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ORIGINAL ARTICLE

Inflammatory Cytokines Are Involved in Focal Demyelination in Leprosy Neuritis

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Abstract

Mycobacterium leprae (ML) infection causes nerve damage that often leads to permanent loss of cutaneous sensitivity and limb deformities, but understanding of the pathogenesis of leprous neuropathy that would lead to more effective treatments is incomplete. We studied reactional leprosy patients with (n = 9) and without (n = 8) acute neuritis. Nerve conduction studies over the course of the reactional episode showed the findings of demyelination in all patients with neuritis. Evaluation of patient sera revealed no correlation of the presence of antibodies against gangliosides and the clinical demyelination. In nerve biopsies of 3 patients with neuritis, we identified tumor necrosis factor (TNF), TNF receptors, and TNF-converting enzyme in Schwann cells (SCs) using immunofluorescence. To elucidate immunopathogenetic mechanisms, we performed experiments using a human SC line. ML induced transmembrane TNF and TNF receptor 1 expression in the SCs; TNF also induced interleukin (IL)-6 and IL-8 production by the SCs; and ML induced IL-23 secretion, indicating involvement of this previously unrecognized factor in leprosy nerve damage. These data suggest that ML may contribute to TNF-mediated inflammation and focal demyelination by rendering SCs more sensitive to TNF within the nerves of patients with leprous neuropathy.

Key Words: Demyelination, IL-23, Leprosy, Mycobacterium leprae, Neuropathy, Schwann cell, Tumor necrosis factor.

INTRODUCTION

Leprosy is an infectious disease characterized by skin lesions and peripheral nerve damage, which often results in serious sensory and motor dysfunctions that lead to the development of permanent deformities and/or disabilities (1). The etiological agent of leprosy, *Mycobacterium leprae* (ML), is an obligatory intracellular pathogen that preferentially infects macrophages and Schwann cells (SCs) (2, 3).

Leprosy remains a major health challenge due to the peripheral nerve damage and irreversible physical deformities it causes (4). Despite advances in knowledge regarding the pathogenesis of the leprosy spectrum, understanding of the mechanisms involved in nerve damage and regeneration in leprosy-associated neuropathy remains incomplete. The natural affinity of ML for peripheral nerves, particularly for SCs, makes it likely that leprosy nerve damage starts at a very early stage of infection but the mechanisms underlying nerve damage in early disease have not been elucidated (5). Nerve conduction studies (NCS) suggest the existence of a defined disease progression pattern despite clinically undetected alterations (6). There are, however, a number of controversies in the literature regarding the most highly affected parameters and the prevalence and evolution of different types of nerve lesions (7).

Studies in experimental animal models and with neuron and SC cocultures indicate that nonmyelinated axonal units are more susceptible to ML infection than myelinated ones, rendering the former the main target of this mycobacterium in the PNS (8). It has recently been reported that SCs in mice undergo genetic reprogramming upon long-term ML infection and that they assume a state of progenitor/stem-like cells uncommitted to the SC lineage (9). Moreover, ML can induce a large number of immune-related genes during the early stages of infection, rendering SC capable of eliciting an inflammatory response in the tissue and thus contributing to nerve damage (10, 11).

Loss of the myelin sheath is among the most damaging effects of nerve injury in many human diseases, and progressive demyelination can lead to permanent neurological dysfunction (12). The identification of antiganglioside antibodies that target neural molecules, including myelin itself, in patients with peripheral neuropathies such as Guillain-Barré syndrome have been described (13). However, there are no

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data linking this mechanism to the nerve injury observed in leprosy patients.

On the other hand, experimental evidence from both in vivo and *in vitro* studies point to the involvement of immune mediators such as tumor necrosis factor (TNF) in the immunopathogenesis of several inflammatory demyelinating disorders in the PNS and CNS (14). This cytokine is one of the first mediators to appear subsequent to experimental peripheral nerve damage (15, 16). High levels of TNF in neurological disorders have been shown to promote demyelination, axonal degeneration, increased nerve blood barrier permeability, and immune cell recruitment to the injury site (17). Moreover, TNF has been detected in the dermis and epidermis of leprosy reactional skin lesions and in the sera of reactional patients (18–21). In patients with reverse reaction and the pure neuritic form of the disease, an increase in the mRNA of this cytokine was observed in the peripheral nerves, suggesting that TNF plays an important role in the pathogenesis of leprosy nerve injury (22).

The lack of knowledge concerning the molecular and immunological mechanisms of ML-induced nerve damage in leprosy precludes the development of effective therapies for leprosy neuropathy. Although immunosuppression does not prevent nerve damage, corticosteroids are able to curb inflammation-associated symptoms such as pain.

The existence of a National Reference Center for Leprosy Neuropathy allowed us to select a group of reactional patients with and without acute neuritis in an attempt to clarify the potential mechanisms involved in the immunopathogenesis of nerve lesions in leprosy patients. We also evaluated the roles of serum antiganglioside antibodies and of TNF, including their possible applications as predictive markers.

MATERIALS AND METHODS

Patients

The study was conducted at the Leprosy Laboratory and Leprosy Out-Patient Unit of the Oswaldo Cruz Foundation in Rio de Janeiro, RJ, Brazil. Reactional leprosy patients were selected and classified according to the Ridley-Jopling scale (23). All patients provided their informed written consent, and the acquisition of all specimens was approved by the Human Ethics Committee of the Oswaldo Cruz Foundation.

Over a 1-year period, patients diagnosed with any type of leprosy reaction were evaluated for neurological complications. Only those who presented leprosy-related neuropathy and no contraindications for steroid treatment were included in the study.

For this work, reactions were defined as: (1) reverse reaction (ie, increased inflammation of existing lesions with or without nontender new lesions and/or acroedema that could be associated or not with neuritis (24)), (2) erythema nodosum leprosum (ie, the sudden appearance of inflamed papules, nodules, and plaques that are tender upon palpation). In this case, patients may be ill with inflammatory systemic symptoms associated or not with neuritis, and (3) neuritis (ie, one or more nerves may be enlarged, painful, or show loss of function). Neuritis may or might not be accompanied by skin lesions, in which case it is referred to as "isolated neuritis" (7).

Out of the 35 leprosy patients diagnosed as having a reaction within the year, 17 were found eligible and were included in the study (Table 1). All of the patients had neurological and neurophysiological examinations to diagnose leprous neuropathy. They were then sorted into 2 groups: patients with neuritis and those without neuritis (Table 1).

Clinical Evaluation

A detailed neurological examination was performed to record the number and distribution of affected nerves. The neurological examination evaluated the following: the motor strength and tactile sensation of large myelinated nerve fibers; thermal and pain sensation; the presence of erythrocyanosis on the palms and/or soles; paresthesia; and nerve pain. Sensory impairment, motor deficit, and disability/deformity status were assessed using standard methods. In brief, the tactile threshold was tested via Semmes-Weinstein monofilaments (25, 26). Thermal sensation was determined by the use of cold metal (15°C) objects, and a safety pin was utilized to ascertain pain perception. Disability was recorded in accordance with the standard World Health Organization grading criteria (27).

Neurophysiological Evaluation

The NCS was performed using the Nihon-Kohden apparatus using standard procedures (28). Amplitude, velocity, and latency were recorded for the median, radial, ulnar, and sural sensory nerves in addition to the median, ulnar, and peroneal motor nerves. The results of the conduction studies were used to identify: (1) normal nerves, (2) axonal lesions, which were defined by a reduction in compound muscle action potentials (CMAP), and/or sensory nerve action potentials (SNAP), with the amplitude being less than 30% of the reference value and/or the maximum conduction velocity (MCV) being above 70% of the reference value (7), (3) demyelination lesions, which were defined in cases when the CMAP and/or SNAP latency were prolonged in comparison to the reference together with a reduction in sensory conduction velocity and/or an MCV below 85% of the reference value, (4) mixed lesions, which were defined when both axonal and demyelinating lesions were detected in the same nerve, and (5) no conduction, defined as an increase in the duration of the proximal distal CMAP of over 30% (29).

Histopathological Analysis

The sural nerves of 3 patients were biopsied for histopathological diagnosis. These biopsies were performed because there was a suspicion of pure neural leprosy or another superimposed neuropathy in addition to leprosy. These patients had a persistent and prolonged course of reactional episodes, refractoriness to antireactional treatment and leprosy relapse. The samples were submitted for routine histopathological processing followed by staining with hematoxylin and eosin and Wade stain for detection of acid-fast bacilli; 500-μm-thick sections were then stained with toluidine blue and examined in detail for detection of fine nerve fiber alterations.

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TABLE 1. Clinical Data

Patient No.	Clinical Form	Reaction	Multidrug therapy	Age (Years)	Gender	Degree of Disability	Mean Bacilloscopy Index
Patients without neuritis							
1	LL	ENL	During	24	M	0	5.0
2	BL	RR	During	39	F	0	3.2
3	BT	RR	After	67	F	1	0
4	LL	RR	During	30	M	1	5.0
5	BT	RR	After	51	F	0	0
6	BB	RR	During	56	F	1	0.5
7	BT	RR	After	57	F	0	0
8	BL	RR	During	66	M	1	0
Patients with neuritis							
9	BT	Neuritis	After	33	M	0	0
10	BL	RR + Neuritis	During	66	M	1	1.5
11	BL	Neuritis	During	57	M	1	2.5
12	BL	Neuritis	After	19	M	0	4.25
13	LL	ENL + Neuritis	After	34	M	2	5.0
14	BT	RR + Neuritis	During	58	M	2	0
15	BB	RR + Neuritis	During	55	F	1	0.75
16	LL	ENL+ Neuritis	After	75	M	1	2.75
17	BT	Neuritis	During	54	M	1	0

ENL, erythema nodosum leprosum reaction; F, female; M, male; RR, reverse reaction.

Schwann Cell Line and Culture

The human SC line ST8814 isolated from a malignant schwannoma of a patient with neurofibromatosis 1 (30) was generously donated by Dr J.A Fletcher (Dana Farber Cancer Institute, Boston, MA). The cells were cultured in RPMI media (Gibco BRL, Grand Island, NY) supplemented with 100 U/mL of penicillin, 100 mg/mL of streptomycin, 2 mM of L-glutamine, and 15% fetal bovine serum (Hyclone Laboratories, South Logan, UT) in a humidified CO_2 incubator at 37°C. For experimental assays, the cells were detached from the culture bottles via trypsin/EDTA (0.25%, 1 mmol/L) (Gibco BRL), washed, suspended in complete medium, and cultured in plates as needed for *in vitro* assays.

Reagents and Stimuli

Irradiated armadillo-derived ML were provided by Dr P. Brennan (Department of Microbiology, Colorado State University, Fort Collins, CO) and used at a dosage (μg/mL) corresponding to a multiplicity of infection of 50 bacteria:cell in each experiment. The endotoxin level, measured using a limulus amoebocyte lysate assay, was found to be below 50 IU/mg (Cambrex, East Rutherford, NJ) . Recombinant human TNF (RhTNF) (Calbiochem, Merck-Millipore, Darmstadt, Germany) was used in a 25 ng/mL concentration.

For protein expression analyses, the following monoclonal antibodies (mAbs) were purchased: anti-TNF (mAb 210, R&D Systems, Minneapolis, MN); anti-TNF receptor 1 (TNFR1) (mAb 225, R&D Systems); anti-TNF receptors 2 (TNFR2) (mAb 226, R&D Systems); anti-TNF converting enzyme (TACE, mAb 9301, R&D Systems); and anti-CD44 (M7082, Dako, Glostrup, Denmark). Polyclonal anti-S100 antibody (Z0311, Dako) was also obtained.

In Vitro Assay Conditions

For TNF protein expression evaluation, ST8814 SCs were cultured in 12-well plates at 37°C/5% CO_2 and stimulated with irradiated ML for 3, 5, and 7 hours. Whole-cell extracts were obtained for Western blot and enzyme-linked immunosorbent assay (ELISA) analyses. TNF surface levels were investigated by immunofluorescence after the cells were cultured on glass coverslips in 24-well plates and stimulated with irradiated ML for 3 and 7 hours. Phenotypic characterization of the ST8814 line was performed in the same way in the absence of stimuli. TNF secretion was evaluated through analysis of the culture supernatants after stimulation of ST8814 SCs seeded in 12-well plates with irradiated ML for 3, 6, and 24 hours by Western blot and ELISA.

TNFR1 and TNFR2 gene expressions were measured in ST8814 SCs cultured in 6-well plates and stimulated with RhTNF and irradiated ML associated or not for 24 hours. The RNA of these cells was obtained for semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, and the culture supernatants were evaluated by ELISA to assess inflammatory cytokine secretion.

Western Blot

Soluble and transmembrane TNF expression was assessed by Western blot analysis. Whole-cell extracts were obtained after detachment of ST8814 SCs from the culture plates. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated for 30 minutes with lysis buffer and protease inhibitor cocktail II (Calbiochem), as described elsewhere (31). Proteins (30 μg) from extracts and culture supernatants were resolved in 12% sodium dodecyl sulfate polyacrylamide gels and blotted onto nitrocellulose membranes

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(Bio-Rad Laboratories, Hercules, CA). Membranes containing supernatant samples were stained with Ponceau S for protein-loading evaluation. After blocking with a solution of 5% bovine serum albumin (BSA) in tris-buffered saline (TBS) 0.15% Tween (TBS-T), blots were incubated for 1 hour at room temperature with anti-TNF (0.3 μg/mL). Blots with the extracts were also probed for α-tubulin (T6074, Sigma-Aldrich, St. Louis, MO) (0.4 μg/mL), as controls of the protein load. Anti-mouse horseradish peroxidase-conjugated immunoglobulin G (IgG) (Dako) was used as a secondary antibody in a 1:2000 dilution. An enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) was used. The densities of the bands were determined using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA).

Immunofluorescence

Immunofluorescence assays were performed in SC cultures and frozen tissue sections to evaluate surface-level TNF and TNF-related molecules. ST8814 SCs were cultured onto 24-well plates containing glass coverslips covered with 4% silane (Sigma-Aldrich). The samples were washed with PBS and fixed with 4% paraformaldehyde. To evaluate surface-level proteins, samples were blocked with 10% goat serum and 10% BSA in PBS for 1 hour at room temperature and were then incubated with anti-TNF (1:50), anti-CD44 (1:100), and anti-S100 (1:200) in 1% BSA/PBS overnight at 4°C.

Frozen sections (5 μm thick) of leprosy nerve tissues (n = 3) were also obtained and fixed with cold acetone. For protein surface-level evaluation the sections were blocked with 5% goat serum and 10% BSA in PBS for 1 hour at room temperature. The samples were then incubated with anti-TNF (1:50), anti-TNFR1 (1:50), anti-TNFR2 (1:100), and anti-TACE (1:10) associated or not with anti-S100 (1:200) in 1% BSA/PBS overnight at 4°C.

After rinsing with PBS, either Alexa 532 goat anti-mouse or Alexa 633 goat anti-rabbit IgG secondary antibody was added to both SC cultures and frozen sections for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (D9542, Sigma-Aldrich) and coverslips were mounted with Permafluor (Thermo Scientific, Waltham, MA). Samples were analyzed by an Axio Observer Z1 Colibri microscope (Zeiss, Oberkochen, Germany) and images were processed via AxioVision software (Zeiss).

RNA Isolation and Semiquantitative RT-PCR

ST8814 SC cultures plated in 6-well plates were suspended in 1 mL of TRIzol® reagent (Life Technologies, Carlsbad, CA). Total RNA was extracted according to the manufacturer's instructions. cDNA was synthesized via Superscript III first-strand RT-PCR Kit (Invitrogen, Carlsbad, CA). Semiquantitative analysis of TNFR1 (*tnfrsf1a*) and TNFR2 (*tnfrsf1b*) mRNA was performed in ST8814 cultures as previously described (32). Sequences for *tnfrsf1a* (gene ID: 7132) were: 5′-ATTTGCTGTACCAAGTGCCACAAAGGAACC-3′ and 5′-TCGATTTCCCACAAACAATGGAGTAGAGC-3′. Sequences for *tnfrsf1b* (Gene ID: 7133) were: 5′-GAATACTATGACCAGACAGCTCAGATGTGC-3′ and 5′-TATCCGTGGATGAAGTCGTGTTGGAGAACG-3′ (Applied Biosystems, Foster City, CA). The primers were designed to avoid genomic DNA amplification. PCR was performed, as previously described (33), and the samples were amplified in a DNA thermocycler 2400 (Perkin Elmer, Waltham, MA). PCR products were subjected to electrophoresis in 1.7% agarose gels at which time the specificity of the amplified bands was validated by their predicted size (α-*actin*, 661 bp; *tnfrsf1a*, 586 bp; and *tnfrsf1b*, 402 bp). Densitometry analysis was performed by scanning the gel images (Video Documenting System, Amersham-Pharmacia Biotech inc., Piscataway, NJ), and values were obtained via Image Master VDS Software (GE Healthcare Life Sciences, UK).

ELISA

IgG and IgM antibodies to the gangliosides asialo-GM1 (GA1), GM1, GM2, GD1a, GD1b, GQ1b were determined by Ganglio Combi ELISA (Bühlmann Laboratories, Schönenbuch, Switzerland) in serum samples from 13 leprosy patients. Results were expressed as a percent ratio of a highly positive control and categorized as either negative (<30%), borderline (30%–50%), or moderately (50%–100%) or strongly positive (>100%). The values obtained from 3 healthy blood donors served as a reference group.

TNF, IL-23, IL-8, IL-6, and IL1-β concentrations in cell-free ST8814 culture supernatants (30 μg of total protein) and patient sera were determined using commercial ELISA kits and processed according to the manufacturer's specifications. Specific kits for IL-23, IL-8, IL-6, and IL1-β were purchased from R&D Systems. Evaluations of TNF expression and secretion in whole-cell extracts and supernatants were performed using a high sensitivity kit (lowest detection limit of 4 pg/mL) from Ebioscience (San Diego, CA).

Statistical Analysis

Results were expressed as mean ± SE (standard error). The Kolmogorov-Smirnov test was performed to evaluate if samples came from a Gaussian distribution as well as to determine the appropriate statistical test. Significant differences between 2 groups were determined by the Wilcoxon nonparametric test or the paired *t*-test in the experimental data. The Kruskal–Wallis test and a post-hoc test were used to compare more than 2 groups. Pearson chi-squared test corrected by Fisher exact test was employed for clinical data evaluation. The adopted statistical significance level was $p \leq 0.05$. Analyses were performed using Windows GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

RESULTS

Leprosy Patients With Neuritis Exhibit Evidence of Nerve Demyelination

The 17 patients (64.7% male, mean age 49.4 ± 1.6 years) evaluated were sorted into 2 groups. The group without neuritis consisted of 8 patients (47.1%) and the group with neuritis included 9 patients (52.9%). The demographic findings of the patients are presented in Table 1. Fifty-nine percent (n = 10)

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were receiving multidrug therapy. Most (64.7%, n = 11) were treated with the multibacillary scheme because they had the POLAR lepromatous (LL), borderline lepromatous (BL), or mid-borderline (BB) forms. Only 2 patients (11.7%) had permanent disabilities and/or acral ulcers at the time of diagnosis (ie, a disability grade 2, according to the World Health Organization grading system (34)).

The histopathological evaluation of the nerve biopsy specimens in the neuritis group revealed a significant reduction in the quantity of myelinated nerve fibers. Moreover, there was endoneurial fibrosis along with an inflammatory infiltrate that consisted of lymphocytes and macrophages distributed across the 3 nerve compartments (Supplementary Data 1). Foamy macrophages forming perivascular clusters were present in 1 section while an excessive extracellular matrix with a fibrotic appearance occupied the whole endoneurium of the fascicles, leaving some residual inflammatory cells and microvessels behind. Wade staining revealed acid-fast bacilli in all of the samples (Supplementary Data 1).

Clinical examination showed that the neuritis group exhibited a greater frequency of nerve enlargement (66.6% vs the group without neuritis [0%], $p < 0.004$), nerve pain (77.8% vs 12.5%, $p < 0.007$), sensory impairment (88.9% vs 50%, $p < 0.07$), and motor impairment (66.7% vs 0%, $p < 0.004$). Paresthesia frequency was not significantly different between the groups (66.7% in the neuritis group vs 37.5% in the non-neuritis group, $p = 0.22$). Seven patients in the neuritis group felt pain during nerve palpation; 1 patient who reported nerve pain had characteristics of neuropathic pain.

The NCS studies indicated that the most frequent pattern in the sensory examinations in the group without neuritis was mononeuropathy multiplex (62.5%). Patients with neuritis showed a greater percentage of polyneuropathy (55.5%). In the motor NCS, mononeuropathy and mononeuropathy multiplex were found at the same frequency (37.5%) in the neuritis group; 25% of patients without neuritis were normal. Among the patients with neuritis, 22.2% displayed polyneuropathy and 66.6% had mononeuropathy multiplex. Importantly, the NCS results of all the individuals in the study were altered.

In the sensory NCS, in the group without neuritis, the number of nerves with no conduction disturbances (normal nerves) was significantly higher than in the neuritis group ($p < 0.000482$). However, in this group, the number of nerves with no conduction was significantly more frequent ($p < 0.00 0001$). The motor NCS showed a higher demyelination rate in the neuritis group (15 motor nerves [27.8%] vs 5 [10.4%] in the group without neuritis, $p = 0.027502$). When the total number of nerves presenting any type of alteration was considered in each group (25% in the group without neuritis vs 61.2% in the neuritis group), the frequency was significantly higher in the neuritis group ($p < 0.0005$; $X^2 = 24.99$) (Table 2).

Demyelination was detected in 3 patients (5 motor nerves) without neuritis in the motor NCS. Demyelination in 2 of these patients could not be attributed to leprosy reaction because both presented with carpal tunnel syndrome in the median nerve; however, the possibility that the third patient was afflicted with silent neuritis cannot be ruled out (Table 2). As in the sensory NCS, the number of nerves without any detectable injury (normal nerves) was significantly greater in the group without neuritis ($p = 0.000246$).

Antiganglioside Antibody Screening Is Not a Reliable Indicator of Peripheral Nerve Demyelination in Leprosy Patients

Serum samples from 13 patients (8 with and 5 without neuritis) were screened for the presence of antibodies against GA1, GM1, GM2, GD1a, GD1b, and GQ1b (Table 3). All of

TABLE 2. Neurophysiological Findings in Patients With and Without Neuritis

	Without Neuritis (n = 8)	Neuritis (n = 9)	Total (n = 17)	p Value
Sensory nerves				
Number of nerves evaluated	n = 64	n = 72	n = 136	
Normal	27 (42.2%)	11 (15.2%)	38 (28%)	<0.000482
Axonal	13 (20.3%)	19 (26.4%)	32 (23.5%)	0.404
Demyelinating	0	0	0	-
Mixed	0	0	0	-
No conduction	6 (9.4%)	30 (41.7%)	36 (26.5%)	< 0.000001
No classification	18 (28.1%)	12 (16.6%)	30 (22%)	< 0.107
Motor nerves				
Number of nerves evaluated	n = 48	n = 54	n = 102	
Normal	36 (75%)	21 (38.9%)	57 (56%)	0.000246
Axonal	1 (2.1%)	3 (5.55%)	4 (3.9%)	0.367194
Demyelinating	5 (10.4%)	15 (27.8%)	20 (19.6%)	0.027502
Mixed	0	0	0	-
No conduction	0	3 (5.55%)	3 (2.9%)	0.097408
No classification	6 (12.5%)	12 (22.2%)	18 (17.6%)	0.198581
Temporal dispersion	0	3		

Compound muscle action potentials (CMAPs) and sensory nerve action potentials (SNAPs) were defined by combining the nerve conduction study parameters as defined in "Materials and Methods." The definition of partial conduction block (CB) depended on a 50% or higher reduction in the proximal compared to the distal amplitudes (29).

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TABLE 3. Antiganglioside Antibodies in Leprosy Patient and Control Sera

Patient No.	Reaction	Nerve Demyelination	IgG						IgM					
			GA1	GM1	GM2	GD1a	CD1b	GQ1b	GA1	GM1	GM2	GD1a	CD1b	GQ1b
					Patients without neuritis									
1	ENL	No	20.7	16.4	18.2	22	23.1	16	59.4*	76.8*	15.1	15.1	31.1#	11
3	RR	No	10.5	9.3	11.6	23.8	22	15.8	4.3	4.8	5.5	4.8	4.5	3.6
4	RR	Yes	21.5	23.5	22.1	24	25.2	19.3	67.2*	47.6#	28.4	11.3	45.1#	16.1
5	RR	Yes	10.2	9.6	10.4	25.3	12.9	11.5	8.7	12.4	4.1	8.2	4.6	4.1
7	RR	No	7.9	7.2	8.3	8.1	8.5	9.6	23.3	12.1	11.5	6.7	8.9	8.1
					Patients with neuritis									
10	RR + Neuritis	Yes	23	16.6	23.1	23.1	31.2#	19.7	64.9*	17.5	23.9	17.6	17.3	19.3
11	Neuritis	Yes	14	12.7	14.9	31.6#	26.9	18	14.1	9.7	5.8	9.7	9.5	6.7
12	Neuritis	Yes	21.2	19.8	19.8	19.8	17.1	17.3	46.4#	74.6*	18.8	15.2	20.5	14.9
13	ENL+ Neuritis	Yes	11.2	8.4	11.6	18.7	8.5	9.7	15.2	18.1	10.8	4.7	5.6	5.5
14	RR + Neuritis	Yes	12	10.2	11.1	15.6	15.5	12.5	9.6	7.2	4	8.8	6	4.2
15	RR + Neuritis	Yes	11.3	10.9	10.9	18.7	13.1	10.5	15	7.1	4.2	8.5	5.8	4.6
16	ENL + Neuritis	Yes	16.9	11.5	14.4	18.2	20.2	31.6#	13	6.1	6.3	6.3	4.7	4
17	Neuritis	Yes	35#	26.5	40.5#	37#	20.7	57*	23.8	19.5	10.4	13.9	10.6	9.3
					Healthy controls									
18	Healthy control	NE	32.1#	23.2	29.2	20.8	19.9	21.1	31.7#	18.8	18.9	18.4	45.9#	35.5#
19	Healthy control	NE	156.9**	18.7	20.0	25.8	22.5	19.9	55.4*	13.4	17.1	15.4	14.1	90.2*
20	Healthy control	NE	31.13#	21.2	52.1	35.9#	21.6	23.5	71.0*	21.3	30.7#	21.7	24.6	39.8#

ENL, erythema nodosum leprosum; NE, not evaluated; RR, reverse reaction.

Levels of antiganglioside antibodies (immunoglobulin G [IgG] and M [IgM]) were assessed in the sera of 13 leprosy patients with or without neuritis by enzyme-linked immunosorbent assay (ELISA). Results of the IgG and IgM groups are expressed as % ratio of a highly positive control and categorized as negative (<30%), #borderline (30%–50%), or *moderately (50%–100%) or **strongly positive (>100%). Values obtained from 3 healthy blood donors served as a reference group.

the patients in this group with neuritis had NCS evidence of demyelination in one or more peripheral nerves even though the frequency of autoantibodies against gangliosides in the serum did not correlate with the conduction disturbances detected in the NCS. With respect to IgM antibodies, the sample from only 1 patient with neuritis (12.5%) (with associated demyelination) was immunoreactive to GA1, and only 1 other (12.5%) to GM1. Similarly, elevated IgG antibody levels to GQ1b were found in 1 patient with neuritis. On the other hand, 2 patients without neuritis (25%) (one with and the other without demyelination) showed elevated IgM anti-GA1 levels. All healthy control sera exhibited different levels of IgM and IgG GA1 and IgM GQ1b antibodies.

TNF Is Present in the Serum and Peripheral Nerves of Leprosy Patients With Neuritis

Our previous work showed that the peripheral nerves of leprosy patients diagnosed with neuritis had higher TNF gene expression than was found in normal nerves (11). Here, we evaluated the protein expression of this cytokine and related molecules in nerve lesions of 3 patients by immunofluorescence. TNF protein, TNF receptors, and TACE were most often expressed by SCs (Fig. 1). This led us to investigate the role of this cytokine in the pathogenesis of leprosy nerve damage via SC cultures.

ML Induces Transmembrane TNF Protein Expression and TNFR1 Gene Expression in the Human SC Line ST8814

We previously found that the human SC line ST8814 is nonmyelinating and expresses S-100, CD44, laminin, and GFAP (35). To evaluate the effects of ML infection on TNF production in human SCs, ST8814 cultures were stimulated with irradiated ML at different time points after which TNF protein expression was assessed by Western blot, ELISA, and immunofluorescence assays. As indicated by the protein expression analysis of whole-cell extracts via Western blot and ELISA, membrane-bound TNF (mTNF) was upregulated by ML stimulation at 3 and 7 hours after exposure (Fig. 2A, B). The evaluation of TNF surface levels in ST8814 cultures after ML stimulation by immunofluorescence illustrated that the bacteria not only induced TNF protein expression, but also promoted molecular insertion in the cell membrane (Fig. 2C). On the other hand, ST8814 culture supernatants after ML stimulation did not demonstrate soluble TNF (sTNF) at either the early or late time points (Fig. 2D, E).

Both the soluble and membrane-bound forms of TNF exercise their biological effects through interaction with TNFR1 or TNFR2 (36). The next step involved evaluating ML modulation of TNF receptors in human SC ST8814. Gene expression analysis of TNFR1 and TNFR2 after stimulation with irradiated ML for 24 hours by semiquantitative RT-PCR showed

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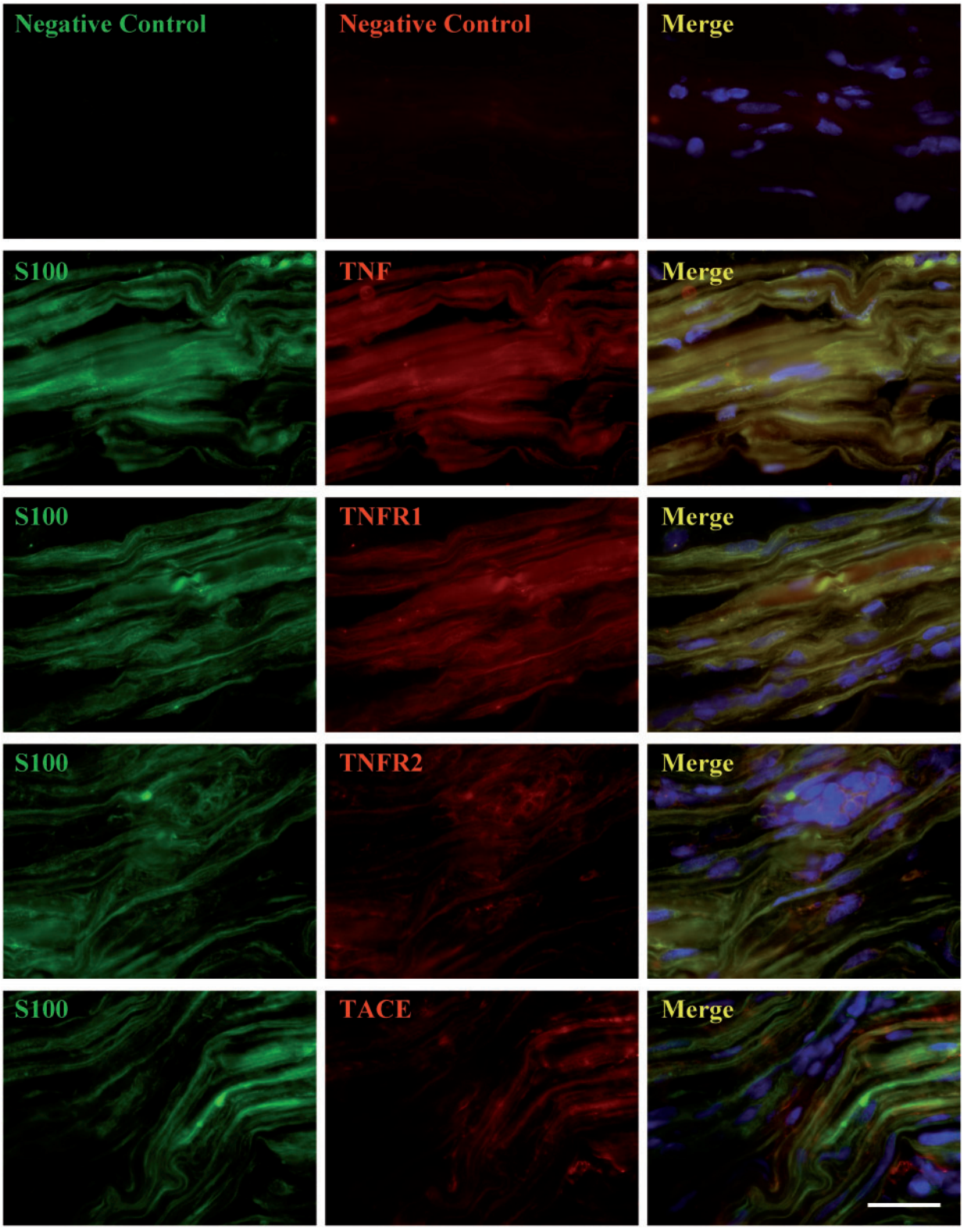

FIGURE 1. Immunofluorescence analysis of peripheral nerves of leprosy patients for tumor necrosis factor (TNF), TNF receptors 1 (TNFR1) or 2 (TNFR2), and anti-TNF converting enzyme (TACE) expression in Schwann cells (SCs). Longitudinal cryosections were stained with anti-S100 (green), a specific phenotypic marker of human SCs, together with anti-TNF, -TNFR1, -TNFR2, or -TACE (red). Nerve lesions of 3 leprosy patients with neuritis were evaluated. Negative controls were made without the primary antibodies. Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar = 50 μm.

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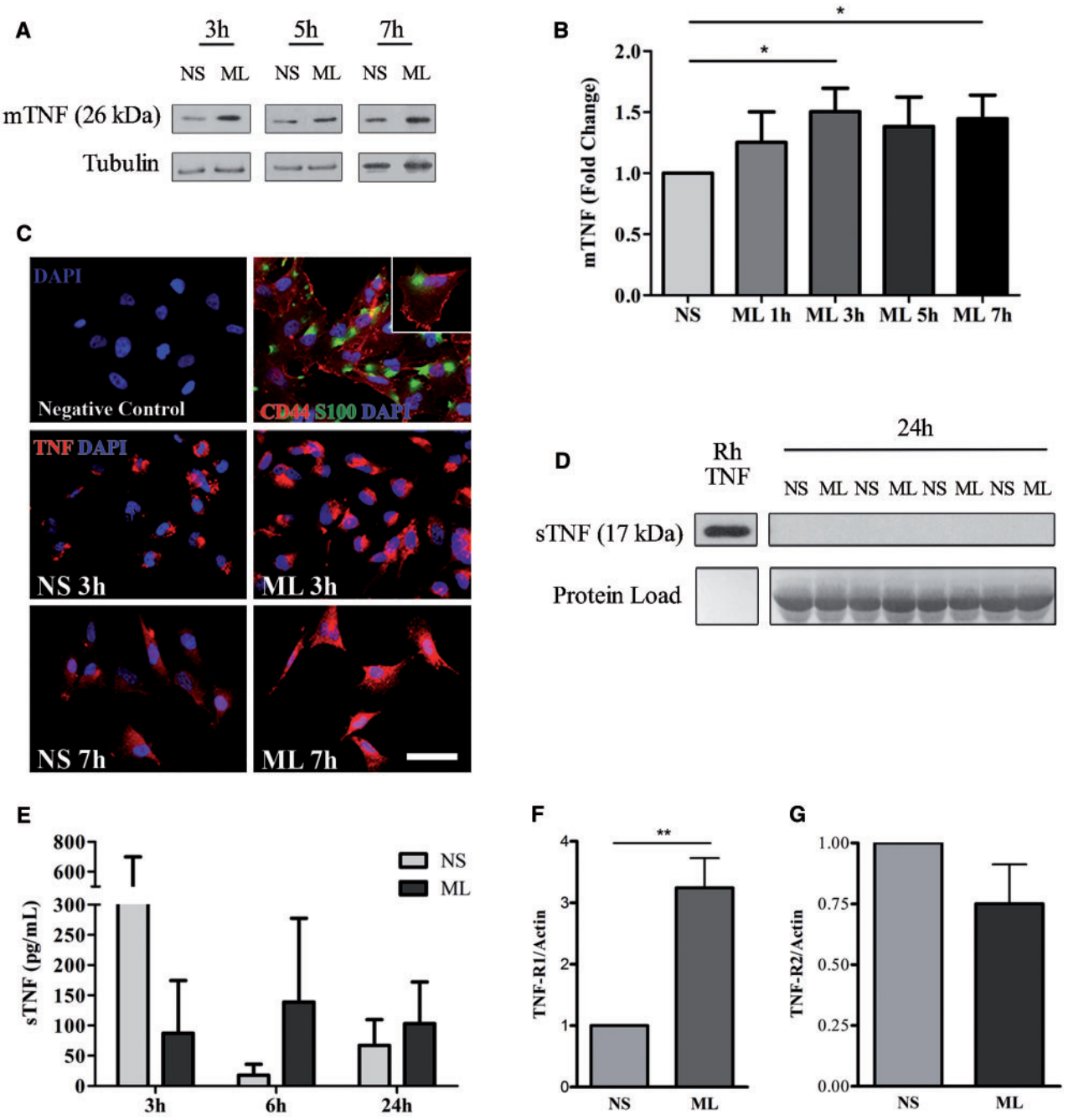

FIGURE 2. *Mycobacterium leprae* (ML) induces the expression of membrane-bound tumor necrosis factor (mTNF) but not its secretion in the human Schwann cell (SC) line ST8814. **(A)** Evaluation of tumor necrosis factor (TNF) protein expression in ST8814 SCs after stimulation with irradiated ML for 3, 5, and 7 hours (h) by Western blot. **(B)** Evaluation of TNF expression in protein extracts from ST8814 SCs after stimulation with irradiated ML for 1, 3, 5, and 7 hours by enzyme-linked immunosorbent assay (ELISA). **(C)** Phenotypic characterization of ST8814 SCs with CD44 (red) and S100 (green) staining (upper panels). Evaluation of TNF surface levels (red) in ST8814 SCs after stimulation with irradiated ML for 3 and 7 hours by immunofluorescence (lower panels). Nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Scale bar = 50 μm. **(D)** Evaluation of TNF secretion in supernatants from ST8814 SC cultures after stimulation with irradiated ML for 24 hours by Western blot; 50 ng of RhTNF were used as a positive control. Protein load was assessed by Ponceau S staining. **(E)** Evaluation of TNF secretion in supernatants from ST8814 SC cultures after stimulation with irradiated ML for 3, 6, and 24 hours by ELISA. The ELISA and Western Blot experiments used 30 μg of total protein. **(F, G)** Evaluation of TNF receptors 1 (TNFR1) and 2 (TNFR2) gene expression in ST8814 SC cultures after stimulation with irradiated ML for 24 hours by reverse transcriptase-polymerase chain reaction (RT-PCR). Results are expressed as mean ± SE and are representative of 4 or more independent experiments. Comparison between 2 groups was performed using paired t-test. NS = nonstimulated.

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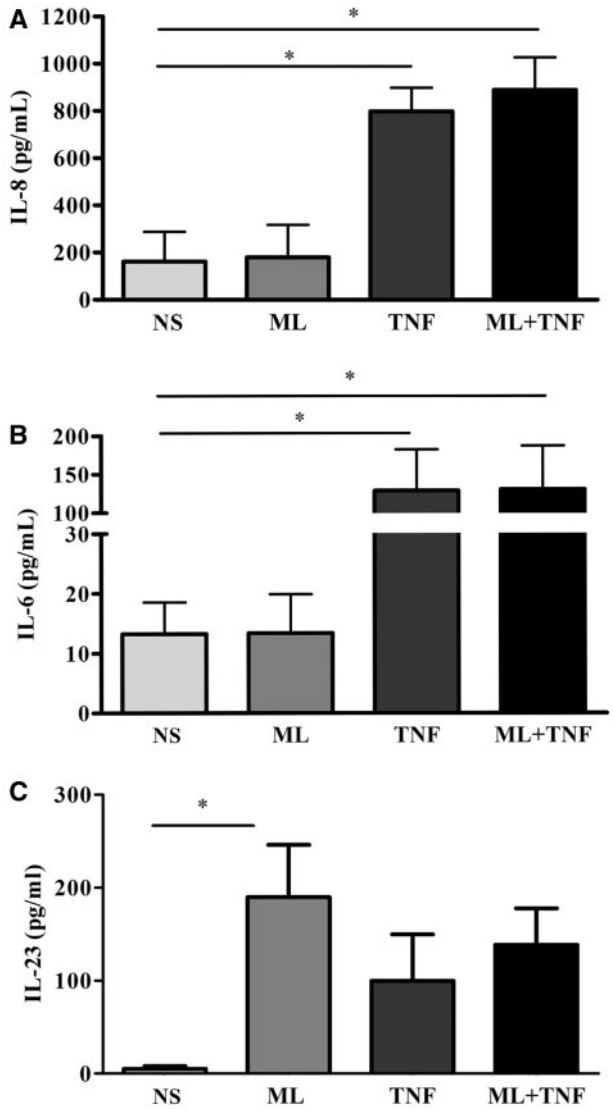

FIGURE 3. *Mycobacterium leprae* (ML) and tumor necrosis factor (TNF) induce inflammatory cytokines in the human Schwann cell (SC) cell line ST8814. **(A–C)** Evaluation of interleukin (IL)-6 **(A)**, IL-8 **(B)**, and IL-23 **(C)** secretion in ST8814 SC cultures after stimulation with RhTNF and irradiated ML associated or not for 24 hours by enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean ± standard error (SE) and are representative of 4 or more independent experiments. Comparison between 2 groups was performed using the paired t-test (IL-8) or the Wilcoxon nonparametric test (IL-6). Comparison among 4 groups was determined by the Kruskal–Wallis test (IL-23). NS = nonstimulated. *$p \leq 0.05$.

that ML was able to induce the upregulation of TNFR1 but not of TNFR2 (Fig. 2F, G).

ML Induces Secretion of IL-23 in the Human Schwann Cell Line ST8814

The capacity to produce inflammatory cytokines allows SCs to modulate immune responses. We next investigated whether ML and/or TNF were able to induce secretion of inflammatory cytokines in the human SC line ST8814. Concentrations of different molecules in the supernatants of ST8814 cultures stimulated with RhTNF and irradiated ML either alone or together for 24 hours were assessed by ELISA. ML was not able to induce the secretion of IL-6 or IL-8 (Fig. 3A, B). Only RhTNF stimulation led to the release of these cytokines in the SC cultures while the combination of TNF and ML did not differ from the effect caused by TNF alone. Interestingly, IL-23 was detected in the supernatants of SC cultures stimulated with irradiated ML for 24 hours (Fig. 3C).

DISCUSSION

To the best of our knowledge, this is the first report linking demyelination and acute neuritis in leprosy. In this study, 17 leprosy patients undergoing reaction with clinical signs of neurological complications were evaluated and divided according to the presence or absence of neuritis. Although reaction has been considered to be an important factor underlying nerve damage, we found that only those patients who had acute neuritis had demyelination in the NCS. In addition, patients diagnosed with neuritis exhibited more nerve dysfunction based on the NCS; motor impairment was more frequent in the neuritis group. Based on the NCS data, the patients with neuritis had more extensive nerve involvement with a greater number of nerves with demyelination and absence of nerve conduction than the group without neuritis. Neuritis patients also had more clinical symptoms than the non-neuritis group (data not shown).

Demyelination in the group without neuritis could be due to the development of silent neuritis in which the patient has no pain symptoms and the diagnosis requires serial neurological examinations, particularly prior to possible initiation of steroid treatment.

It is not known whether demyelination is a consequence of the inflammatory process in neuritis or if it is directly induced by ML, as reported by Rambukana et al (8). Persistent demyelination is associated with axonal damage, which progressively compromises large fibers, leading to motor impairment (7). Damage of the myelin sheath may be a consequence of the inflammatory process resulting either from humoral immunity or the release of immune mediators (37, 38), but the precise mechanisms of demyelination in leprosy neuropathy are still unclear.

It is worth emphasizing that nerve samples taken for diagnosis in pure neural leprosy patients show advanced stages of nerve damage with severe fiber loss, inflammatory infiltrate, and/or endoneurial fibrosis (39); thus, demyelination may not be identified in histopathological analyses in earlier stages. However, the patient nerve samples we analyzed here showed remyelinated fibers indicating that there had previously been demyelination. NCS are clinically more efficient in detecting demyelination because extensive nerve histopathological analyses are not feasible. It is plausible that demyelination occurs in acute neuritis and that it is followed by remyelination in some patients who recover nerve function.

We found that antiganglioside autoantibodies were not significantly associated with the detection of demyelination by NCS. Thus, they could not be employed as a predictive

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marker for demyelination. We also evaluated serum levels of TNF, IL1-β, and IL-10 but found no significant differences between patients with and without neuritis (data not shown); this is likely because of the small numbers of samples evaluated. On the other hand, evaluation of TNF and related molecules in leprosy nerve lesions showed expression of the cytokine together with both the receptor and shedding enzyme TACE in the SC populations, in agreement with our previous study (22). These data suggest that the possible role of TNF in leprous neuropathy is local and restricted to the site of the injury with little systemic effects.

In painful neuropathy caused by chronic constriction injury, 2 peaks of TNF production were identified: the first promoted by local cells such as SCs and resident macrophages, and the second most likely caused by recruited macrophages from the blood (15). TNF gene expression was also detected in the sciatic nerves of mice after 1 hour of chronic constriction injury (16), and 24 hours after injury in a model of enhanced axon regeneration (40). Thus, endogenous TNF released by resident populations in the PNS occurs early after nerve damage.

An immediate increase in TNF expression is known to induce massive macrophage infiltration (41), which is probably enhanced by metalloproteinases (MMPs) (42). TNF is also an upstream activator of MMP-9, a molecule involved in myelin basic protein degradation. Therefore, TNF expression is likely also related to inflammation-mediated demyelination (15). Indeed, poor macrophage recruitment and delayed myelin removal in following sciatic nerve transection have been reported in TNF-deficient mice (41).

High expression of TNF and related molecules in SCs in the nerves of leprosy patients led us to speculate about the effect of TNF on SCs upon exposure to ML *in vitro* and the possibility of TNF contributing to nerve damage. Although ML exposure resulted in upregulated protein expression of mTNF with cell membrane staining, ML did not stimulate the release of sTNF into the extracellular space at either the early or late time points analyzed. These results are in agreement with a previous study, which found that ML induces TNF gene expression at early time points in ST8814 cultures, despite no evidence of cytokine detection in the supernatants (32). Conversely, ML activated the expression of a diverse set of innate immunity-related genes in reprogrammed SCs after long-term infections; that is, high levels of a large range of chemokines and cytokines such as TNF were found in culture supernatants of SCs after 28 days of ML infection (10).

Even without inducing TNF secretion in SCs, ML seems to elicit TNF-mediated mechanisms through the transmembrane form of the cytokine. While sTNF exerts its effects at sites remote from the site where it is produced, the transmembrane form acts in a cell-to-cell contact fashion, functioning as a ligand by binding to TNF receptors and, as a receptor, transmitting signals back into the mTNF-bearing cells (43). The importance of mTNF in the immune responses against several pathogens, including HIV, has been reported (44). In addition, mTNF was shown to provide protection against infections by *Mycobacterium tuberculosis* and the less virulent *Mycobacterium bovis* bacillus Calmette-Guerin; initiating T-cell and macrophage migration as well as granuloma formation (45–47).

In transgenic mice that only express mTNF and not sTNF, the initiation and progression of experimental autoimmune encephalomyelitis (EAE) occurs with the maintenance of autoimmune properties and resistance to infection similar to what is found among wild-type mice (48). On the other hand, Ruuls et al demonstrated that mTNF is incapable of promoting an effective inflammatory response in the brain in the absence of sTNF (49).

TNFRs seem to play divergent roles in neuroinflammatory responses, with TNFR1 being involved in inflammation and demyelination and TNFR2 in remyelination and pathology limitation (50). Here, we show that ML induced TNFR1 gene expression in ST8814 cultures, a finding also reported by Oliveira et al (32). We also evaluated TNFR1 secretion by ML in ST8814 cultures finding that the pathogen was not able to induce TNFR1 secretion in the supernatants (data not shown). TNF/TNFR1 signaling has also been associated with many human diseases, including multiple sclerosis; this could be a mechanism by which ML contributes to leprosy neuropathy (51).

Clinical signs of EAE were reduced in single or double TNFR1 knockout mice compared to wild-type mice with EAE, whereas TNFR2-deficient mice exhibited enhanced disease severity accompanied by higher clinical scores and severe inflammation and demyelination (52, 53). It was further demonstrated that TNFR2 mediated protective effects, which included oligodendrocyte regeneration and suppression of activated lymphocytes (52).

Accumulating evidence indicates that SCs can secrete inflammatory cytokines, reinforcing their potential role in the modulation of the immune response in the PNS (54). Our data show that stimulation with TNF can induce the secretion of IL-6 and IL-8 in ST8814 cultures, which is in agreement with a previous study demonstrating that the ST8814 cell line is responsive to TNF because the cell line expresses constitutive levels of TNFR1 and TNFR2 (32). Previous studies have shown that IL-6 plays a role in axon regeneration following injury by increasing the expression of regeneration-associated genes in neurons (55). On the other hand, IL-8, a proinflammatory chemokine, has been detected in the CSF of patients with optic neuritis, and the presence of IL-8 has been associated with persistent demyelination and final axonal loss (56).

Of the molecules investigated in ST8814 supernatant cultures, ML only induced IL-23. It has been suggested that IL-23 is critically involved in the pathogenesis of various immune-mediated disorders. High IL-23 gene expression has been detected at the onset and during the acute phase of experimental autoimmune neuritis, an animal model of Guillain-Barré syndrome, which is characterized by multifocal demyelination and mononuclear cellular infiltration of peripheral nerves (57). Macrophages expressing IL-23 have also been found in sural nerves and CSF of Guillain-Barré patients (57). Furthermore, IL-23 is upregulated in EAE, and mice lacking IL-23 are resistant to EAE, and the presence of this cytokine in the CNS at the effector phase of EAE is required for disease induction (58, 59). These data underscore the potential pathogenic relevance of IL-23 in the early phases of immune-mediated demyelination in leprous neuropathy.

Overall, our data indicate that TNF seems to be an important cytokine that participates in the early stages of SC

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infection by ML. In addition, we demonstrated that ML is capable of contributing to a TNF-mediated response by inducing mTNF expression and upregulating TNFR1, thus rendering SCs more sensitive to the exogenous TNF levels in the nerve, which likely originates from resident macrophages in the early stages of injury and, later, from inflammatory cells. TNF seems to act on SCs by inducing IL-6 and IL-8 production, contributing not only directly, but also indirectly to neuroinflammation. Moreover, ML induces IL-23 secretion in SCs, a cytokine believed to be involved in demyelinating processes. Importantly, the type of focal demyelination seen in leprosy is most likely caused by the initial inflammatory response triggered by ML. Thus, focal demyelination could potentially become a prime target for therapeutic interventions aiming to improve nerve function in leprosy.

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