in 2020, a reduction in new case detection by 37% in 2020 compared with 2019, as an important consequence of the COVID-19 pandemic for HD control programs (2). According to WHO guidelines (2018), in addition to the cardinal clinical signs for the diagnosis of HD, the only remaining microbiological diagnosis test of HD is based on the presence of acid-fast bacilli in a slit-skin smear (SSS) even though this test is associated with low diagnostic accuracy for paucibacillary (PB) HD. Although the guidelines also reported polymerase chain reaction (PCR)-based assays as being associated with higher diagnostic accuracy, they lack standardization, are not commercially available, and would be difficult to perform in most primary health-care settings (3). Thus, the need to intensify the development and improvement of current laboratory methodologies for the early and satisfactory diagnosis of the disease is evident (4, 5). However, different clinical, bacteriological, and immunopathological characteristics constitute the spectrum of the disease and express the relationship between the pathogenicity of the bacilli and the host's immune response, which makes it difficult and unfeasible to control the disease (6). Additionally and focusing on strategies to break the chains of HD transmission, WHO included a recommendation to implement HD post-exposure prophylaxis with single-dose rifampicin (SDR) for healthy close contacts of patients with HD aged 2 years or older, after excluding HD and tuberculosis diagnosis. However, the introduction of SDR has not yet been widely implemented and still being evaluated the benefits, costs, and risks of such interventions (7).

The SSS is practically the exclusive etiological diagnostic test for HD, and it is a low sensitivity method with numerous risks of misunderstandings and requires technical expertise in collection, fixation, staining, and reading (3). The SSS consists of a microbiological examination through Ziehl-Neelsen staining in smears of dermal scrapings from the earlobes, elbows, and knees as well as skin lesions to detect *M. leprae*, and its result is dependent on the patient's bacillary load (8). The exam allows for classifying patients as multibacillary (MB) and monitoring the treatment when they are positive in the test (9). In general, the SSS is negative in the initial forms of the disease, pure neural cases, and some borderline cases, and it is strongly positive in the borderline lepromatous and lepromatous clinical forms (8). A negative SSS does not rule out the diagnosis of HD, and its sensitivity varies between 10 and 50% while its specificity is 100% (10). The performance of the test is based on the quality of the collection, the expertise of the performing professional and the laboratory protocol used to identify the entire bacteria (11).

The limitations in the diagnosis of PB and household contacts of HD patients (HDP) generate the need to incorporate techniques with greater technological performance with the aim of identifying groups of difficult bacilloscopic and histopathological diagnosis as a way to obtain an early diagnosis with high specificity (12). Currently, different gene targets are studied to support the specific detection of *M. leprae*. The gene sequence of specific regions located along the mycobacteria genome has become a potential and promising target for the molecular diagnosis of HD. Therefore, conventional or quantitative PCR assays provide fast and reliable results for molecular detection and/or quantification (13). PCR is sensitive and 100% specific due to negative results in samples of different mycobacteria, healthy individuals, and other granulomatous diseases (14). Thus, PCR can be used as a complementary test for the diagnosis of HD regardless of the clinical form of the disease (13, 14). The type of material collected, means of transport, technique chosen, and targets for detection of the bacteria are the main variables influencing the results (15). PCR of dermal scrapings stored in 70% alcohol or from skin biopsies has been highlighted, but its transport poses risks. Therefore, new ways of collecting and storing the collected sample are being validated, such as PCR of dermal scrapings on filter paper, as we have used at our hospital over the last 10 years.

Considering that for both the SSS and PCR the dermal scrapings come from the same closed place, without contact with the environment and other contaminants, such as from nasal and/or oral mucosa, the search for specific *M. leprae* DNA (PCR) tends to offer better positivity than the search for morphologically complete bacilli (SSS), even in low-load early cases (16).

Additionally, by serology, high titers of IgM anti-phenolic glycolipid-I antibodies (APGL-I) are associated with dissemination and progressive infections by the bacilli, making the test positive preferentially in MB cases of high burden, but its detection for PB patients is of limited value (4). Although very widespread in the literature, serological studies with PGL-I antigen have shown an average sensitivity of 63.8 and an average specificity of 91% (17). Thus, our study aimed to measure accuracy and performance among the SSS and PCR exams of dermal scrapings collected on filter paper and APGL-I serology in the diagnosis of HD.

Materials and methods

Design and study population

A cross-sectional study was conducted at the National Reference Center in Sanitary Dermatology and HD, Clinical Hospital of Ribeirão Preto Medical School (HCFMRP-USP), University of São Paulo, Brazil. Medical records (n = 345) of the HD outpatient-dermatology division from 2014 to 2021 were reviewed. The inclusion criterion for the selected patients was the execution of the three exams evaluated (SSS, PCR, and APGL-I) at the same time. The medical records were classified according to diagnosis into two patient groups by clinical screening of HD, as new cases of HD without multidrug therapy (MDT) and patients without diagnosis of HD who presented some skin lesions or neuropathy. Both groups underwent the three tests compared in the study (SSS, PCR, and APGL-I) and clinical evaluation at the same time.

Hansen's disease patients

HDP (n = 286) were diagnosed by clinical evaluation according to the WHO guidelines and recommended cardinal signs (3). The dermatological and neurological evaluation of the patients was the confirmatory exam for diagnosis of HD. Complementary tests to the clinical diagnosis were used when available as serology, molecular exam, bacilloscopy, ultrasound of peripheral nerves, electroneuromyography, and assessment of tactile sensation by Semmes-Weinstein esthesiometer. Negative results for complementary exams did not rule out the clinical diagnosis of HD. Clinical evaluations were performed by dermatologists and leprologists. Considering that none of the classifications for HD include all of the clinical manifestations of HD, particularly those involving macular and pure neural forms, we classified the patients considering the guidelines adapted by Madrid (Congress of Madrid 1953) and the Indian Association of Leprology (IAL 1982) classifications as follows: indeterminate (I), polar tuberculoid (TT), borderline (B), polar lepromatous (LL), and pure neural (N).

Non-Hansen's disease patients

Non-Hansen's disease patients (N-HDP) (n = 59) were defined as patients presenting some skin lesions or neuropathy who were referred due to the suspicion of HD. The signs and symptoms of HD suspicion were dermatological such as skin lesions with altered thermal, painful and/or tactile sensation, nodules, loss of eyelashes and/or eyebrows, as also, neurological findings such feel numbness in hands or feet, tingling (pricking), stinging sensation, pain in the nerves, weakness in the hands and/or feet, swelling of hands, feet and/or face. After dermato-neurological evaluation and complementary laboratory tests, these patients had the diagnosis of HD excluded and were classified as N-HDP.

Bacilloscopy

The SSS remains the reference standard of HD detection and is taken from 4 routine sites of dermal scraping samples from earlobes and at least one elbow and/or typical skin lesion. According to the Brazilian Ministry of Health guidelines, bacterial index (BI) counting and morphological analysis were used in a common optical microscope. The Ziehl–Neelsen technique was applied on the intradermal scraping slide containing four smears from each patient. The BI is an index of the bacillary load in the patient. This is expressed on a semilogarithmic scale: (1 +) 1–10 bacilli per 100 high-power (oil immersion) fields, (2 +) 1–10 bacilli per 10 high-power fields, (3 +) 1–10 bacilli per high-power field, (4 +) 10–100 bacilli per high-power field, (5 +) 100–1,000 bacilli per high-power field and (6 +) > 1,000 bacilli per high-power field (8).

Anti-phenolic glycolipid-I serology

Indirect ELISA was used as index test to measure the APGL-I IgM titer of every serum sample according to a previously reported protocol (4). Serology was performed with ND-O-BSA (PGL-I) based glycoconjugate of bovine serum albumin (NR-19346. BEI Resources). The respective index was calculated by dividing the optical density (O.D. 450 nm) of each sample by the cutoff, and indices above 1.0 were considered positive.

Polymerase chain reaction for detecting *Mycobacterium leprae* DNA

PCR was used as second index test. Dermal scraping samples from earlobes and at least one elbow and/or lesion obtained in the same collection performed for the SSS were transferred and stored on filter paper. Samples were refrigerated at 2–8°C until processing. Filter paper with samples was cut with a scalpel blade and transferred to a microtube, and 100 μL of sterile Milli-Q water was added. For material elution, the tube was incubated at 95°C for 15 min. Total DNA extraction was performed with commercial DNA extraction according to the manufacturer's protocol. DNA was used to perform conventional or quantitative PCR with primers specific to *M. leprae* according to a previous study (4). For conventional PCR the band was used to identify the PCR product with molecular weight relative to positive control with 148 bp. The quantitative PCR (qPCR) result was considered positive to detect *M. leprae* DNA with amplification until 40.0 cycle threshold (Ct) and melting temperature at 87.5°C.

Statistical analysis

All data were analyzed by GraphPad Prism v. 9.0 software (GraphPad Inc., La Jolla, CA, United States). The chi-squared test was used to assess associations among categorical variables and the positivity of exams. Study population characteristics were analyzed by a *t*-test and chi-squared test. Spearman's correlation was used to compare the immunoglobulin index APGL-I and BI in the SSS. The kappa coefficient (κ) was used to measure the reliability and concordance of the SSS, molecular test (PCR), and serology (APGL-I). The interpretation used for kappa value was slight (0–0.2), fair (0.21–0.4), moderate (0.41–0.6), substantial (0.61–0.8) and almost perfect (0.81–1.0). The level of statistical significance was set by alpha value (5%). Venn diagrams were generated using the online tool Draw Venn Diagram[1] to represent the overlap in the number of positive exams in the HDP. According to Bossuyt et al. (9), the Standards for Reporting of Diagnostic Accuracy Studies (STARD statement) was used to improve the completeness and transparency of results of diagnostic accuracy available at: https://www.equator-network.org/reporting-guidelines/stard/ (9). According to Whiting et al. (10), risk of bias and applicability judgments was applied using Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) available at: https://www.bristol.ac.uk/population-health-sciences/projects/quadas/quadas-2/ (10). The study was developed with pre-specified tests and considering SSS as reference standard and, PCR and APGL-I as index tests.

1 http://bioinformatics.psb.ugent.be/webtools/Venn/

Role of the funding source

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all of the data in the study and had final responsibility for the decision to submit for publication.

Results

Assessment of completeness, transparency, and risk of bias

To determine the quality and standardize the developed accuracy study, the STARD and QUADAS-2 were applied. STARD diagram to report flow of participants through the study was designed to summarize the selection of the population and performance of the index tests (APGL-I and PCR) as compared to the standard reference test (SSS) (**Figure 1**). All participants performed the standard reference test and the two index tests. In our study, inconclusive results were not reported. The STARD checklist was completed to assess the transparency of the study (**Supplementary Table 1**). The analysis of risk of bias and regarding applicability showed a concern classified as low for bias (**Supplementary File**).

Clinical and demographic characteristics

The study included 345 medical records grouped as HDP (n = 286; 82.9%) and N-HDP (n = 59; 17.1%). No significant difference was observed among ages (P = 0.363) and between genders (P = 0.645) of the groups. Among the 286 HDP, 197 (68.9%) were classified as B clinical forms of HD, 16 (5.6%) were classified as I and N forms, 9 (3.1%) as TT form, and 48 (16.8%) as LL form. The descriptive characteristics of the study population are summarized in **Table 1**.

Performance of laboratory tests in diagnosis of Hansen's disease

An analysis of the performance parameters of laboratory tests was performed to assess the concordance for the SSS, PCR on filter paper, and APGL-I serology for the diagnosis

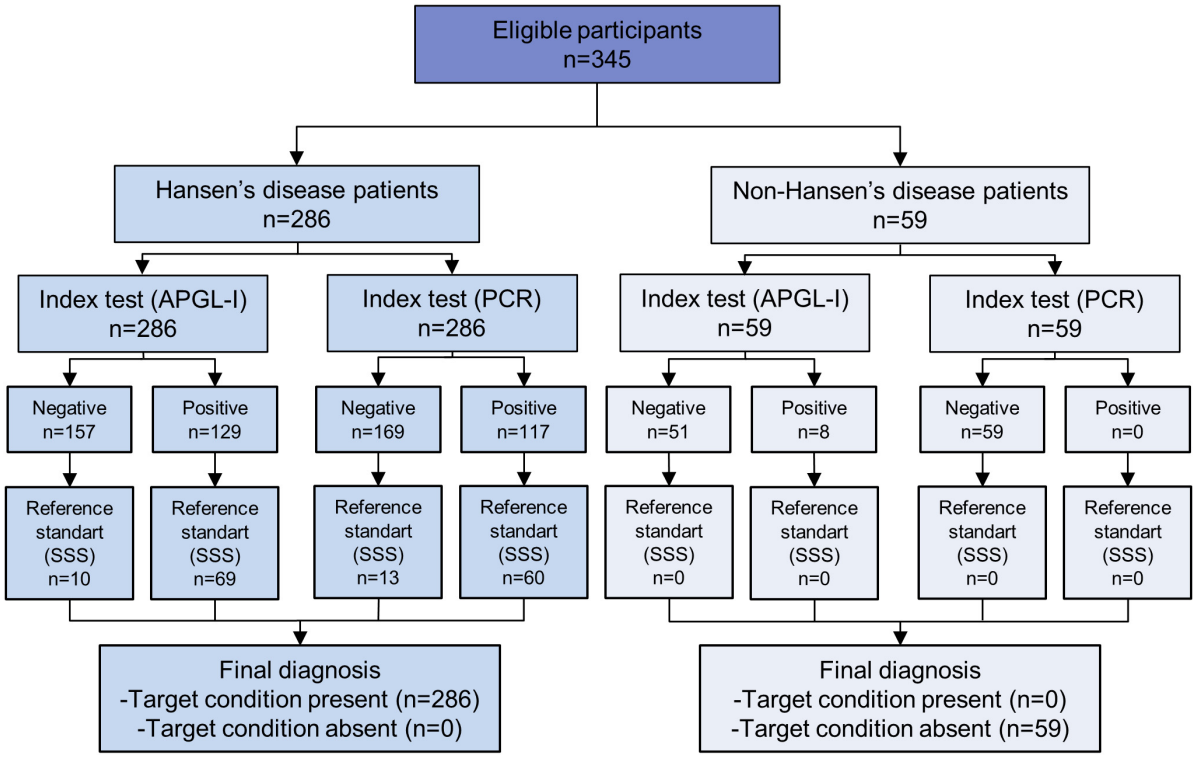

FIGURE 1
STARD diagram to report flow of participants through the study.

TABLE 1 Study population characteristics (N = 345).

	HDP n = 286	N-HDP n = 59	P
Age, years, mean (SD)	49.4 (16.1)	49.8 (15.9)	0.363[a]
Sex, *n* (%)			
Male	180 (62.9)	39 (66.1)	0.645[b]
Female	106 (37.1)	20 (33.9)	
Clinical form	***n* (%)**	***n* (%)**	
Indeterminate	16 (5.6)	-	
Tuberculoid	9 (3.1)	-	
Borderline	197 (68.9)	-	
Lepromatous	48 (16.8)	-	
Neural Pure	16 (5.6)	-	

[a]Comparison of two groups using the t-test. [b]Comparison of two groups using the chi-squared test. HDP, HD patients; N-HDP, non-HD patients; SD, standard deviation.

of HD patients (Table 2). The SSS presented a probability of case detection of only 24.5 and 100% specificity. Thus, it presented the lowest negative predictive value (21.5%), accuracy (37.4%), and positivity for evaluated cases (24.5%). The PCR assay had the best significant performance ($P < 0.0001$), with 41, 100, and 51% sensitivity, specificity, and accuracy, respectively. APGL-I had the highest probability of detecting cases (41%) and providing correct results (52.2%) and the highest rate of positive tests in patients (45.1%) despite yielding a positive test in individuals without diagnosis of HD (13.6%). The use of dermal scrapings for PCR and APGL-I serology increased the detection of HD cases to 16 and 20.6%, respectively. The SSS and PCR are the only tests that showed 100% probability of the disease when the test was positive. Positivity for all exams separately and in parallel was significantly different compared with the N-HDP ($P < 0.0001$).

The evaluation of tests in parallel showed that the inclusion of the SSS with PCR reduced by 20% the detection of cases and had 29 and 44.7% of sensitivity and accuracy, respectively. The absence of positive results for SSS and PCR in N-HDP maintained 100% specificity and positive prediction of the tests. PCR positivity and APGL-I serology combined demonstrated the best sensitivity (39.6%), negative predictive value (33.7%), accuracy (53.8%), and positivity (26.6%), when compared to performance including SSS (Table 2).

Serial analysis with positive SSS when the results were negative for PCR and APGL-I had the lowest sensitivity (7.7%; 6.4%) and accuracy (31.6%; 33.5), respectively. However, PCR performance in negative SSS results was 27.8% for probability of case detection and 43.3% of accuracy. Negative SSS results when the APGL-I serology was positive had the best sensitivity (50%); on the other hand, it had the lowest negative predictive value (25.9%). The results were similar for

TABLE 2 Comparison of the performance for the SSS, PCR and APGL-I as diagnostic tests in HD.

Exams	Se%	Sp%	PPV%	NPV%	Acc%	Positivity		P^a
						HDP *n* (%)	N-HDP n (%)	
SSS	24.5	100	100	21.5	37.4	70 (24.5)	0 (0)	<**0.0001**
PCR	41.0	100	100	25.9	51.0	117 (41)	0 (0)	<**0.0001**
APGL-I	45.1	86.4	94.2	23.8	52.2	129 (45.1)	8 (13.6)	<**0.0001**
SSS and PCR	26.8	100	100	27.4	42.6	57 (19.9)	0 (0)	<**0.0001**
SSS and APGL-I	29.0	100	100	28.6	44.7	60 (21.0)	0 (0)	<**0.0001**
PCR and APGL-I	39.6	100	100	33.7	53.8	76 (26.6)	0 (0)	<**0.0001**
SSS + PCR-	7.7	100	100	27.4	31.6	13 (4.5)	0 (0)	**0.028**
SSS- PCR +	27.8	100	100	27.4	43.3	60 (21.0)	0 (0)	<**0.0001**
SSS + APGL-I -	6.4	100	100	28.6	33.5	10 (3.5)	0 (0)	**0.047**
SSS- APGL-I +	50.0	86.4	89.6	25.9	43.8	69 (24.1)	8 (13.6)	**0.005**
PCR + APGL-I -	25.6	100	100	33.7	40.3	40 (14.0)	0 (0)	<**0.0001**
PCR- APGL-I +	31.4	86.4	86.9	30.5	45.6	53 (18.5)	8 (13.6)	**0.007**

[a]Chi-squared test between HD patients (HDP; n = 286) and non-HD patients (N-HDP; n = 59) positivity. For the serial evaluation, the + and - signs were used to represent positive and negative results, respectively. SSS, slit-skin smear; PCR, polymerase chain reaction; APGL-I, IgM anti-phenolic glycolipid-I; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; Acc, accuracy.

positive results for APGL-I serology when PCR was negative and reached the best accuracy (45.6%). The positivity in HDP for SSS when serology was negative had the lowest rate (3.5%; $P = 0.047$) (Table 2).

Concordance among the slit-skin smear, polymerase chain reaction, and anti-phenolic glycolipid-I

Analysis of concordance among the three tests was applied to evaluate the positivity of these exams in HD cases (Table 3). The overlap of the SSS results with PCR and APGL-I achieved moderate concordance, with kappa values ranging from 0.43 (CI: 0.33–0.55) and 0.41 (CI: 0.31–0.53), respectively. The analysis between PCR and APGL-I serology showed a concordance classified as fair with a kappa value of 0.33 (CI: 0.22–0.44).

Positivity overlap for the slit-skin smear, polymerase chain reaction, and anti-phenolic glycolipid-I exams

The performance generated by the Venn diagram showed the positivity overlap of the SSS, PCR, and APGL-I tests (60.5%; 173/286 HDP), which was 28.9% (50/173) for all assays; 1.7% (3/173) were only positive for the SSS, and the highest number of positive results isolated were for PCR (19.7%; 34/173) and APGL-I (24.9%; 43/173). PCR and APL-I were superior methodologies for the diagnosis of HD as compared with the SSS exam, which showed less than 61.5 and 73.1% overlap with APGL-I and PCR, respectively, compared with APGL-I and PCR overlap (Figure 2).

Correlation among anti-phenolic glycolipid-I serology, polymerase chain reaction, and bacillary load bacterial index from slit-skin smear

Correlation analyses were performed to assess the antibody levels and bacillary load. There was a moderate positive correlation between the index of APGL-I IgM and BI resulting from the SSS ($r = 0.53$; $P < 0.0001$) (Figure 3). The comparative evaluation between the positive PCR (PCR +) and negative PCR (PCR-) results in relation to BI-SSS showed that 92.3% (156) of HD PCR cases were also negative BI-SSS. PCR was positive in 51.3% (60) of the HDP when the BI-SSS was negative (BI = 0). The PCR results ranged from 1.2 to 2.4% in patients with BI-SSS scores between 1+ and 4+. HDP with BI-SSS 5 + and 6 + showed only PCR + results (Table 4).

Discussion

Our study was designed to evaluate the performance of three different laboratory assays (SSS, PCR, and APGL-I) to aid in the diagnosis of HD. The results obtained allowed the comparison between patients with HD (HDP) and patients referred to the outpatient clinic of dermatology with suspected HD due to dermato-neurological signs and symptoms, and the diagnosis of HD was ruled out through clinical and/or laboratory evaluation

TABLE 3 Concordance among the SSS, PCR and APGL-I results in HDP.

Overlap	kappa (*k*) value	95% CI	Standard error	Interpretation
SSS vs. PCR	0.437	0.33–0.55	0.057	Moderate
SSS vs. APGL-I	0.418	0.31–0.53	0.056	Moderate
PCR vs. APGL-I	0.331	0.22–0.44	0.057	Fair

SSS, slit-skin smear; PCR, polymerase chain reaction; APGL-I, IgM anti-phenolic glycolipid-I; HDP, HD patients; CI, confidence interval.

(N-HDP). The majority of HDP (68.9%) were classified as B clinical forms, and 85.7% were classified as MB forms (B and LL). Similarly, 67.3% of the cases reported worldwide by the WHO and 80.1% reported by the Brazilian Ministry of Health were MB diagnoses in 2020 and 2021, respectively (2, 18).

According to the WHO (3), the SSS is the only laboratory technique considered a cardinal sign used as a diagnostic criterion in bacteria identification, and it operationally classifies individuals between MDT PB and MB schemes when positive (3). In addition to being a tool that is highly dependent on the expertise of the performing professional and the patient's bacillary load, the main difficulty for its routine use is the low sensitivity in the diagnosis of initial cases with low bacterial load and exclusively neural HD (16). Thus, the goals proposed by the WHO (2) of having early diagnosis and laboratory techniques that identify subclinical infection are unfeasible (19). The SSS showed sensitivity and positivity of only 24.5%, despite its high specificity (100%), and the technique is classified as having low accuracy (37.4%) for the diagnosis of HD. Siwakoti et al. (13) demonstrated that the SSS sensitivity can be 18%, and microscopy has the advantage of being easily available at peripheral and referral centers; however, as its detection limit is 10^4 bacilli/ml, it suffers from low sensitivity (13). Additionally, Gurung et al. (20) reported that the SSS can have a sensitivity of 24–41% and a specificity of 93–100% (20).

The use of PCR and APGL-I techniques allowed an increase of 16.5 and 20.6%, respectively, for the identification of HD patients as compared with the SSS, although the SSS showed moderate concordance with the results of PCR and APGL-I serology. The quantitative evaluation of the SSS by BI shows a moderate positive correlation with APGL-I serology. On the other hand, 51.3% of the SSS results with negative BI had a positive PCR test. Thus, 38.5% of negative BI-SSS became positive in the PCR assay, a considerable improvement.

In the diagnosis of patients presenting only hypochromatic macular lesions investigations focusing almost exclusively on cutaneous signs, the inability of widely used tests to detect the different clinical forms and reaction states, and the identification of subclinical infection are challenges in controlling the magnitude of the disease and result in a non-timely diagnosis (14, 21, 22). The PCR technique consists of extracting, amplifying, and identifying *M. leprae* DNA in clinical samples, such as intradermal scrapings, with higher

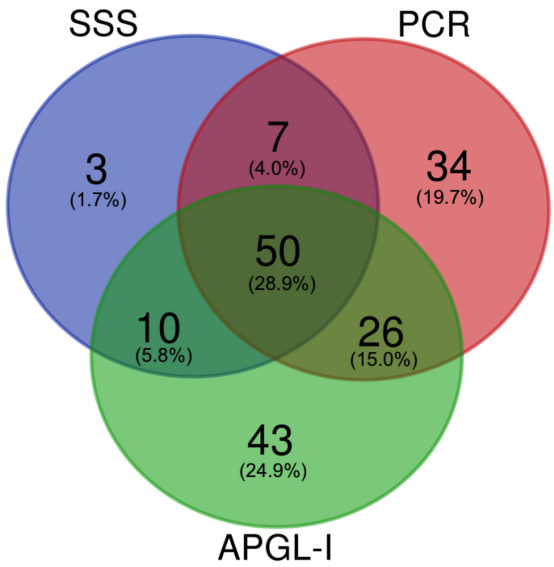

FIGURE 2
The Venn diagrams represent the overlap in the number of positive SSS, PCR, and APGL-I index examinations in HD patients. Data were represented in absolute value and percentage relative to the total of positive tests (n = 173/286). SSS, slit-skin smear; PCR, polymerase chain reaction; APGL-I, IgM anti-phenolic glycolipid-I.

laboratory accuracy, and it mitigates the main gaps with the use of the SSS alone in laboratory diagnosis across the clinical spectrum of HD. A compilation of published studies reports PCR sensitivity ranging from 51 to 91% and specificity ranging from 46 to 100% (20). Currently, different gene targets are being studied to support the specific detection of *M. leprae*. The gene sequence of specific repetitive element regions (RLEP) located along the mycobacteria genome is an established target for the molecular diagnosis of HD. The application of molecular assays with RLEP allows greater sensitivity by providing multiple copies throughout the genome. Therefore, PCR-RLEP assays provide fast and reliable results for molecular detection and quantification (16, 23).

In addition to presenting a 16.5% greater ability to diagnose new cases compared to SSS, PCR showed 100% specificity and accuracy with a 36.5% increased chance of diagnosis when positive and compared with controls (N-HDP). Corroborating the findings of other published studies, Azevedo et al. (16)

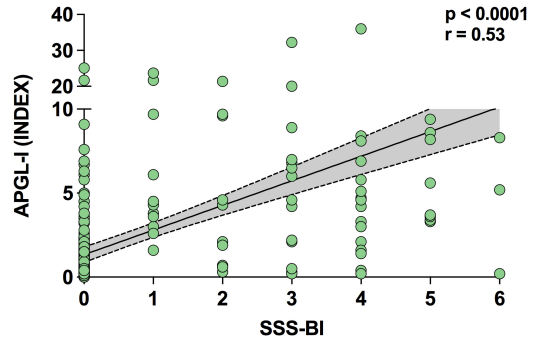

FIGURE 3
The APGL-I serology index shows a moderate positive correlation with the SSS bacillary load. Spearman's correlation coefficient was used to compare the bacterial index in the SSS (BI-SSS) with the APGL-I index. SSS, slit-skin smear; APGL-I, IgM anti-phenolic glycolipid-I.

TABLE 4 Comparison between PCR and bacillary load results in HDP.

BI-SSS	PCR– *n* (%)	PCR+ *n* (%)
0	156 (92.3)	60 (51.3)
1+	4 (2.4)	7 (6.0)
2+	4 (2.4)	9 (7.7)
3+	3 (1.7)	11 (9.4)
4+	2 (1.2)	19 (16.2)
5+	0 (0)	8 (6.8)
6+	0 (0)	3 (2.6)
Total	169 (100)	117 (100)

HD patients (HDP; n = 286). PCR, polymerase chain reaction; BI-SSS, bacterial index from slit-skin smear.

demonstrated that PCR was sensitive and 100% specific (16), while the detection of RLEP DNA in the SSS was higher when using qPCR, with 84% sensitivity, 75% specificity, and 77% accuracy (23). Thus, PCR-RLEP can be used as a complementary test for the diagnosis of HD regardless of the clinical form of the disease. The lower and variable sensitivity and accuracy of the molecular assay can be associated with the technique used (conventional or qPCR), gene target for identification of the bacteria, protocol used, and patients' bacillary load. In our study, 93.9% of the results were obtained by conventional PCR. The evaluation of the test performance to identify bacteria DNA showed a difference of only 4% less positivity with the use of the qPCR technique.

Despite showing a fair concordance between PCR and serology results for the detection of APGL-I, we found a difference of only 4.1% in the ability to detect the disease between these tests. On the other hand, APGL-I serology was 13.6% positive in cases without a clinical diagnosis of HD. APGL-I positivity has been mainly correlated with MB forms and with higher BI-SSS scores (24). Thus, laboratory assays using APGL-I and anti-LID-1 by ELISA and rapid test platforms with NDO-LID show low values of specificity and sensitivity and are not recommended for isolated use in the diagnosis of HD, considering the complexity of immunological presentations, and disease clinics (25). The study published by Frade et al. (25) demonstrated a sensitivity ranging between 48 and 62% and a specificity of 70% for APGL-I and anti-LID ELISA and 40% for NDO-LID. Also, 30% positivity for APGL-I serology among individuals with no history of contact with an HD patient, indicative of the generally high rate of exposure in the endemic region (25). Worobec (26) showed that subclinical *M. leprae* infection in endemic regions with APGL-I seropositivity was detected in 1.7–35% of all individuals, in view that the PGL-I antigen used in serology is species-specific (26). In our study, N-HDP showed 13.6% APGL-I ELISA positive, considering that the city was classified in the last year (2021) as very high endemicity, even in a low endemic state since 2006 as São Paulo.

Most of the published studies use IgM as a target molecule in serological assays because the seroprevalence of anti-PGL-I IgM is higher than the seroprevalence of IgA and IgG in endemic areas (27). IgM seropositive individuals are at increased risk of developing the disease (28); however, IgM seropositivity is not predictive of disease, as demonstrated with IgG APGL-I (29, 30). Therefore, the APGL-I serological assay is limited because it cannot be used as a predictive criterion for the diagnosis of the disease when there is no association with the neurological and/or dermatological diagnosis of HD.

The use of the same collection sites (intradermal scraping) for PCR processing provides the same interpretation of the SSS as a microbiological diagnostic criterion, which is the identification of bacilli in the host. However, detecting *M. leprae* DNA using a modern technique with greater sensitivity and 100% specificity allows us to rethink the use of the SSS in outpatient routines, as it is a methodology that presents low positivity. It could not detect 75.5% of the HD cases evaluated in our study despite 85.7% of the patients being classified as having clinical forms B and LL considered operationally MB. Similarly, the low accuracy occurred even with cases with higher bacillary load or more advanced stages of the disease and who were diagnosed in tertiary-level centers and highly specialized research centers in a national reference service comprising dermatologists and leprologists.

As a way of assisting in the assessment of bacillary load and therapeutic monitoring of cases, the use of qPCR offers a quantitative method, making it possible to assess the amount of DNA in the sample through a calculation considering the *Ct*-value, volume of DNA extract, volume of template, and mean RLEP copy number (31). The presence of *M. leprae* DNA was best detected in skin biopsies and skin scrapings independent of the extraction method or the clinical form. Interestingly, skin scrapings are less invasive samples and are the second-best clinical sample type for *M. leprae* detection (32). A study published by Gobbo et al.

(33) showed that increasing the number of Ct (>40) and using qPCR can provide greater sensitivity (86% positivity in HD cases), increasing the identification of early cases of the disease, household contacts and oligosymptomatic individuals (23, 33).

In view of the findings evidenced in the study, the use of the SSS as a way of diagnosing HD is presented as a low sensitivity and accuracy method due to its low performance in case detection. The use of molecular biology methodologies (PCR) and association with serological techniques allow for a more accurate clinical diagnosis of patients as well as identifying a greater number of individuals regardless of the clinical form or operational classification. Additionally, the need for implementation of serological techniques and PCR as complementary tests is not restricted to referral and research centers because we are delaying the diagnosis and treatment and consequently increasing the disability and stigma of HD patients. Therefore, bacilloscopy should be urgently rethought as the exclusive criterion for the laboratory diagnosis of HD as proposed by the WHO guidelines.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Review Board for Human Research of the HCFMRP-USP (MH-Brazil Project—Protocol number 16620/2014). The ethics committee waived the requirement of written informed consent for participation.

Author contributions

FL and MF substantially contributed to manuscript conception and design, acquisition of data, and analysis and interpretation of data. HL, FP, and AW contributed to the clinical care of patients. FL, MS, GA, GF, and VA contributed to acquisition of clinical data and review of the medical records. FL and NP contributed to execution and interpretation of the laboratory tests. FL, GM, and MF contributed to the statistical analysis and interpretation of the data. MF gave final approval of the final submitted version and any revisions, as well as provided supervision and orientation of the study. All authors contributed to the interpretation of the results and critical revision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer SV declared a shared affiliation with the authors to the handling Editor at time of review.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.972244/full#supplementary-material

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