CONTINUED LEARNING OF TISSUE-SPECIFIC IMMUNITY FROM THE IMMUNO-PATHOLOGICAL SPECTRUM OF LEPROSY

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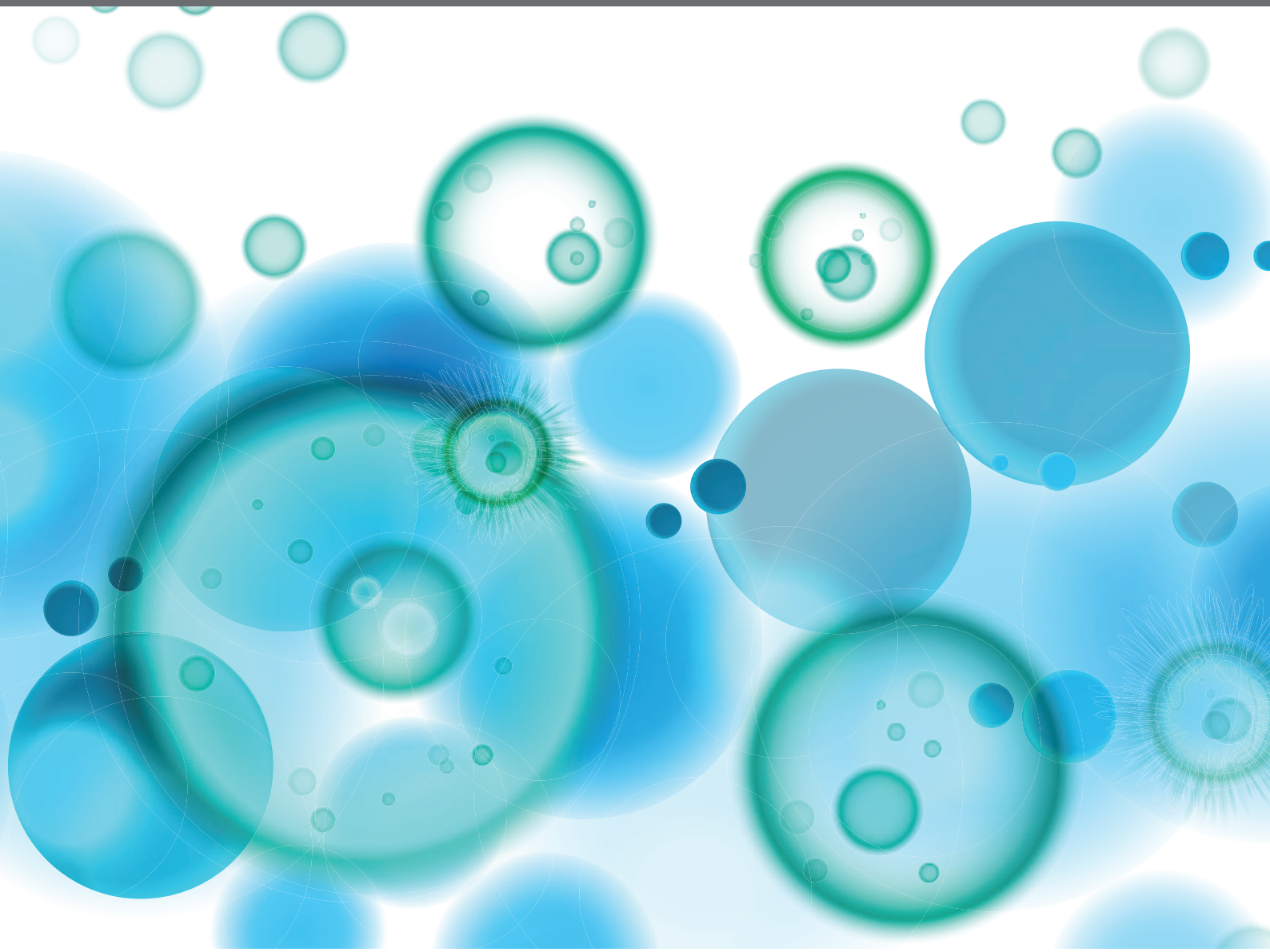

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CONTINUED LEARNING OF TISSUE-SPECIFIC IMMUNITY FROM THE IMMUNO-PATHOLOGICAL SPECTRUM OF LEPROSY

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Leprosy is one of the oldest recorded debilitating diseases affecting mankind, the immunopathology of which is characterized by fluctuating granulomatous inflammation that targets mainly skin and peripheral nerve. The disease is caused by infection with Mycobacterium leprae, a slow growing obligatory intracellular and non-cultivatable organism. The disease is manifested with diverse pathology due to varied immune (both innate and adaptive) responses of the hosts as a result of cognate interaction with the organism. Of note, leprosy can be regarded as a unique model to elucidate the complexity of host immunity at both skin and systemic levels.

New cases of leprosy continue to emerge despite the availability of effective multi drug therapy, suggesting continued M. leprae transmission. Although the immunopathology of leprosy has been elucidated considerably by studying the relationship between host immunity and disease activity, many facets of the disease pathology remain speculative because of the lack of any defined animal or in vitro experimental models. Furthermore, a remaining challenge lies in the development of immunological predictive tools that would help prevent extensive tissue destruction, in particular nerve damage, the latter being a hallmark of leprosy.

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Differential Expression of MicroRNAs in Leprosy Skin Lesions

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Leprosy, a chronic infectious disease caused by *Mycobacterium leprae*, is a major public health problem in poor and developing countries of the Americas, Africa, and Asia. MicroRNAs (miRNAs), which are small non-coding RNAs (18–24 nucleotides), play an important role in regulating cell and tissue homeostasis through translational downregulation of messenger RNAs (mRNAs). Deregulation of miRNA expression is important for the pathogenesis of various neoplastic and non-neoplastic diseases and has been the focus of many publications; however, studies on the expression of miRNAs in leprosy are rare. Herein, an extensive evaluation of differentially expressed miRNAs was performed on leprosy skin lesions using microarrays. Leprosy patients, classified according to Ridley and Jopling's classification or reactional states (R1 and R2), and healthy controls (HCs) were included. Punch biopsies were collected from the borders of leprosy lesions (10 tuberculoid, 10 borderline tuberculoid, 10 borderline borderline, 10 borderline lepromatous, 4 lepromatous, 14 R1, and 9 R2) and from 9 HCs. miRNA expression profiles were obtained using the Agilent Microarray platform with miRBase, which consists of 1,368 *Homo sapiens* (hsa)-miRNA candidates. TaqMan quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was used to validate differentially expressed miRNAs. Sixty-four differentially expressed miRNAs, including 50 upregulated and 14 downregulated (fold change ≥2.0, p-value ≤ 0.05) were identified after comparing samples from patients to those of controls. Twenty differentially expressed miRNAs were identified exclusively in the reactional samples (14 type 1 and 6 type 2). Eight miRNAs were validated by RT-PCR, including seven upregulated (hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-146b-5p, hsa-miR-342-3p, hsa-miR-361-3p, hsa-miR-3653, and hsa-miR-484) and one downregulated (hsa-miR-1290). These miRNAs were differentially expressed in leprosy and several other diseases, especially those related to the immune response. Moreover, the integration of analysis of validated mi/mRNAs obtained from the same samples allowed target pairs opposite expression pattern of hsa-miRNA-142-3p and AKR1B10, hsa-miRNA-342-3p and FAM180b, and hsa-miRNA-484 and FASN. This study identified several miRNAs that might play an

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important role in the molecular pathogenesis of the disease. Moreover, these deregulated miRNAs and their respective signaling pathways might be useful as therapeutic markers, therapeutic targets, which could help in the development of drugs to treat leprosy.

Keywords: leprosy, expression, microarray, microRNA, signaling pathways

INTRODUCTION

Leprosy, a chronic infectious disease caused by *Mycobacterium leprae*, is a major public health problem in poor and developing countries in the Americas, Africa, and Asia (1). Sporadic cases have been reported in developed countries, but usually in immigrants from endemic areas (2). *M. leprae* is an obligate intracellular parasite with tropism for the peripheral nervous system, and thus neural involvement is a feature of all forms of leprosy. The bacillus has slow replication, a long incubation period, and few genes controlling its metabolism (3). As a consequence, the disease evolves slowly for years or even decades, resulting in different clinical presentations and mimicking numerous other diseases; thus, treating this disease is very challenging. Leprosy is classified according to Ridley and Jopling (R&J), using two polar forms, tuberculoid (TT) and lepromatous (LL), and an intermediate group that is subdivided into borderline tuberculoid (BT), borderline borderline (BB), and borderline lepromatous (BL) (4). During the course of the disease, reactional episodes can occur, some of which are intense and destructive (5).

There are basically two types of reactions in leprosy. A type 1 reaction (R1) occurs with relative preservation of specific cellular immunity against *M. leprae*. A type 2 reaction (R2) occurs with poor preservation or absence of cellular immunity. R1 is an immunocellular response that occurs mainly in patients with TT and the borderline forms of the disease (BT, BB, and BL). The clinical signs of R1 include swelling and erythema of preexisting lesions, and when very intense, there can be necrosis and ulceration. Histopathological examination typically reveals more extensive, confluent, and poorly delimited granulomas associated with interstitial and intracellular edema, fibrin deposition, focal or confluent necrosis, and varying degrees of epithelial hyperplasia (5). R2 is clinically and histopathologically different from R1; it occurs in individuals with the lepromatous forms of leprosy (BL and LL) and is clinically characterized by erythematous papules, nodules, or plaques on specific lesions (usually during regression), and depending on the intensity of the reaction can evolve with suppuration, necrosis, and ulceration. The cutaneous lesions are accompanied by general systemic manifestations such as fever, myalgia, asthenia, inappetence, and often inflammatory manifestations in all parts of the body that contain bacillary antigens. In addition, neuritis, arthritis, painful lymphadenopathy, buccopharyngeal lesions, laryngitis, hepatomegaly and splenomegaly, bone lesions, iridocyclitis, uveitis, orchitis, and glomerulitis associated with proteinuria, and hematuria can occur. At any of these locations, the histological presentation is characterized by an acute or subacute non-granulomatous inflammatory reaction with vascular proliferation, endothelial swelling, venocapillary thrombi, serous–fibrin–neutrophil exudation, disorganization of preexisting granulomas, and the formation of microabscesses (5).

There is no specific treatment to prevent the occurrence of these reactional phenomena and no blood marker that identifies reactional episodes or their intensity. In addition, there is no any effective treatment regimen for all cases. During episodes, neurological lesions often worsen, typically resulting from direct aggravation of the neural branches and other tissues, which can lead to permanent functional disabilities.

The pathophysiological mechanisms of leprosy progression and reactional episode onset are virtually unknown (5). It is also not known how the bacillus, initially present in only a few lesions and predominantly in the peripheral neural branches (indeterminate form), is able to spread throughout the entire body, parasitizing different types of cells such as macrophages, Schwann cells, smooth muscle cells, endothelial cells, fibroblasts, melanocytes, and potentially even epithelial cells. It is likely that *M. leprae* is able to subvert host immune mechanisms by modifying the expression of genes in parasitized cells, making these cells an environment conducive to the survival of the bacilli; in addition, it is speculated that a similar strategy is used for their spread to adjacent tissues and distant organs.

There are a large number of studies showing that non-coding RNAs are important for the maintenance of cell and tissue homeostasis and that their dysregulation is involved in the development of diseases (6). Among these non-coding RNAs, microRNAs (miRNAs) play an important role in the downregulation of gene expression at the translational level, through specific binding to messenger RNA (mRNA), which results in translational repression and/or degradation of target mRNA (7).

It is well known that altered expression of miRNAs occurs in various types of diseases, but most often this has been described in neoplasms. Studies have provided a better understanding of the pathophysiological mechanisms of different diseases at the molecular level, some of which have displayed peculiar miRNA expression patterns allowing for their molecular classification (8). The miRNAs that are differentially expressed in these diseases have also been the subject of studies to discover new biomarkers with prognostic, predictive, or therapeutic potential (9). However, there have been few studies related to the expression of miRNAs in infectious diseases, particularly with respect to leprosy (10, 11).

In a recent study on the expression of mRNAs in skin samples using microarrays, hundreds of differentially expressed mRNAs were identified in the spectrum of leprosy and its reactional states suggesting their participation in the pathophysiology of these conditions (12). Some of these differentially expressed mRNAs might be regulated in cells that comprise the granulomas of the leprosy lesions.

Leprosy is a disease that is difficult to treat, and this is especially true of the reactional episodes. Drugs currently used, including corticosteroids and thalidomide, significantly disrupt homeostasis, leading to difficult-to-control disorders such as obesity,

diabetes, immunodeficiency, and teratogenesis, among others (13). Thus, this disease is challenging in all aspects, and there is an urgent need for new drugs to treat reactional episodes; it is also important to discover markers that can predict or identify these reactional states (14). miRNAs play an important role in triggering and maintaining many diseases, and therefore they could be important in the pathophysiology of leprosy. Thus, this study sought to evaluate the expression of miRNAs in the skin lesions of patients comprising the entire spectrum of leprosy.

This study utilized microarrays to identify differentially expressed miRNAs that might be involved in the pathophysiology of leprosy. These candidate miRNAs were hypothesized to represent novel markers and therapeutic targets for leprosy and its reactional states, which will be the focus of future research.

MATERIALS AND METHODS

Project Design, Sample Collection, and Classification

The events in this study occurred in the following order: patients who were consulted at the leprosy Outpatient Clinics of the Lauro de Souza Lima Institute (ILSL—Bauru, São Paulo) and Rondonópolis (Mato Grosso) were examined by leprologists and underwent two skin biopsy procedures. One biopsy was processed by histopathological analysis and bacilloscopy. The other was stored immediately after collection in RNAlater solution for future extraction of RNA. This study was performed using the same RNA material extracted from samples used in a recent publication reporting mRNA expression in leprosy (12).

After clinical and histopathological assessments and bacilloscopy, the patients were classified according to Ridley and Jopling's criteria of disease and reactions (TT, BT, BB, BL, LL, R1, and R2) (4, 5). Sixty-seven samples of leprosy lesions (TT = 10, BT = 10, BB = 10, BL = 10, LL = 4, R1 = 14, and R2 = 10), and nine skin biopsies from healthy subjects used as controls (healthy control; HC = 9), were collected. To avoid large variations in histological patterns that could interfere with miRNA expression, only samples from lesions on the trunk and upper and lower limbs were used. No samples were collected from the scalp, face, palms, and soles.

The data for all patients, including age, gender, and ethnicity, as well as the identification of each sample, are listed in Table S1 in Supplementary Material. It is important to mention that ethnic factors were not considered since previous studies demonstrated the high individual ancestral variability was observed in Brazilian population (independently of different geographical regions) which reflects a singular proportion of Amerindian, European, and African ancestries in its mosaic genome, and that in this population it is not possible to predict the color of persons from their genomic ancestry nor the opposite. The classic skin color stratification for additional analysis is not useful in the population investigated (15). This study was approved by the Research Ethics Committees of Hospital A.C. Camargo (no. 1535/11) and the Instituto Lauro de Souza Lima (ILSL—no. 033/2011).

The following comparisons were used to identify differentially expressed miRNAs in terms of the leprosy spectrum and different reactional states: (1) disease (TT + BT + BB + BL + L L + R1 + R2) vs. HC; (2) clinical forms (TT + BT + BB + BL + LL) vs. HC; (3) between polar forms (TT vs. LL); (4) tuberculoid (TT + BT) vs. HC; (5) lepromatous (BL + LL) vs. HC; (6) TT vs. HC; (7) LL vs. HC; (8) borderline leprosy (BT + BB + BL) vs. HC; (9) reactional type 1 (R1) vs. its respective clinical forms (TT + BT + BB + BL); (10) R1 vs. HC; (11) reactional type 2 (R2) vs. its respective clinical forms (BL + LL); (12) R2 vs. HC.

Extraction and Analysis of RNA Quality and Integrity

Skin biopsy samples (stored in RNAlater) were individually fragmented using a scalpel and transferred to a ceramic bead tube (CK28-Bertin Technologies). QIAzol reagent (700 µL; Qiagen) was added, and the samples were processed (homogenization and lysis) in a Precellys 24 homogenizer (Bertin Technologies) with three cycles of 10-s pulses, and further incubated at 4°C for 5 min. Total RNA (including miRNA) was extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions in a QIAcube apparatus (Qiagen). The RNA was recovered in 30 µL of RNase-free water. RNA quantification was performed using a Nanodrop 2000 (ThermoScientific), and integrity was evaluated using a Bioanalyzer 2100 electrophoresis system (GE Healthcare Bio-Sciences). Samples with low quality or insufficient RNA for analysis were excluded.

Labeling and Hybridization of miRNA

To evaluate the expression of miRNA, 300 ng of each total RNA sample was subjected to RNA (Cy3) labeling, performed according to the manufacturer's instructions (miRNA microarray system—complete labeling and hyb kit; protocol: version 2.4, September 2011; Agilent Technologies). The slides used in these assays were miRNA—human miRNA microarray (G4872A-031181—8 × 60 K) (G&E Healthcare Bio-Sciences). The oligoarrays were hybridized with fluorescent targets at 55°C for 20 h in a hybridization oven. After hybridization, the slides were processed using buffers provided by the manufacturer (Agilent Technologies) to eliminate non-specific targets and background interference, and then were subjected to a drying process that included washing in acetonitrile for 1 min and in a washing and stabilization solution for microarrays for 1 min. The arrays were digitized using the Agilent Bundle Scanner (Agilent, USA) with a resolution of 3 µm and Feature Extraction software (Version 11.0). The digitized images of each array were submitted for data quality analysis using Agilent Genespring software version 11.0 (Agilent®).

Selection of miRNAs for Reverse Transcription Polymerase Chain Reaction (RT-PCR) Validation

After analyzing differentially expressed miRNAs among diverse samples representing the leprosy spectrum and different reactional forms, 51 miRNAs were selected for reverse transcription polymerase chain reaction (RT-PCR) validation; some were highly upregulated or downregulated based on most comparisons, whereas others were expressed in either type 1 or type

2 reactional states. An unpaired asymptotic *T* test was employed with a Bonferroni FWER correction for statistical analysis, and those with fold change ≥2.0 and a *p*-value ≤ 0.05 were validated by RT-PCR.

Validation of miRNAs by RT-PCR

Complementary DNA (cDNA) was synthesized from total RNA by adding 0.5 μg of oligo-dT15 to 2 μg of total RNA in a final volume of 5 μL, and incubating the sample at 70°C for 10 min and cooling on ice for oligo annealing. Reverse transcription was performed using SuperScript III enzyme (Invitrogen) according to the manufacturer's instructions in a final volume of 20 μL. The sample was incubated at 50°C for 1 h, followed by 15 min at 70°C. Aliquots of the obtained cDNA were diluted 10-fold and stored at −20°C.

After selecting genes, custom PCR plates were ordered in a 96 × 1 format (Qiagen—miRNA #CMIHS02125). Twenty-four miRNA samples, representing the entire spectrum of disease, the reactional forms, and controls, were validated.

Complementary DNA synthesis from miRNA samples was performed using the miScript II RT Kit and HiSpec Buffer (Qiagen). The obtained cDNA was subjected to the RT-PCR protocol of the miScript miRNA PCR Array with the miScript SYBR Green PCR Kit (Qiagen), using an ABI VIIA 7 device (Applied Biosystems).

After completing the reaction, the quality of data was analyzed using SDS 2.3 software (Applied Biosystems). The dissociation curves were analyzed for amplification of genomic DNA, primer dimers, and splicing variants. Using amplification plots, the fluorescence intensity threshold was adjusted in the exponential phase of the graph in which the cycle threshold values of each reaction were considered. Duplicates with SDs of less than 0.5 were considered acceptable.

The relative expression in each group was compared using a non-parametric Mann–Whitney test.

Filtering miRNA/mRNA Target Pairs Opposite Expression Pattern

We identify from our list of differentially expressed miRNAs and mRNAs those pairs that (i) were previously described as a validated interaction partners and (ii) show opposite expression pattern in our data. For this analysis, we consider only miRNA/mRNAs found as differentially expressed in each of the following group comparisons (Clinical forms vs. HC, TT vs. HC, LL vs. HC, R1 vs. HC and R2 vs. HC). The mRNAs were selected from a previous already published study by the same authors, in which the same set of samples used in this study were analyzed (12). Each group comparison was analyzed separately. Basically, the computational filtering strategy consisted in collecting from the MirWalk database (16) all validated mRNA targets for all differentially expressed miRNAs found in the comparisons mentioned earlier. After that, by using an in-house R script, we search for validated pairs with opposing expression pattern in our data, i.e., for each miRNA downregulated we searched for upregulated mRNA targets and for each miRNA upregulated we searched for downregulated mRNA in our data.

Pathway Enrichment Analysis

The genes selected from the miRNA/mRNA analysis were submitted to pathway enrichment analysis by using the ReactomeFIViz plugin from the Cytoscape software[1] that performs searches in the Reactome Database.[2] The pathway enrichment analysis was applied separately to the gene sets selected from each group comparison (Clinical forms vs. HC, TT vs. HC, LL vs. HC, R1 vs. HC and R2 vs. HC) and also to a single gene set consisted of all selected genes found in these comparisons.

Microarray Data Accession Number

The microarray data set has been submitted to the Gene Expression Omnibus database at NCBI[3] and assigned accession number GSE102314.

RESULTS

Differentially Expressed miRNAs in Disease and Reactional Episodes

Comparing disease (TT + BT + BB + BL + LL + R1 + R2) vs. HC groups, 64 miRNAs (50 upregulated and 14 downregulated) were differentially expressed (Table S2 in Supplementary Material; **Table 1**). Upon comparing clinical forms (TT + BT + BB + BL + LL) vs. HC groups, 20 miRNAs (14 upregulated and 6 downregulated) were differentially expressed (Table S3 in Supplementary Material; **Table 2**). Only one downregulated miRNA (hsa-miR-181a*) was differentially expressed between the polar forms (TT vs. LL) (Table S4 in Supplementary Material). Tables S5–S9 in Supplementary Material list all miRNAs

[1]http://www.cytoscape.org/.
[2]http://www.reactome.org.
[3]http://www.ncbi.nlm.nih.gov/geo/.

TABLE 1 | The 10 most upregulated or downregulated microRNAs differentially expressed, based on microarray, in disease (TT + BT + BB + BL + LL + R1 + R2) vs. HC, fold change (FC) ≥ |2| and $p \leq 0.05$.

Gene symbol	Regulation	FC microarray	*p* Value
hsa-miR-181a*	Up	35.59919	8.81E−12
hsa-miR-155	Up	26.33272	5.46E−19
hsa-miR-21*	Up	14.50879	1.88E−10
hsa-miR-3607-5p	Up	11.07103	6.08E−05
hsa-miR-3926	Up	11.01895	1.39E−05
hsa-miR-4261	Up	9.929722	1.51E−04
hsa-miR-378*	Up	9.214098	5.55E−05
hsa-miR-423-3p	Up	9.161276	9.57E−07
hsa-miR-501-3p	Up	9.157262	2.58E−05
hsa-miR-31	Up	9.013615	0.001011495
hsa-miR-141	Down	−2.25298	7.14E−06
hsa-miR-193b	Down	−2.35208	5.69E−09
hsa-miR-205	Down	−2.4154	2.55E−06
hsa-miR-200c	Down	−2.4379	1.58E−06
hsa-miR-486-5p	Down	−2.65039	0.006190634
hsa-miR-203	Down	−2.84448	0.002550727
hsa-miR-200b	Down	−2.91643	1.71E−04
hsa-miR-338-3p	Down	−2.94236	0.011949077
hsa-miR-429	Down	−3.37992	0.010056388
hsa-miR-224*	Down	−4.27994	0.006564327

TABLE 2 | The 10 most upregulated and six most downregulated microRNAs differentially expressed, based on microarray, in the different forms (TT + BT + BB + BL + LL) vs. healthy control, fold change (FC) ≥ |2| and $p \leq 0.05$.

Gene symbol	Regulation	FC microarray	*p* Value
hsa-miR-155	Up	16.85744	3.84E−20
hsa-miR-146a	Up	7.594823	8.38E−14
hsa-miR-142-5p	Up	6.288545	1.96E−13
hsa-miR-21*	Up	5.760914	8.59E−08
hsa-miR-142-3p	Up	5.004883	7.57E−11
hsa-miR-21	Up	4.018055	6.73E−12
hsa-miR-146b-5p	Up	3.947622	4.07E−06
hsa-miR-150	Up	3.921194	6.15E−09
hsa-miR-181a*	Up	3.792876	9.06E−09
hsa-miR-342-5p	Up	3.128596	4.68E−09
hsa-miR-429	Down	−2.14509	−2.145087
hsa-miR-141	Down	−2.17806	−2.1780634
hsa-miR-205	Down	−2.21269	−2.2126882
hsa-miR-193b	Down	−2.22064	−2.2206442
hsa-miR-200c	Down	−2.26256	−2.2625608
hsa-miR-224*	Down	−2.26869	−2.2686923

differentially expressed when making the following comparisons: tuberculoid form (TT + BT) and HC (17 miRNAs—Table S5 in Supplementary Material), lepromatous form (BL + LL) and HC (18 miRNAs—Table S6 in Supplementary Material), TT and HC (17 miRNAs—Table S7 in Supplementary Material), LL and HC (18 miRNAs—Table S8 in Supplementary Material), and borderline leprosy (BT + BB + BL) and HC (18 miRNAs—Table S9 in Supplementary Material).

In relation to reactional status, we observed that 37 miRNAs were differentially expressed in reactional type 1 samples (Tables S10 and S12 in Supplementary Material). The 10 most upregulated or downregulated miRNAs differentially expressed in type 1 reaction and HC are shown in **Table 3**. In the comparison between R1 vs. R1 respective clinical forms, the hsa-miRNA-378* is exclusive. Regarding type 2 reactions, 26 miRNAs were differentially expressed in these samples (Tables S11 and S13 in Supplementary Material). The 10 most upregulated and 7 downregulated miRNAs differentially expressed in type 2 reaction and HC are shown in **Table 4**. When comparing type 2 reaction (R2) samples with their respective clinical forms (BB + BL), one miRNA (hsa-miR-20a*) was differentially expressed (upregulated) (Table S11 in Supplementary Material).

In general, miRNAs were heterogeneously expressed. The majority of these miRNAs were differentially expressed in different disease forms or reactional states compared to expression in the HC group, but with higher or lower magnitude (hsa-miR-142-5p, hsa-miR-155-5p, hsa-miR-181a*, and hsa-miR-21-3p, among others). However, some were expressed only in specific groups (type 1 reactions: hsa-miR-1290, hsa-miR-200a, hsa-miR-200b, hsa-miR-205*, hsa-miR-34a, hsa-miR-501-3p, hsa-miR-27a, hsa-miR-27b, hsa-miR-133b, hsa-miR-224, hsa-miR-96, hsa-miR-203, hsa-miR-378, and hsa-miR-500a*; type 2 reactions: hsa-miR-125b-2*, hsa-miR-214, hsa-miR-7, hsa-miR-629, hsa-miR-20a*, and hsa-miR-223).

Of the 51 miRNAs subjected to RT-PCR validation, 8 were validated (hsa-miR-1290, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-146b-5p, hsa-miR-342-3p, hsa-miR-361-3p, hsa-miR-3653, and hsa-miR-484). All miRNAs subjected to validation and the associated values for the comparisons are shown in **Table 5**.

TABLE 3 | The 10 most upregulated or downregulated microRNAs differentially expressed in reaction type 1 (R1) vs. healthy control, fold change (FC) ≥ |2| and $p \leq 0.05$.

Gene symbol	Regulation	FC microarray	*p* Value
hsa-miR-155	Up	21.27233	5.77E−12
hsa-miR-21*	Up	11.91278	8.96E−11
hsa-miR-146a	Up	9.099005	2.64E−08
hsa-miR-142-5p	Up	8.484986	1.66E−09[a]
hsa-miR-150	Up	5.275174	6.62E−09
hsa-miR-181a*	Up	5.255039	1.32E−08
hsa-miR-142-3p	Up	5.105462	5.94E−06[a]
hsa-miR-21	Up	4.870885	7.12E−09
hsa-miR-146b-5p	Up	4.820066	2.46E−04[a]
hsa-miR-342-5p	Up	4.810751	1.59E−10
hsa-miR-133b	Down	−3.04698	5.46E−06
hsa-miR-205	Down	−3.06662	1.09E−07
hsa-miR-1290	Down	−3.24258	1.07E−04[a]
hsa-miR-224*	Down	−3.48345	7.73E−06
hsa-miR-224	Down	−3.75265	7.50E−05
hsa-miR-200b	Down	−4.04193	5.27E−07
hsa-miR-200a	Down	−4.24384	8.55E−06
hsa-miR-429	Down	−4.24633	5.53E−08
hsa-miR-96	Down	−5.11292	2.55E−05
hsa-miR-203	Down	−5.17564	6.86E−05

[a]*Validated miRNA.*

TABLE 4 | The 10 most upregulated and seven most downregulated microRNAs differentially expressed in reaction type 2 (R2) vs. healthy control, fold change (FC) ≥ |2| and $p \leq 0.05$.

Gene symbol	Regulation	FC microarray	*p* Value
hsa-miR-21*	Up	19.92985	3.25E−10
hsa-miR-155	Up	9.499085	1.63E−07
hsa-miR-223	Up	8.636663	2.38E−04
hsa-miR-142-5p	Up	7.731227	5.34E−08[a]
hsa-miR-142-3p	Up	5.971354	1.98E−04[a]
hsa-miR-146b-5p	Up	5.863311	2.11E−04[a]
hsa-miR-21	Up	5.585104	2.01E−09
hsa-miR-150	Up	5.319824	1.94E−05
hsa-miR-181a*	Up	5.028419	3.16E−07
hsa-miR-7	Up	4.940581	1.29E−05
hsa-miR-214	Down	−2.14012	1.71E−04
hsa-miR-125b-2*	Down	−2.17508	7.85E−05
hsa-let-7b	Down	−2.40185	2.46E−05
hsa-miR-193b	Down	−2.41444	1.08E−05
hsa-miR-205	Down	−2.54304	2.15E−04
hsa-miR-429	Down	−2.64966	2.54E−04
hsa-miR-200c	Down	−2.68127	7.31E−06

[a]*Validated miRNA.*

miRNA/mRNA Target Pairs Opposite Expression Pattern

MicroRNA/mRNAs found as differentially expressed in each of the following group comparisons (Clinical forms vs. HC, TT vs. HC, LL vs. HC, R1 vs. HC and R2 vs. HC) are detailed in Table S14 in Supplementary Material. In summary, in the comparison Clinical forms vs. HC, six miRNAs (hsa-miRNA-429, hsa-miRNA-142-3p, hsa-miRNA-142-5p, hsa-miRNA-146b, hsa-miRNA-342-3p, and hsa-miRNA-342-5p) are associated with 39 mRNAs.

TABLE 5 | Results of microRNA validation with respective target genes comparison.

Symbol	Real time	Microarray	*p* Value	Regulation	Validated form	Target gene	miRBase
hsa-miR-1290	−6.418556575	−3.2425804	0.0319	Down	R1 vs. healthy control (HC)		MIMAT0005880
hsa-miR-142-3p	72.40788202	5.157617	0.0001	Up	Disease vs. HC	AKR1B10	MIMAT0000434
hsa-miR-142-3p	46.32763259	5.105462	0.0248	Up	R1 vs. HC		MIMAT0000434
hsa-miR-142-3p	50.73379106	5.9713535	0.0046	Up	R2 vs. HC		MIMAT0000434
hsa-miR-142-5p	13.50652073	6.894877	0.0001	Up	Disease vs. HC		MIMAT0000433
hsa-miR-142-5p	11.24670109	8.484986	0.0095	Up	R1 vs. HC		MIMAT0000433
hsa-miR-142-5p	7.586663505	7.731227	0.0198	Up	R2 vs. HC		MIMAT0000433
hsa-miR-146b-5p	7.640099529	4.3596835	0.0001	Up	Disease vs. HC		MIMAT0002809
hsa-miR-146b-5p	7.006850053	4.8200655	0.0030	Up	R1 vs. HC		MIMAT0002809
hsa-miR-146b-5p	8.340527806	5.8633113	0.0002	Up	R2 vs. HC		MIMAT0002809
hsa-miR-342-3p	6.380637592	2.780538	0.0001	Up	Disease vs. HC	FAM180B	MIMAT0000753
hsa-miR-361-3p	5.662851322	2.0504081	0.0001	Up	Disease vs. HC		MIMAT0004682
hsa-miR-361-3p	6.351012385	2.0587645	0.0185	Up	R2 vs. HC		MIMAT0004682
hsa-miR-3653	2.298881007	2.0732837	0.0122	Up	Disease vs. HC		MIMAT0018073
hsa-miR-484	2.195647906	2.6891153	0.0056	Up	R2 vs. HC	FASN	MIMAT0002174

Disease = (TT + BT + BB + BL + LL + R1 + R2).

In the comparison TT vs. HC, 6 miRNAs (hsa-miRNA-429, hsa-miRNA-142-3p, hsa-miRNA-142-5p, hsa-miRNA-342-3p, hsa-miRNA-342-5p, and hsa-miRNA-361-3p) are associated with 27 mRNAs. In the comparison LL vs. HC, 4 miRNAs (hsa-miRNA-429, hsa-miRNA-142-5p, hsa-miRNA-342-3p, and hsa-miRNA-484) are associated with 44 mRNAs. In the comparison R1 vs. HC, 9 miRNAs (hsa-miRNA-429, hsa-miRNA-133b, hsa-miRNA-142-3p, hsa-miRNA-142-5p, hsa-miRNA-146b-5p, hsa-miRNA-342-3p, hsa-miRNA-342-5p, hsa-miRNA-361-3p, and hsa-miRNA-501-3p) are associated with 62 mRNAs. In the comparison R2 vs. HC, 6 miRNAs (hsa-miRNA-429, hsa-miRNA-142-3p, hsa-miRNA-142-5p, hsa-miRNA-146b-5p, hsa-miRNA-361-3p, and hsa-miRNA-484) are associated with 57 mRNAs. After comparing the miRNAs validated in this study with the mRNAs validated by Belone et al.'s study, the following mi/mRNAs were identified: hsa-miRNA-142-3p and AKR1B10, hsa-miRNA-342-3p and FAM180b, and hsa-miRNA-484 and FASN (**Table 5**).

Pathway Analysis

The pathways obtained by the different comparisons are detailed in Table S16 in Supplementary Material. The following signaling pathways were obtained by the comparisons: fatty Acyl-CoA Biosynthesis, ChREBP activates metabolic gene expression, Triglyceride Biosynthesis, metabolism of water-soluble vitamins and cofactors, Gap junction trafficking, fatty Acyl-CoA biosynthesis, Gap junction trafficking and regulation, ChREBP activates metabolic gene expression, activation of gene expression by SREBF (SREBP), regulation of cholesterol biosynthesis by SREBP (SREBF), synthesis of very long-chain fatty acyl-CoAs, linoleic acid metabolism.

DISCUSSION

Analysis of the human genome has indicated that many genomic sequences encode RNA that does not encode protein. Many longer non-coding RNAs, small nucleolar RNAs, miRNAs, and other small regulatory RNAs are included among the non-coding RNAs. miRNAs (20–24 nucleotides) have been extensively studied. They consist of a group of small non-coding RNA molecules related to small interfering RNAs. miRNAs play important roles in key biological processes such as gene regulation, cell growth, apoptosis, and hematopoietic lineage differentiation. As such, miRNAs are involved in various human diseases such as cancer, vascular disease, immune disease, and infections (17). Regarding neoplasms, it is clear that miRNAs are differentially expressed between normal and cancerous cells, that they reflect tissue-specific expression signatures, and that they can either promote ("oncomiRs") or suppress tumor development and progression, thereby influencing all of the hallmarks of cancer. Many miRNAs have been used for early detection, diagnosis, and prognosis in different types of cancer (9, 18). miRNAs are also deregulated in several non-neoplastic diseases. There are many publications showing that they play an important role in the dysregulation of the immune response in inflammatory/autoimmune diseases (such as systemic lupus erythematous, rheumatoid arthritis, and multiple sclerosis) and infectious diseases (including tuberculosis and leprosy).

In this study, a large number of differentially expressed miRNAs were identified mainly due to the varied and polymorphous composition of the cells that contributed to the inflammatory processes in leprosy skin lesions (4, 5, 14). In the tuberculoid forms (TT and BT), bacilli are absent or rarely found in neural branches, macrophages, or mononuclear cells of the papillary dermis. In contrast, in the lepromatous forms (BL and LL), bacilli are abundant and can parasitize virtually all tissues. Therefore, it is likely that these differentially expressed miRNAs are involved in bacillary proliferation and dissemination.

Many miRNAs described in the literature are associated with the regulation of the immune response in other diseases; for example, hsa-miR-34, hsa-miR-142-3p, hsa-miR-146a, hsa-miR-150, hsa-miR-155, hsa-miR-214, hsa-miR-223, and hsa-miR-424 were also present in leprosy tissues (Tables S2, S3, and S13 in Supplementary Material; **Table 5**). Recently, several miRNAs were described as being important in neural diseases. Leprosy, due to the tropism of *M. leprae* for peripheral neural

branches, is a predominantly neural infectious disease. Initially, in its indeterminate phase, before the development of lesions within the R&J spectrum, the peripheral neural branches of the skin or subcutaneous tissue are involved, and this is characterized by minimal inflammatory infiltration, which is predominantly lymphocytic, without well-formed granulomas. Subsequently, with complete disease establishment, granulomas involving neural branches become a histological feature present in all forms and both reactional states of leprosy. Therefore, there is constant interaction between neural branches and the inflammatory process during the disease.

Some studies showed that miRNAs play important roles in the development of mycobacterial diseases (tuberculosis, leprosy, and *Mycobacterium avium* infection), probably by regulation of the immune response of the hosts. Functional profiles and experiments generated evidence suggesting that regulation of specific miRNAs during infection might stimulate the immune response or facilitate immune evasion by the pathogens (19, 20). They have also been involved in the regulation of the host immune response in relation to other bacteria such as *Salmonella*, *Helicobacter pylori*, *Francisella tularensis*, and *Listeria monocytogenes* (21). miRNA17 participates in the regulation of autophagy in macrophages in tuberculosis. *Mycobacterium tuberculosis* (Mtb) infection leads to the downregulation of miRNA17 and consequently the upregulation of its targets Mcl-1 and STAT3 (22). In addition, miRNA expressed in Mtb-infected macrophages revealed the downregulation of miR-let-7f, which was dependent on the Mtb-secreted effector ESAT-6 (23). It was shown that let-7f targets A20, a feedback inhibitor of the NF-κB pathway. Experimental studies in mice infected with Mtb showed decrease in let-7f expression and increase in A20 during progression of the infection. A20-deficient macrophages result in decreased Mtb survival. Moreover, production of tumor necrosis factor (TNF), interleukin (IL-1β), and nitrites, which are mediators of immunity to Mtb, is consequently increased. Furthermore, the overexpression of let-7f diminishes Mtb survival and augments the production of the TNF and IL-1β cytokines. These results suggest let-7f and its target A20 play a role in regulation of the immune response against Mtb and control of mycobacterial burden (23). To ensure their survival and replication, bacterial pathogens manipulate a wide range of host cell functions by providing effector proteins to host cells. Regulation of miRNA expression by bacterial pathogens is emerging as an essential part of the host's response to infection, as is the discovery of molecular mechanisms exploited by bacteria to control the microenvironment of host cells (11).

There have been a few studies on the expression of miRNAs specifically related to leprosy. Liu et al. identified 13 miRNAs that were differentially expressed in the lesions of subjects with LL in comparison to expression in the self-limiting TT disease. Bioinformatics analysis revealed marked enrichment of LL-specific miRNAs, which target key immune genes shown to be downregulated in LL tissue compared to TT lesions. The most differentially expressed miRNA in LL lesions, hsa-mir-21, was upregulated in *M. leprae*-infected monocytes. hsa-mir-21 inhibited gene expression of vitamin D-dependent antimicrobial peptides, CAMP and defensin Beta 4A, through downregulation of toll-like receptor 2/1 (TLR2/1)-induced cytochrome p450 27B1 and IL-1β, and upregulation of IL-10. Thus, the ability of *M. leprae* to upregulate hsa-mir-21 could result in the regulation of multiple genes associated with the LL disease form, providing an effective mechanism to escape from vitamin D-mediated antimicrobial pathway (24). Our results show that miRNA21 and miRNA21* are upregulated both in the leprosy spectrum and in the reactional states (Tables S1, S2, S13, and S14 in Supplementary Material). Although we did not find significant differences in terms of miRNA 21/miRNA21* expression between TT and LL, there were significant similarities with the work of Liu et al. We observed that the expression of miRNA21/miRNA21* was higher in lepromatous form samples and type 2 reactions [LL vs. HC; (BL + LL) vs. HC and R2 vs. HC] when compared to that in the tuberculoid forms and type 1 reactions [TT vs. HC; (TT + BT) vs. HC and R1 vs. HC] (Tables S4–S7, S12, and S13 in Supplementary Material).

Jorge et al. recently published a study on the expression of miRNAs in leprosy polar forms. After evaluating the expression of 377 miRNAs by TaqMan Low Density Array (TDLA) in skin samples of patients with the polar leprosy forms (TT and LL) and HC, the authors identified four validated miRNA (hsa-miR-101, hsa-miR-196b, hsa-miR-27b, and hsa-miR-29c) that can be used to discriminate HC from leprosy patients with 80% sensitivity and 91% specificity, respectively. In addition, the same miRNAs can discriminate, with 83% sensitivity and 80% specificity, LL from TT patients (25). In this study, one miRNA (hsa-miR-181a*) was found differentially expressed in the comparison TT vs. LL (Table S4 in Supplementary Material). None of the miRNAs validated by Jorge et al. were identified in this study when comparing TT vs. HC (Table S7 in Supplementary Material) and LL vs. HC (Table S8 in Supplementary Material); however, hsa-miR-27b was upregulated in R1 reaction (Table S12 in Supplementary Material). Cezar-de-Mello et al. reported that pre-miR-146a, which is known to modulate TNF levels, exhibits polymorphisms that are associated with susceptibility to leprosy. In this study, which was performed on skin samples, although we observed increased levels of miR-146a, it was not possible to correlate this with any specific form of leprosy (26). We observed that miR-146a is upregulated in all forms of leprosy and exhibits higher expression in the lepromatous form compared to that in the tuberculoid form (Tables S2, S4–S6, and S7 in Supplementary Material). There were also no significant differences when expression values were compared between the forms. Between the two types of reactions, only R1 presented with significantly different expression compared to that of HC samples (Table S13 in Supplementary Material). Regarding inflammatory/infectious diseases, hsa-miR-146a is downregulated in peripheral blood mononuclear cells at different stages of chronic hepatitis B virus infection (27). The miRNA hsa-miR-146a is crucial for the progression of Alzheimer's disease and functions through the hsa-miR-146a/STAT1/MYC pathway (28). In Kaposi's sarcoma (KS), this miRNA is upregulated and is associated with the downregulation of CXCR4; this might contribute to the development of KS by promoting the premature release of KS-associated herpes virus-infected endothelial progenitors into the circulation (29).

A study by Kumar et al. showed that immunological dysregulation might lead to hyporeactivity or anergy in T cells in *M.*

leprae-infected patients. It also identified Cbl-b overexpression and loss of miR-181 expression as important characteristics for the progression of leprosy (30). In this study, we determined that miR-181a* is upregulated throughout the disease spectrum and reactional states, indicating that it takes part in the pathophysiological processes of the disease. However, we found higher expression in the lepromatous forms (LL) than in the tuberculoid forms (TT) (Tables S3, S6, and S7 in Supplementary Material). The regulation of T cell sensitivity by miR-181a allows mature T cells to recognize antagonists (inhibitory peptide antigens) as agonists. These effects may be achieved by the downregulation of multiple phosphatases, which results in elevated steady-state levels of phosphorylated intermediates and reduction in the signaling threshold of T cell receptor. It is important to mention that higher miR-181a expression correlates with greater T cell sensitivity in immature T cells. This suggests that miR-181a acts as an intrinsic antigen sensitivity "rheostat" during development of T cells (31).

Another very relevant aspect of leprosy is its reactional episodes. However, there were no previous references in the literature regarding miRNA expression in leprosy reactions. In respect to type 1 reactions, we observed that 14 miRNAs were differentially expressed only in these samples. Several of these miRNAs are described in the literature related to diseases such as lung, breast, and kidney cancer (32–34). Upregulation of hsa-miR-34a and hsa-miR-500a is associated with the development of neuroblastoma and is related to poor response to chemotherapy in non-small cell lung carcinoma (NSCLC) (35, 36). In the comparison R1 vs. R1 respective clinical forms (Table S9 in Supplementary Material), the miRNA-378* is exclusive. The literature shows that miRNA-378* is associated with lipids metabolism (37).

Regarding type 2 reactions, six miRNAs were differentially expressed exclusively in these samples. Based on the literature, downregulation of hsa-miR-125b-2* and hsa-miR-214 is associated with miscarriages, development of gastric adenocarcinoma in the elderly (compared to that in young individuals), glioma cell proliferation, and germ cell tumor growth in the testis (34, 38, 39). In addition, the upregulation of hsa-miR-223 is directly related to periodontitis and gastric cancer (40, 41). It is not currently known how these miRNAs participate in the initiation and/or maintenance of type 1 and type 2 reactions. Clinical and histopathological characteristics are different between reactions and clinical forms. There are also important changes that occur in the composition of granulomas, in the phenotype of interstitial cells, and in angiogenesis between these cell types. Thus, the clinical and histopathological characteristics specific to the various forms and reactions of the disease could be explained by differentially expressed miRNAs. The role of these miRNAs in leprosy is unknown.

Of the eight miRNAs validated by RT-PCR in this study (hsa-miR-1290, hsa-miR-142-3p, hsa-miR-142-5p, hsa-miR-146b-5p, hsa-miR-342-3p, hsa-miR-361-3p, hsa-miR-3653, and hsa-miR-484), there are no references in the literature regarding their expression in leprosy skin lesions. hsa-miR-1290, validated as downregulated in type 1 reactions compared to that in HCs, is associated with the suppression of proliferation and invasion in NSCLC and is significantly downregulated in luminal-A breast tumors (42). Its potential target, arylamine *N*-acetyltransferase 1, is correlated with increased survival in patients with these tumor subtypes (33). hsa-miR-139-5p, which was downregulated in disease samples compared to expression in HC tissues, plays a pivotal role in lung cancer and breast cancer. It inhibits cell proliferation and metastasis and promotes apoptosis by targeting oncogenic c-Met (43, 44). hsa-miR-142-3p, which was upregulated in disease samples vs. HC, R1 vs. HC, and R2 vs. HC, is associated with bromocriptine-resistant prolactinoma and is present in inflamed gingival tissue but not in healthy gingival tissue (45, 46). hsa-miR-142-5p, which was upregulated in disease vs. HC, R1 vs. HC, and R2 vs. HC, in combination with hsa-miR-375, was reported to be a predictor of disease progression, showing potential to predict recurrent gastric cancer; hsa-miR-142-5p is involved in the regulation of several oncogenic signaling pathways such as vascular endothelial growth factor hsa-miR-142-5p, TP53, MAPK, and Wnt (47). hsa-miR-146b-5p, which was upregulated in disease vs. HC, R1 vs. HC, and R2 vs. HC, is also upregulated in recurrent glioblastoma compared to expression in primary glioblastoma, suggesting that it might be related to disease relapse (48). hsa-miR-342-3p, which was found to be upregulated in disease vs. HC and R1 vs. HC, might play a general role in late stage prion disease and this way be used as a marker for animal and human spongiform encephalopathies (49). hsa-miR-361-3p, which was found to be upregulated in disease vs. HC and R2 vs. HC, is upregulated in the inflamed mucosa of Crohn's disease patients compared to expression in non-inflamed mucosa (50). The function of hsa-miR-3653, found to be upregulated in disease vs. HC, is virtually unknown. hsa-miR-484, which was upregulated in R2 vs. HC, is associated with chemoresistance in ovarian cancer due to enhanced angiogenesis, resulting from modulation of the tumor vasculature, through regulation of VEGFB and VEGFR2 pathways (51). Interestingly, increased angiogenesis occurs in skin lesions in the entire spectrum of leprosy and in both reactional states compared to that in HCs; the number of vessels was observed to be higher in R2 tissues than in any of the other disease forms or the R1 stage (52).

MicroRNAs are increasingly described as important players in the regulation of cellular functions. However, the differential expression of miRNAs and target mRNAs in tissues, fluids, or cultured cells does not necessarily mean that there is a related disorder in cell metabolism. Most miRNAs were validated only experimentally with studies directed at the target mRNA. As a single miRNA species can regulate hundreds of mRNAs and because several miRNAs can be regulated by a single mRNA, the broad analysis of multiple mRNA/miRNA interactions becomes very complex, and often these interactions remain unknown as a result. Recently, we published a study on the expression of mRNAs in leprosy using the same samples and types of comparisons used in this study (12). Comparing the mi/mRNAs present in both studies, we observed several miRNAs opposed to mRNAs with different functions. The signaling pathways obtained indicate predominant changes in the lipid metabolism, cellular trafficking, and metabolism of soluble vitamins and its cofactors. They have been associated with different diseases and immunological processes in the literature (53–57).

In summary, this study has uncovered miRNAs that are deferentially expressed in leprosy skin lesions, several of which were described for the first time in this disease. miRNA expression profiles as they relate to the spectrum of leprosy and the reactional states might provide a solid basis for the understanding of the pathophysiological mechanisms of leprosy. These miRNAs will require validation and functional analyses to evaluate their role in the pathogenesis of leprosy. Thus, this study represents the initial stages of an important field of research to identify molecular markers of this disease or its reactional states. Furthermore, these deregulated miRNAs and their respective signaling pathways could be used as therapeutic targets, consequently enabling the development of novel drugs for the treatment of leprosy.

AUTHOR CONTRIBUTIONS

CS, AB, DC, and FS conceived the project. CG and CS performed clinical evaluation of patients and performed biopsy procedures. CS performed histopathological and immunohistochemical analysis. AB, AC, MP, LF, and AT were involved with RNA sample preparation, hybridization, and PCR. CS, MP, AC, AB, AT, LF, RR, and PR were responsible for data analysis. CS and PR wrote the manuscript. All the authors critically revised the paper for intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.01035/full#supplementary-material.

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Immune Checkpoints in Leprosy: Immunotherapy As a Feasible Approach to Control Disease Progression

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Leprosy remains a health problem in several countries. Current management of patients with leprosy is complex and requires multidrug therapy. Nonetheless, antibiotic treatment is insufficient to prevent nerve disabilities and control *Mycobacterium leprae*. Successful infectious disease treatment demands an understanding of the host immune response against a pathogen. Immune-based therapy is an effective treatment option for malignancies and infectious diseases. A promising therapeutic approach to improve the clinical outcome of malignancies is the blockade of immune checkpoints. Immune checkpoints refer to a wide range of inhibitory or regulatory pathways that are critical for maintaining self-tolerance and modulating the immune response. Programmed cell-death protein-1 (PD-1), programmed cell death ligand-1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4, and lymphocyte-activation gene-3 are the most important immune checkpoint molecules. Several pathogens, including *M. leprae*, are supposed to utilize these mechanisms to evade the host immune response. Regulatory T cells and expression of co-inhibitory molecules on lymphocytes induce specific T-cell anergy/exhaustion, leading to disseminated and progressive disease. From this perspective, we outline how the co-inhibitory molecules PD-1, PD-L1, and Th1/Th17 versus Th2/Treg cells are balanced, how antigen-presenting cell maturation acts at different levels to inhibit T cells and modulate the development of leprosy, and how new interventions interfere with leprosy development.

Keywords: immunotherapy, leprosy, T-regulatory cells, immune checkpoint blockade, PD-1:PD-L1, cytotoxic T-lymphocyte-associated protein 4

INTRODUCTION

Leprosy remains a relevant health problem in Brazil and India even after the introduction of multidrug therapy and has spread worldwide (1–3). Leprosy presents different clinical features that are determined by the host immune response against *Mycobacterium leprae*; at the pole of this spectrum are tuberculoid and lepromatous disease. In tuberculoid leprosy (TT), Th1 polarization, characterized by the production of IFN-γ, which activates CD8 T cells, macrophages and

bactericidal mechanisms that control *M. leprae* growth, is critical for the protective response (2, 4, 5). By contrast, lepromatous leprosy (LL) presents with impaired specific cellular immunity. The immune response often differentiates into a Th2 profile, with abundant production of IL-4 and predominant B cell activation, which allows for evasion by the bacillus. *M. leprae* shows strategies to limit the host protective immune response leading to chronic infection (6, 7). In chronic infections, T cells are exposed to persistent antigen stimulation as a gradual loss of effector functions and cytokine production as well as persistently increased expression of multiple inhibitory receptors (6, 8). The immunomodulatory properties from mycobacteria have been explored to understand macrophage function (5, 9). In addition, *M. leprae* antigens interfere with T-cell proliferation (10) and are involved in Treg-cell expansion through HSP-60 (11). Evidence has indicated that Treg cells, besides expression of immune checkpoint molecules with inhibitory activity, such as PD-1, PD-L1, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), induce specific T-cell anergy, leading to disseminated and progressive disease (7, 12, 13). Immune checkpoint (ICP) molecules play an important role in T-cell activation and determine the functional outcome of T cells, reducing the proliferation and secretion of inflammatory cytokines, such as IL-2, IFN-γ, and TNF-α (14, 15). Those molecules also interfere with dendritic cell (DC) maturation and macrophage effector function (5, 16). ICP, particularly PD-1/PD-L1 and CTLA-4, have been widely explored as therapeutic targets in cancer because these biomarkers are also highly expressed in the tumor microenvironment (14, 15). In infectious diseases, this therapeutic approach has been applied against HIV, HCV, and tuberculosis as an adjuvant of antimicrobial drugs (17–19).

Herein, to discuss new approaches for leprosy monitoring and treatment, we reviewed some of the ICP for leprosy persistence and mechanisms associated with T-cell lymphocyte anergy to *M. leprae* antigens as well as the role of Treg cells to modulate disease development.

IMMUNE CHECKPOINTS IN LEPROSY

Although ICP have been studied for approximately two decades, many features of their biology and signaling pathways remain unknown. ICP receptors are associated with autoimmunity, suggesting that these molecules play a critical role in immune tolerance and homeostasis (7, 8). In chronic infections, T lymphocytes are under persistent exposure to antigens, and this stimulus is commonly associated with T exhaustion (20). Various ICP molecules are highly expressed on exhausted T cells (14, 20), and this literature indicates that ICP blockade can restore immunity after reversion of the exhaustion phenotype of T cells (8). In leprosy, some recent data have shown a strict relationship between ICP expression and disease persistence.

Cytotoxic T-lymphocyte-associated protein 4 is an important molecule that controls lymphocyte activation (21). This molecule binds to CD80/CD86, antagonizing CD28 signaling, on antigen-presenting cell (APC) cells, leading $CD4^+$ and $CD8^+$ T cells to assume an anergic phenotype (14, 22). Some CTLA-4 signaling pathways are still unknown, and it is unclear how this receptor interferes with lymphocyte activation as well as how CD3 phosphorylation, ZAP-70 activation, or tyrosine phosphatase SHP-2 act as intracellular mediators of those pathways (21). Indeed, CTLA-4 is essential for Tregs function. Treg cells highly express CTLA-4, which controls DC maturation, leading to internalization of CD80 and/or CD86 in addition to indoleamine-2,3-dioxygenase (IDO) activation, leading to expression of the immunosuppressive mediator kynurenin (16, 21, 23). These signals can also promote nuclear localization of Foxo, a transcriptional factor that suppresses transcription of the genes encoding IL-6 and TNF-α, both of which are crucial effector cytokines for the control *M. leprae* infection (6, 22). In LL patients, CTLA-4 has been described as a biomarker in blood and inflammatory infiltrating cells (24, 25). Increased expression of CTLA-4 was detected in LL lesions compared with that in TT lesions (24). Our group has found increased expression of CTLA-4 on lymphocytes and Treg cells from LL patients in contrast to reduced CTLA-4 expression on the same cell populations of TT patients[1] (**Figure 1**). We also observed that $CD4^+CD25^-$ T cells obtained from LL patients suppressed allogenic proliferation in functional tests (**Figure 1**). A suppressive role of CTLA-4 has also been demonstrated in $FoxP3^-$ T cells, and these data might explain the suppressive profile presented by LL patients (26). We also observed that $CD4^+CD25^-$ T cells obtained from LL patients suppressed allogenic proliferation on functional tests (**Figure 1**). In TT patients, *in vitro* blockade of CTLA-4 restored peripheral blood mononuclear cell (PBMC) proliferation (12), but there is no clinical trial showing those effects on LL patients. Immunotherapy (IT) with CTLA-4 blockade has mostly been conducted against tumoral cells; nonetheless, recent evidence has shown that CTLA-4 expression is associated with reduced secretion of TNF-α and IFN-γ and enhanced frequency of memory $CD8^+$ lymphocytes in experimental *L. monocytogenes* infection (27). Similarly, CTLA-4 blockade induced higher production of IFN-γ and NO when T cells were stimulated with *Trypanosoma cruzi* antigens, although it did not restore lymphocyte proliferation (28). Furthermore, despite few clinical trials concerning CTLA-4 blockade to control infectious diseases, HCV patients demonstrated promising results after this therapy (29). Taken together, these data suggest that immunotherapies might modulate the immune system in patients with a latent leprosy infection or active disease, enabling better control of *M. leprae* replication. Therefore, new discoveries concerning the role of CTLA-4 in the immune response during *M. leprae* infection could provide critical insight that can be applied to other infectious diseases.

Programmed cell-death protein-1 (PD-1) and its ligands PD-L1/L2 have also been identified as relevant ICP that promote immune evasion of tumor cells and infected cells (8, 14, 15). Those molecules are promising targets in anticancer therapy and

[1] Peripheral mononuclear blood cells were obtained from untreated patients diagnosed with leprosy at the Dermatology Clinic of the Lauro de Souza Lima (ILSL) in Bauru, São Paulo, Brazil. The experimental protocol used was approved by Ethical Comitee of Bauru School of Dentistry of Bauru (protocol number #148/2009), University of São Paulo, and informed, and written consent was obtained from all subjects before performing the studies.

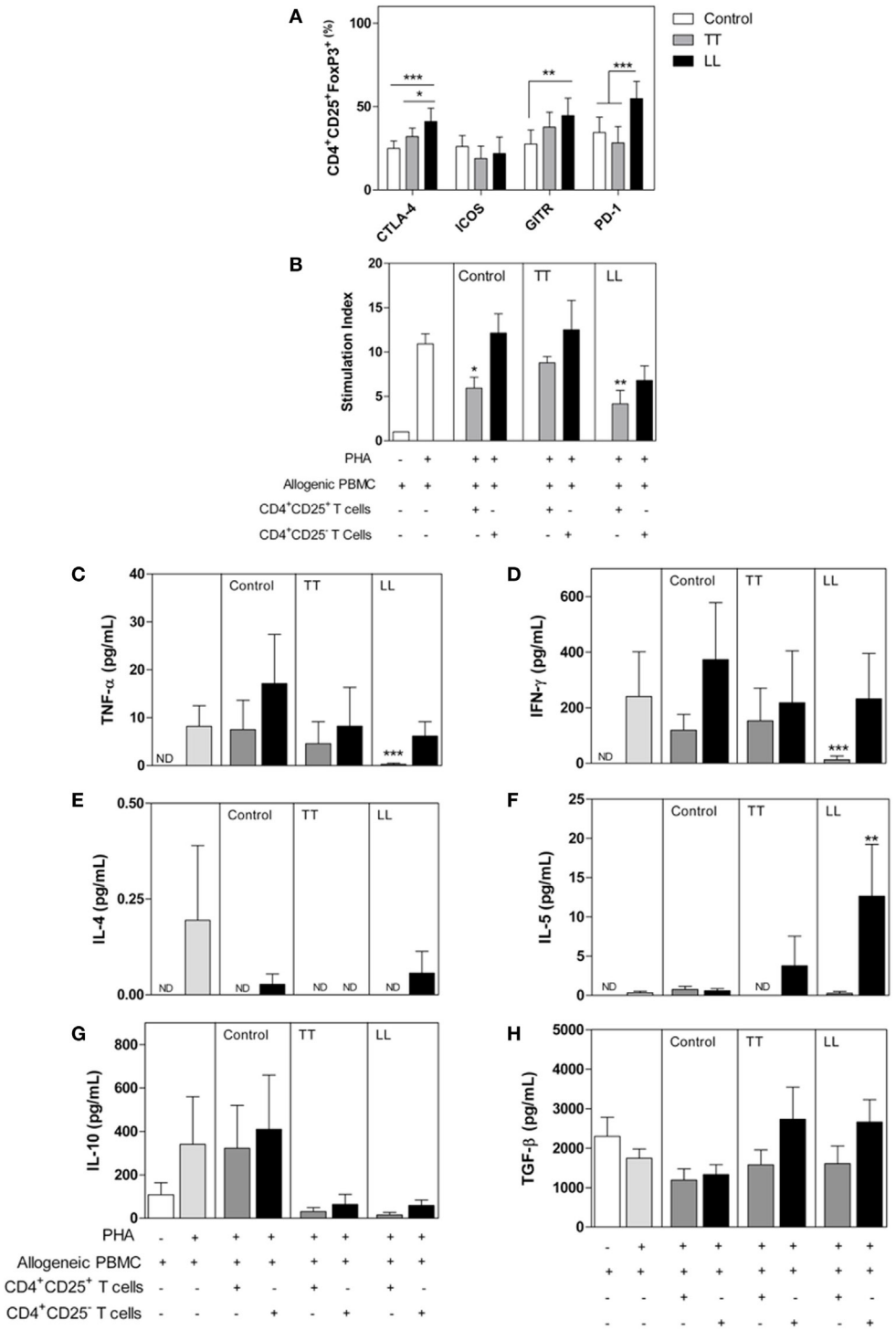

FIGURE 1 | Phenotype and functional characterization of $CD4^+CD25^+$ T cells in leprosy patients. Peripheral blood mononuclear cells (PBMCs) were isolated from patients with tuberculoid (TT, $n = 12$) and lepromatous leprosy (LL, $n = 12$), as well as from healthy control subjects ($n = 12$). **(A)** The frequency of $CD25^+$ and $FoxP3^+$ cells and expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), GITR, ICOS, and PD-1 were determined by flow cytometry. **(B)** Allogeneic PBMC (1×10^5 cells/well) was cultured with medium only, PHA, PHA plus $CD4^+CD25^+$ T, or $CD4^+CD25^-$ T cells (1×10^4 cells/well) from patients or control subjects. Proliferation was determined after 4 days of culture by CFSE dilution analyzed by flow cytometry. The results are expressed as the means ± SEM of the stimulation index of proliferation. IFN-γ **(C)**, TNF-α **(D)**, IL-4 **(E)**, IL-5 **(F)**, IL-10 **(G)**, and TGF-β **(H)** levels were determined in supernatants from cultures of suppression assays. The results are presented as the means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared with control subjects using ANOVA and the Bonferroni posttest. For the suppressive assay **(B)**, the results are expressed as the means ± SEM; $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, compared with the proliferation of allogeneic PBMCs cultured with PHA. ND, not detected.

are implicated in dysfunctional acquired immune responses, reducing the TCR signal to lymphocyte proliferation through ITIM (immune receptor tyrosine-based inhibition) motifs (30). The PD-1 signaling axis has been strongly related to T-cell anergy, pathogen persistence, and peripheral immune tolerance (14, 30). Although not yet targeted clinically, PD-1 is a promising target for leprosy IT. In leprosy, patients have presented with increased expression of PD-1 and PD-L1 on $CD4^+$, B cells, and $CD11^+$ cells (12, 13, 24, 31, 32), and *in vitro* blockade of PD-1 increased IFN-γ and IL-17 production by T cells (33). In accordance, our group found increased expression of PD-1 and GITR on lymphocytes and Tregs from LL patients (**Figure 1**). Blockade of PD-1 signaling in infectious disease has been associated with pathogen control in animal models for HBV, HIV, *Plasmodium* spp., *Leishmania* spp., *Trypanossoma* spp., and *M. tuberculosis* infection (34–38). These results suggest that ICP might be an important mechanism to regulate the immune response of LL patients. Thus, antibodies targeting the PD-1 pathways may improve the clinical outcome by restoring T-cell-mediated *M. leprae* immunity. However, in the infectious diseases context, immunotherapies based on ICP have not been tested or developed to the same extent as they have in cancer (39).

More recently, the signaling pathways and inhibitory mechanisms of lymphocyte-activation gene-3 (LAG-3) and TIGIT (T-cell immunoglobulin and ITIM domain) have also been explored as suitable new targets for immune blockade (14, 40, 41). TIGIT, a member of the CD28 family, is expressed on effector and memory T cells, Tregs, and natural killer (NK) cells, and its ligands, CD155 and CD122, are expressed on APC, T cells, and non-hematopoietic cell types, such as tumor cells (14, 40). TIGIT blockade might influence both adaptive and innate immune responses. $TIGIT^+$ Treg cells seem to control the Th1/Th17 ratio through enhanced IL-10 secretion, leading to a Th2 phenotype in animal models (42). In addition, TIGIT also controls NK cell function, limiting IFN-γ secretion and granule production (43–45); however, there are no available data concerning the role of this molecule on NK cells during leprosy. In HIV-infected subjects, PD-1, TIGIT and LAG-3 are considered to be biomarkers of persistent infection because $CD4^+$ T cells expressing these molecules present viral markers, even under antiretroviral therapy (41). Analysis of TIGIT expression on Th2 lymphocytes and Tregs from LL patients as well as its correlation with disease progression are highly warranted.

Regarding LAG-3, this molecule is also expressed on Treg cells and has been associated with increased suppressive events, such as in the immune response against HIV and *Plasmodium* spp., as well as many types of cancers (14, 41, 46). In leprosy patients, the role of LAG-3 remains unknown, although $LAG\text{-}3^+CD8^+$ T cells were detected when human PBMC cells were cultured with *M. leprae* as well as in human mycobacteria-induced granulomas (47). Some clinical trials have explored LAG-3 blockade to achieve tumor reduction and control cancer progress. Although the LAG-3 signaling pathway is not completely understood in *M. leprae* immunity, its homology to CD4 and cross-linking with MHC class II lead to impaired maturation of DC and Treg development (48, 49), suggesting LAG-3 as a new target for therapeutic intervention. Therefore, new discoveries concerning the role of this molecule in the immune response during *M. leprae* infection could provide insights that can be applied to other infectious diseases.

Recently, some evidence has indicated that combined ICP blockade might be a better strategy to explore the synergic effect of multiple immune checkpoints. Single-agent ICP approaches seem to induce compensatory upregulation of other ICPs as a cell mechanism to evade IT effects, and its failure index has been observed in one-half of oncology patients under this therapy (50, 51). One possibility would be to associate independent and non-redundant inhibitory pathways of these molecules, as observed in the CTLA-4 or PD-1 combination (50, 51). In addition, each infectious disease has its own ICP pattern of expression, as observed in HBV patients whose PD-1 expression is higher than that of CTLA-4. Therefore, combined ICP blockade might be a relevant mechanism for immune response regulation in leprosy and, likely, a feasible pathway to be explored as therapeutic targets.

T REGULATORY CELLS (Tregs)

T regulatory cells (Tregs) are a heterogeneous subset of T $CD4^+$ lymphocytes that might be developed at the thymus or on peripheral tissues under the control of many different signals from the microenvironment, such as TGF-β and IL-10 cytokines, retinol, and pathogen-associated molecular patterns (52, 53). Treg cells control many innate and adaptative immunological events, limiting tissue damage and maintaining homeostasis. Tregs explore many different mechanisms that control the immune response, such as increasing expression of CD25, to reduce lymphocyte proliferation through IL-2, increasing secretion of anti-inflammatory cytokines and increasing expression of granzyme and perforin, as well as ICPs, such as PD-1, CTLA-4, GITR, TIGIT, and LAG-3 (14, 40, 52–54). Treg cells in the infection site seems to be associated with the immune hyporesponsiveness observed after infection with many parasites, including *T. cruzi*, *P. brasiliensis*, *L. brasiliensis*, and *S. mansoni* (52–58). Some studies have shown the potential of Treg-cell depletion to augment antitumor immune responses (59) and infectious disease outcome (60) and have indicated that Treg-mediated T-cell suppression is an important mechanism by which pathogens evade immune responses (39, 53). In leprosy, an increased frequency of Treg cells was observed, and those lymphocyte subsets seem to contribute to pathogen persistence (13, 31–33, 61, 62). Our group assessed the suppressor features of Treg cells isolated from leprosy patients (**Figure 1**). Functional suppressive assays demonstrated impaired proliferation of allogenic PBMCs that were CFSE-labeled when cocultured with $CD25^+$ T cells isolated from LL patients. In addition, the IFN-γ and TNF-α production levels were reduced in the presence of $CD4^+CD25^+$ T cells from LL patients. Moreover, our results showed that Treg cells ($Foxp3^+CD25^+$ cells) express high levels of CTLA-4 and PD-1. Such regulatory features were not hallmarks of Treg cells from TT patients (**Figure 1**). Expression of CTLA-4 by Treg cells serves as a mechanism of Treg cells to suppress excessive T-cell responses. Blocking CTLA-4 *in vivo* has been shown to inhibit Treg cells and promote antitumor immunity (63).

Activated Tregs produce IL-10, IL-35, and TGF-β, which act to suppress the immune response (64). Tregs downregulate the immune reactions through production of anti-inflammatory cytokines, lowering the antigen-presenting function in DCs, and macrophages with correspondingly decreased counts of Th1, Th2, Th17 $CD4^+$ T cells, and cytotoxic $CD8^+$ T cells, as well as the cytokines produced by them, and induction of apoptosis [reviewed in Ref. (65)]. High levels of TGF-β and IL-10 producing $Foxp3^+$ T cells were reported to be increased in the lepromatous state in the circulation and skin lesions (61). Recent work has demonstrated that Tregs play a role in *M. leprae*-specific Th1 unresponsiveness during lepromatous disease (33). In LL, Th2/Treg polarization seems to be important to disease progression, and multiple factors may be responsible for these events, such as antigen exposure and innate immune activation (7). Otherwise, TT patients present a cellular immune response polarized to Th1/Th17 (31–33). Recent work has shown that in patients with a type 2 reaction, downmodulation of Tregs favors the development of Th17 responses (66, 67). The $FoxP3^+$ Treg phenotype seems to be reverted into Th17, exploring the signaling through IL-12 and IL-23 (31). Th17 and Treg cells are new players associated with immunopathology in leprosy and its reactions (62). In this context, the ideal treatment for LL patients seems to require modulation of the T lymphocytes subsets to expand Th17 lymphocytes and control Treg cells, favoring the cellular immune response (5, 33). This strategy to shift the immune response to Th1/Th17 probably might achieve better outcomes in leprosy treatment; however, it might be associated with an increased risk of developing reactional states, such as erythema nodosum (66, 67). Reactional episodes have been associated with immune stimulation and can occur at any moment during leprosy infection and represent one of the most adverse events associated with disease (2, 5). Future work will need to confirm the efficacy of Treg cells for IT of infectious diseases. In addition, it is important to analyze the combinations of Treg cell targeting with ICP blockade to make IT more effective.

ANTIGEN-PRESENTING CELLS

Because leprosy is an intracellular infection, T-cell activation and responses are important for protective immunity. It is well known that macrophages and DCs regulate the activity of lymphocytes in adaptive immune responses, which could allow them to play important roles in IT (68). This capacity makes them potent adjuvants for the induction of antigen-specific T cells in infected hosts. In leprosy, data have shown that a delicate balance of costimulatory pathways between T-cell and APCs is essential for T-cell activation.

Dendritic cells play important roles in both innate and acquired immunity responses to *M. leprae* infection. These cells induce Th1 immunity and CTL responses (2, 69). However, *M. leprae* have evolved mechanisms to inhibit the ability of DCs to present antigens, thereby promoting a protective immune response. Exposure of DCs to *M. leprae* impairs its maturation and inhibits CD80, CD86, HLA-DR and CD40 expression (70–72). Recognition of *M. leprae* antigens, such as LAM, through DC-SIGN has also been described as an important signaling pathway to control DC maturation, leading to increased IL-10 secretion, increased lipid metabolism and bacterial persistence (73, 74). Furthermore, IDO is another molecule associated with DC maturation and its tolerogenic phenotype. IDO has also been detected at high levels in LL patients (75). IDO catalyzes the conversion of tryptophan to *N*-formyl-kynurenine, and this molecular messenger controls cell proliferation, induces apoptosis, and shifts T-naïve cells to develop into Tregs (52, 75). DC-based IT has been used to improve the immune response against tumor cells using DC vaccines and blocking ICP associated with DC tolerogenic phenotypes (76). To improve the immune response in chronic infectious diseases, such as HBV infection, PD-L1 blockade has been used to restore the production of Th1 cytokines, such as TNF-α, IL-2, and IFN-γ (34). On the other hand, DC vaccines have also been used to improve the immune response to the Th17 profile against *Leishmania* spp. infection (77). Application of DCs in IT against *M. leprae* has not been explored despite the potential for the stimulation of an efficient antibacterial immunity. In other disease, the results indicate that DC-based IT might be more effective in combination with conventional treatments because the association should modulate the immune system in a way that helps the host control or eliminate pathogens (68, 77, 78). Therefore, exploring strategies to shift the immune response to Th1 might achieve better outcomes in leprosy treatment, leading to reduced expression of *M. leprae* virulence factors, such as LAM, PGL-I, and lipid metabolism (9, 10). Future studies should also address the possible advantage of combining DC-based IT with ICP blockade or other therapeutic approaches, such as antimicrobial and anti-inflammatory drugs.

CONCLUDING REMARKS AND PERSPECTIVES

Altogether, evidence indicates that multiple factors are responsible for antigen-specific unresponsiveness in leprosy. We summarized some of these features and showed how ICP interfere with T-cell activation. We suggest that ICP blockade might interfere with leprosy pathogenesis (**Figure 2**). In our opinion, leprosy has been shown to have many interesting features concerning regulation that can be explored to better understand immunological mechanisms (11).

Immune checkpoint blockade has been widely applied in oncology as an adjuvant to chemotherapy and radiotherapy. In infectious diseases, ICP blockade is still a recent approach. In leprosy, it is even more critical because it is a neglected disease and probably ICP blockade might not be used as a large-scale therapy. There are some different strategies that can be used to achieve better treatment outcomes and improve the cellular response against *M. leprae*. In this context, BCG (re)vaccination for LL patients has been fulfilled without predictive results (79). For refractory patients, IT might be an additional strategy to control chronic disabilities.

Despite these promising results, IT based on ICP blockade has been associated with autoimmune and inflammatory

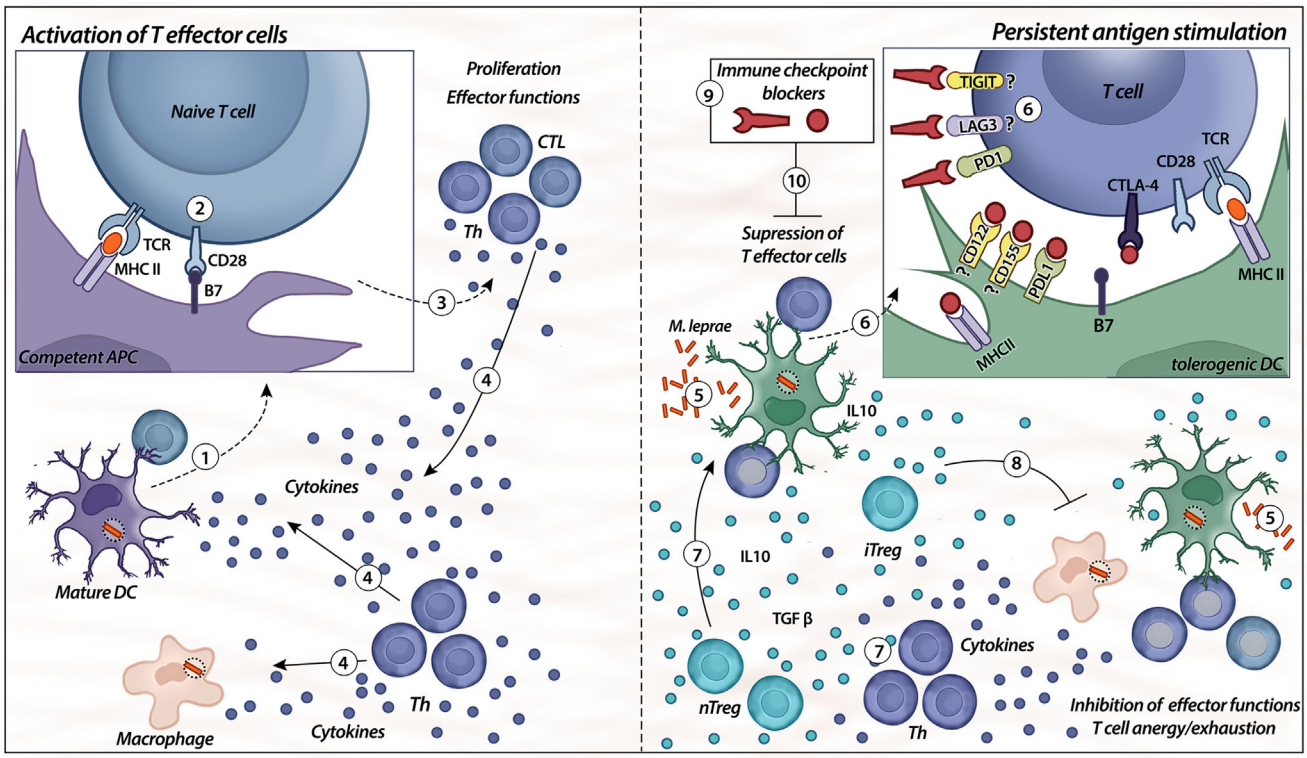

FIGURE 2 | Immune checkpoints. Activation of T effector cells is initiated with competent/mature antigen-presenting cells (APCs), such as mature dendritic cells (DC) (1, 2). For the first signal, APC displays the antigen to the naïve T cell *via* a complex with MHC II on their surfaces that is recognized by TCR on the surface of T cells; the second signal is nonspecific, resulting from the binding of B7 ligand on the APC with its receptor, CD28, on the T cell (2). When both signals are provided (3), T cells (different types of T helper and CTLs) exert their effector functions, such as release of cytokines by different Th cells (IL-6, IL-2, IFN-γ, IL-12, and TNF-α) and cytotoxicity from CTL (4). The presence of chronic immune stimulation due to persistent microbial antigens impairs specific cellular immunity (5, 6). Expression of co-inhibitory molecules, such as PD-1, TIGIT, lymphocyte-activation gene-3 (LAG-3), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), on lymphocytes and their respective ligands on the APC surface (PD-L1, CD122/155, MHC class II, and B7) induce specific T-cell anergy, leading to disseminated and progressive disease. In addition, there is higher differentiation of natural and induced types of Treg cells (nTreg/iTreg), as well as an imbalance of Th cells (7). The release of IL-10 and TGF-β from heterogeneous Treg cell subsets controls the immune response by the inhibition of effector functions, as well as induces tolerogenic phenotypes in DCs (8). The blockade of immune checkpoints, such as PD-1, CTLA-4, LAG-3, and TIGIT, might be a strategy to control the tolerogenic features observed in lepromatous leprosy patients (9, 10).

events, such as oral mucositis and hepatitis (80, 81). In leprosy, increased immune stimulation has been associated with reactional states in LL patients and might be a disadvantage of this therapy. Certainly, more studies and clinical trials are needed to determine the role of Treg cells, ICPs and DCs as therapeutic targets to control *M. leprae* and leprosy progression.

ETHICS STATEMENT

The experimental protocol used was approved by Ethical Committee of Bauru School of Dentistry, University of São Paulo and acquainted by Lauro of Souza Lima Institute Research Ethics Committee (protocol #148/2009). All subjects assigned informed consent that was obtained before performing the studies.

AUTHOR CONTRIBUTIONS

Conception and design: AC, JS, and GG. Development of methodology and formal analysis: HL, TG, TM, VM, MV, MN, FS, EM, and JB. Acquisition of data (provided animals, provided facilities, etc.): AC, JS, VS, and GG. Analysis and interpretation of data (e.g., statistical analysis) and writing of original draft: HL. Writing, review, and/or editing: HL, TG, VS, and AC. Funding acquisition and study supervision: AC.

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REVIEW
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Host Lipid Mediators in Leprosy: The Hypothesized Contributions to Pathogenesis

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The spectrum of clinical forms observed in leprosy and its pathogenesis are dictated by the host's immune response against *Mycobacterium leprae*, the etiological agent of leprosy. Previous results, based on metabolomics studies, demonstrated a strong relationship between clinical manifestations of leprosy and alterations in the metabolism of ω3 and ω6 polyunsaturated fatty acids (PUFAs), and the diverse set of lipid mediators derived from PUFAs. PUFA-derived lipid mediators provide multiple functions during acute inflammation, and some lipid mediators are able to induce both pro- and anti-inflammatory responses as determined by the cell surface receptors being expressed, as well as the cell type expressing the receptors. However, little is known about how these compounds influence cellular immune activities during chronic granulomatous infectious diseases, such as leprosy. Current evidence suggests that specialized pro-resolving lipid mediators (SPMs) are involved in the down-modulation of the innate and adaptive immune response against *M. leprae* and that alteration in the homeostasis of pro-inflammatory lipid mediators versus SPMs is associated with dramatic shifts in the pathogenesis of leprosy. In this review, we discuss the possible consequences and present new hypotheses for the involvement of ω3 and ω6 PUFA metabolism in the pathogenesis of leprosy. A specific emphasis is placed on developing models of lipid mediator interactions with the innate and adaptive immune responses and the influence of these interactions on the outcome of leprosy.

Keywords: leprosy, *M. leprae*, resolvin, leukotriene, lipoxin, prostaglandin, immune responses, clinical spectrum

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INTRODUCTION

Leprosy is a chronic granulomatous disease driven by interactions of the human host with *Mycobacterium leprae* an obligate intracellular pathogen that infects macrophages and Schwann cells of the peripheral nervous system. *M. leprae* is the only mycobacterial infection that causes widespread demyelinating neuropathy, which results in severe and irreversible nerve tissue damage. The prevalence of leprosy is gradually decreasing in many countries due to multidrug therapy (MDT) (1). However, the rates of new case detection remain relatively stable in developing countries (1). India and Brazil are the countries that exhibit the highest incidence and account for 60 and 13% of the global new cases of leprosy, respectively (1).

Leprosy is well known for its bi-polarization of the immune response, and it is established that the nature and magnitude of the host immune response against *M. leprae* are critical factors for the pathogenesis of leprosy and its varied clinical manifestations. At one end of the spectrum, tuberculoid

(TT) disease is typified by strong T-helper type 1 (Th1) cellular immunity and low bacterial load (2–4). This response promotes the protection against the pathogen via interferon-gamma (IFN-γ) activation of macrophage anti-microbicidal mechanisms (5). These patients also present robust T-helper type 17 (Th17) activity (6) that stimulates macrophages and enhances Th1 responses (7). The other end of the spectrum, lepromatous leprosy (LL), is characterized by a low or even absent Th1 response (8) but robust T-helper type 2 (Th2) and humoral responses. The diminished Th1 response in LL is partially explained by the highly suppressive activity of T regulatory (Treg) cells and the reduced frequency of Th17 cells (4, 6). Consequently, these patients manifest the most severe form of the disease and are unable to control *M. leprae* growth (2). Between these two clinical forms, patients with intermediate immune responses develop borderline clinical forms: borderline tuberculoid (BT), borderline-borderline (BB), and borderline lepromatous (BL). BT patients present with a dominant IFN-γ response, and also a higher activity of Th17 cells (6), while BL patients exhibit T-cell anergy, because of the higher frequency of Treg cells (4, 6), and a higher production of interleukin-4 (IL-4) (9–11). Peripheral neuropathy can occur in all clinical forms of leprosy but is most pronounced in patients who present with an exacerbated acute immune-inflammatory response, designated type 1 reaction (T1R). Multiple studies indicate that pathogenic $CD8^+$ and $CD4^+$ T cell responses (12–14) and production of nitric oxide (NO) in *M. leprae*-infected macrophages are related with nerve injury in leprosy patients (15). Thus, the human immune response against *M. leprae* is involved with key aspects of leprosy pathogenesis.

Metabolomic-based studies reveal that *M. leprae* infection promotes several modifications in human metabolism. The most prominent of these metabolic changes is a correlation between the spectrum of clinical forms of leprosy and the metabolism of ω3 and ω6 polyunsaturated fatty acids (PUFAs) (16–18). Of particular interest are the PUFA-lipid mediators: prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), leukotriene B_4 (LTB_4), lipoxin A_4 (LXA_4), and resolvin D1 (RvD1). Both PGE_2 and PGD_2 are found in elevated levels in the sera of LL patients as compared to BT patients (17). Additionally, PGD_2 levels are increased in leprosy patients with T1R, while PGE_2 levels decrease in patients with a T1R (18). BT and LL patients have similar levels of the pro-resolving lipid mediators, LXA_4 and RvD1 (17). However, when compared with healthy individuals, the levels of LXA_4 and RvD1 are elevated in the sera of BT and LL patients. In patients with T1R, the level of RvD1 is significantly decreased, as is the ratio of LXA_4/LTB_4 (18).

It is well established that lipid mediators derived from the metabolism of ω3 and ω6 PUFAs are able to modulate the innate and adaptive immune responses (19–26). Thus, we posit that the PUFA-derived lipid mediators are important factors in the pathogenesis of leprosy. The objectives of this review are to bring together metabolic and immunological data that support our hypothesis and to provide an understanding of how lipid mediators potentially function across the spectrum of disease. Specifically, we will focus the review on the five lipid mediators (PGE_2, PGD_2, LTB_4, LXA_4, and RvD1) found to be differentially produced in leprosy patients (17, 18).

A BRIEF REVIEW OF THE RELEVANT LIPID MEDIATORS

The ω6 PUFA, arachidonic acid (AA), is the precursor for a variety of lipid mediators (prostaglandins, leukotrienes, lipoxins, and thromboxanes) that exhibit immune-inflammatory functions (**Figure 1**; **Table 1**) (26–28). Importantly, AA can be metabolized by three separate pathways: cyclooxygenase (COX) pathway, lipoxygenase (LO) pathway, and epoxygenase pathway (the latter is not discussed in this review) (**Figure 1**) (29).

The COX pathway converts AA into prostaglandins via two isoforms of COX, COX-1 and COX-2 (**Figure 1**) (29). Both enzymes convert AA into PGG_2, which is reduced to PGH_2 and then converted to PGD_2 or PGE_2 by PGD or PGE synthase, respectively (**Figure 1**) (74). PGE_2 and PGD_2 are involved with the early stages of inflammation, and it is well established that both lipid mediators exhibit a dual role in immune-inflammation due to their capacities to exert pro- and anti-inflammatory responses (**Table 1**) (38, 75). This might be partially explained by the fact that both prostaglandins are recognized by more than one prostaglandin receptor (PGE_2 – EP1, EP2, EP3, and EP4; PGD_2 – DP1 and CRTH2) (see **Table 1**) (19, 37, 51, 52). Moreover, PGD_2 and its metabolites (e.g., 15d-PGJ_2) are ligands for the peroxisome proliferator-activated receptor gamma (PPAR-γ) (76, 77).

The LO pathway converts AA to leukotrienes and lipoxins (29). The production of LXA_4 and LTB_4 is dependent on 5-LO that converts AA to leukotriene A_4 (LTA_4) via 5-hydroperoxyeicosatetraenoic acid (5-HPETE) (**Figure 1**) (78–82). Subsequently, LTA_4 hydrolase (LTA_4H) catalyzes the conversion of LTA_4 to LTB_4 (83) and platelet-derived 12-LO or 15-LO uses LTA_4 as a substrate for the production of LXA_4 (**Figure 1**) (84, 85). LTB_4 is involved in the initiating steps of the immune-inflammatory response and exerts its pro-inflammatory functions through two G-protein-coupled receptors BLT1 and BLT2 (**Table 1**) (86). More specifically, LTB_4 has the capacity to act as a chemoattractant for leukocytes, activate inflammatory cells (30), and favor Th1 and Th17 responses (**Table 1**) (21, 32, 33, 87–89). In contrast, LXA_4 is a specialized pro-resolving lipid mediator (SPM) that acts via the G-protein-coupled receptors ALX/FPR2 and GPR32 (**Table 1**) (63). An imbalance between the levels of LXA_4 and LTB_4 exacerbate the immune-inflammatory response and/or favor pathogen survival, including mycobacterial infections (21, 90). Importantly, the SPMs promote the resolution phase of inflammation by impairing the recruitment of leukocytes, stimulating the engulfment of apoptotic cells by phagocytes (known as efferocytosis) and inducing tissue repair (28, 69).

Lipid mediators derived from the essential ω3 PUFAs, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) include the resolvins, maresins, and protectins, all of which are SPMs (**Figure 2**) (28). The E-series resolvins (resolvins E1 to E3) are synthesized directly from EPA, while maresins (maresin-1 and maresin-2), protectins (protectin-1 and neuroprotectin-1), and D-series resolvins (resolvins D1 to D6) are produced from DHA (**Figure 2**). However, DHA itself can be produced from EPA by two elongation steps, desaturation and

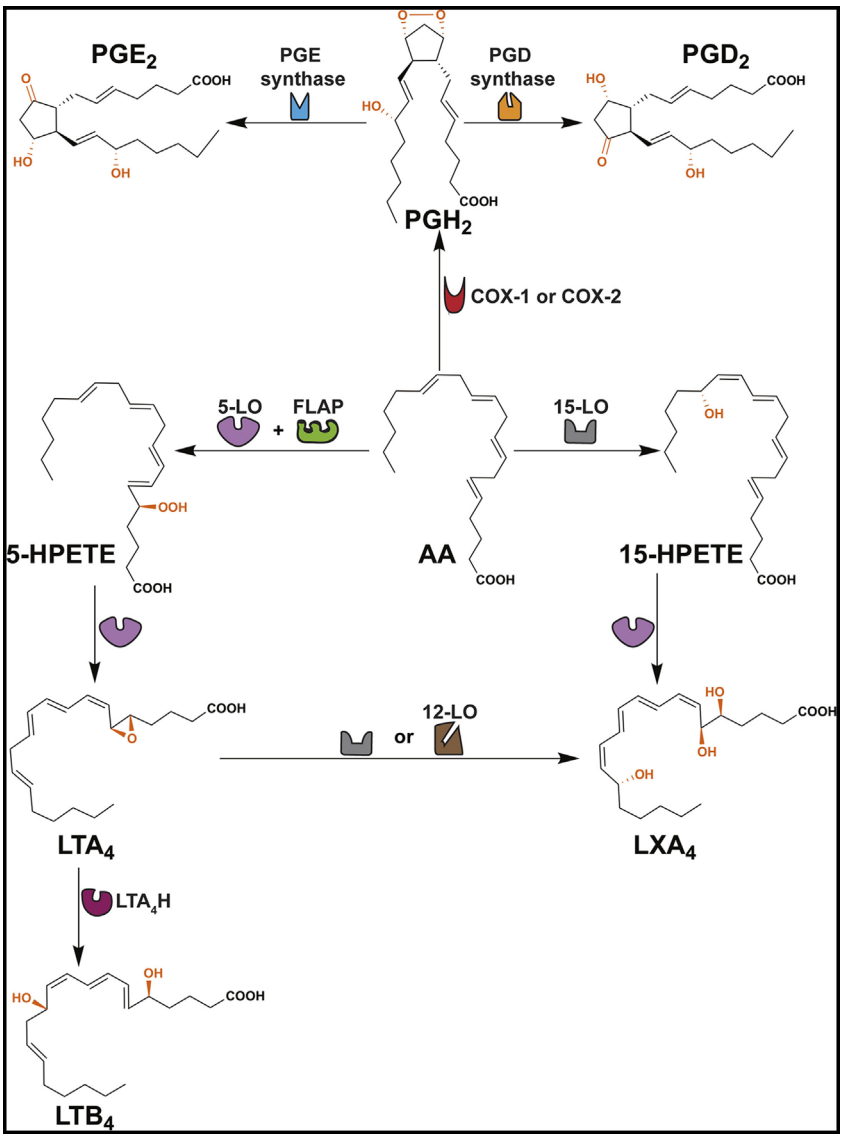

FIGURE 1 | Formation of PGD_2, PGE_2, LTB_4 and LXA_4. This scheme shows that arachidonic acid (AA) is converted to several ω6 PUFA-derived lipid mediators through cyclooxygenase (COX) and lipoxygenase (LO) pathways. COX enzymes (constitutive COX-1 or inducible COX-2) exhibit a COX activity that incorporates two molecules of oxygen into AA to form PGG_2 (not shown) and peroxidase activity that catalyzes a 2-electron reduction of PGG_2 to PGH_2. PGH_2 is the direct precursor of PGD_2 and PGE_2. Formation of LTB_4 occurs via the precursors 5-HPETE and LTA_4. LXA_4 is derived from 15-HPETE and/or LTA_4. FLAP, 5-lipoxygenase-activating protein; LTA_4H, leukotriene A_4 hydrolase.

subsequent β-oxidation in the peroxisome (91, 92). Important in this review is the D-series resolvins and specifically RvD1. This SPM has overlapping activities with LXA_4 and acts via the same G-protein-coupled receptors, ALX/FPR2 and GPR32 (**Table 1**) (63).

ANALYTICAL APPROACHES TO IDENTIFY AND MEASURE LIPID MEDIATORS

The identification and quantitation of PUFA-derived lipid mediators have been a challenge due to the small quantities produced within tissues and cells. Thus, highly sensitive methods of gas and liquid chromatography-based separations coupled with detection by mass spectrometry (e.g., GC–MS, GC–MS/MS, LC–MS, and LC–MS/MS) and immunology-based assays [enzyme-linked immunosorbent assay (ELISA)] have played a pivotal role in the analysis of lipid mediators (93, 94).

The separation of individual lipid mediators by GC or LC allows the analyses of multiple lipid mediators in a single biological sample, and the detection of the lipid mediators by MS or MS/MS provides a means for their identification and quantification (95). It is noted that many of the ω3 and ω6 PUFA-derived lipid mediators are isomers, therefore the fragmentation patterns

TABLE 1 | Functions of the lipid mediators discussed in this review.

Lipid mediators	Receptor(s) and cell expression	Functions
Leukotriene B_4	BLT1 – neutrophils, monocytes/macrophages, dendritic cells, mast cells, effector $CD8^+$ T cells, naive $CD4^+$ T cells, differentiated T-helper type 1 (Th1), T-helper type 2 (Th2), and T-helper type 17 (Th17) cells, and endothelial cells (30, 31)	Recruit neutrophils, monocytes and macrophages (30)
		Enhance Th1 response (22)
		Recruits Th1, Th2, and Th17 cells (32, 33)
	BLT2 – expressed ubiquitously (30, 31)	Enhances TNF-α expression and also the production of pro-inflammatory cytokines associated with Th1 responses [interferon-gamma (IFN-γ) and interleukin (IL)-12] (21, 22)
Prostaglandin E_2	EP1 – endothelial cells (34)	Promotes local vasodilation, attraction and activation of neutrophils, macrophages, and mast cells at early stages of inflammation (38)
	EP2 – mast cells, neutrophil, naive T cells, monocytes, macrophages, Th17 cells, and endothelial cells (34–37)	Regulates the production of IL-23 in dendritic cells (23)
		Inhibits the synthesis of IL-12 in dendritic cells (19)
	EP3 – platelets, mast cells, monocytes, and endothelial cells (34, 37)	Impairs the proliferation of T cells (39, 40)
		Regulates the production of nitric oxide (41)
	EP4 – mast cells, eosinophils, monocytes, dendritic cells, naive T cells, Th1 cells, Th17 cells, B lymphocytes, and endothelial cells (35–37)	Modulates Th1 cells differentiation (24, 42–44)
		Promotes the expansion of T regulatory (Treg) cells (45)
		Up-regulates the transcription factor FOXP3 (46)
		Inhibits the activation of macrophages by IFN-γ (47)
		Induces apoptosis (48, 49)
Prostaglandin D_2	DP1 – mast cells, monocytes, and immature and mature dendritic cells (19, 50)	Promotes the myelination of neurons (55)
		Induces vasodilation, erythema, edema and induration (56–58)
	CRTH2 – Th2 cells, basophils, eosinophils, mast cells, macrophages, and dendritic cells (19, 51–54)	Down-modulates the synthesis of IL-12 in dendritic cells (19, 59)
		Enhance the ability of Th2 cells to produce IL-2, IL-4, IL-5, and IL-13
		Reduces the numbers of $CD4^+$ and $CD8^+$ T cells that produces IL-2 and IFN-γ (60, 61)
		Induces chemotaxis of Th2 cells, eosinophils, and basophils (62)
Lipoxin A_4	ALX/FPR2 and GPR32 – monocytes macrophages, neutrophils, and T cells (Th1, Th17, and Tregs) (26, 63)	Inhibits the recruitment of neutrophils (64)
		Promotes macrophage efferocytosis (65)
		Down-regulates Th1-derived cytokines like IFN-γ, TNF-α, and IL-6 (20, 21, 66, 67)
		Induces the synthesis of the anti-inflammatory cytokine IL-10 (66)
		Inhibits the synthesis of LTB_4 (68)
Resolvin D1	ALX/FPR2 and GPR32 (see Lipoxin A4) (26, 63)	Shortens resolution of inflammation
		Inhibits the recruitment of leukocytes (28, 69)
		Down-modulates the production of TNF-α, IL-6, IL-8, IFN-γ, and IL-12 (70–72)
		Up-modulates the production of IL-10 (70)
		Efferocytosis (73)
		Inhibits LTB_4 production (68)
		Decreases the capacity of Th1 and Th17 cells to produce IFN-γ and IL-17, respectively; prevents Th1 and Th17 generation from naive CD4 T cells; promotes the *de novo* generation of Treg cells; and induces the expression of CTLA-4 (26)

generated my MS/MS provide additional structural information over what is obtained with an accurate mass measurement (MS) (96). However, some isomeric lipid mediators produce similar fragment ion profiles. Thus, it is important to apply authentic standards with rigorous chromatographic separation to confirm the identity of specific lipid mediators. A major advantage of LC–MS or LC–MS/MS as compared to GC–MS or GC–MS/MS is that derivatization to ensure volatility of the lipid mediators is not required (97). Nevertheless, GC-based approaches remain an important tool for confirming the structure and abundance of lipid mediators obtained via LC–MS or LC–MS/MS analyses (93, 94, 98).

Enzyme-linked immunosorbent assay is an orthogonal approach for the quantification of lipid mediators and offers relatively high sensitivity and selectivity (97). However, ELISA-based assays are commercially available for only certain lipid mediators, typically those that are best characterized for their biological activity. Cross-reactivity of antibodies between lipid mediators is a potential limitation of this technique; thus, antibody specificity should be checked with authentic standards (99).

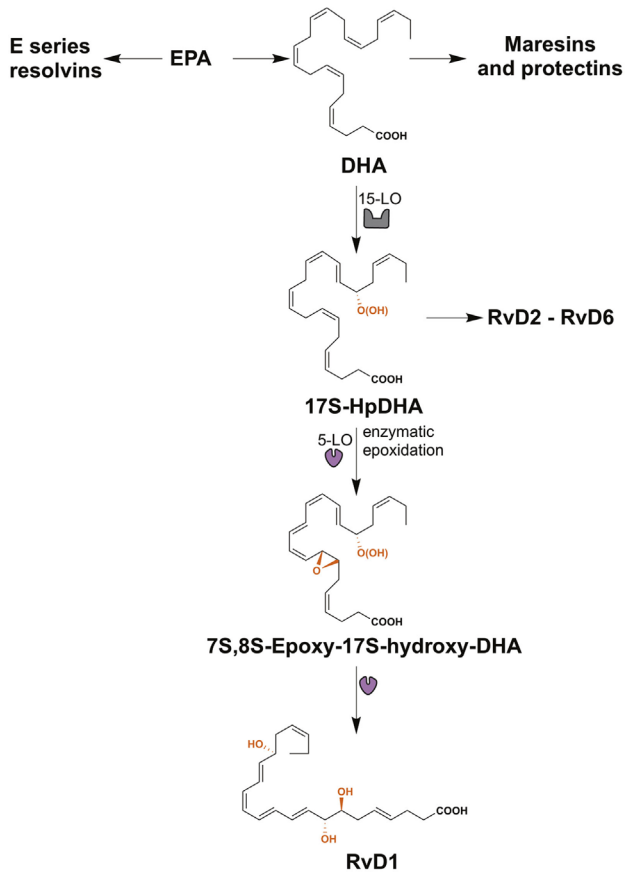

FIGURE 2 | The biosynthesis of resolvin D1 (RvD1). The resolvins from the E-series (resolvins E1–E3) are synthesized from eicosapentaenoic acid (EPA), while maresins (maresin-1 and maresin-2), protectins (protectin-1 and neuroprotectin-1), and resolvins of the series-D (resolvins D1–D6) are produced from docosahexaenoic acid (DHA). RvD1 is generated from the sequential oxygenation of DHA, a process catalyzed by 15-lipoxygenase (15-LO) and 5-lipoxygenase (5-LO). The initial conversion of DHA to 17S-HpDHA is catalyzed by 15-LO, followed a second lipoxygenation via 5-LO, which gives a peroxide intermediate that is transformed to 7*S*-,8*S*-epoxid-17*S*-hydroxy-DHA. Subsequently, the enzymatic hydrolysis of this compound generates the trihydroxylated product RvD1.

THE SPECIALIZED PRO-RESOLVING RvD1 IN LEPROSY: BAD WITH IT, WORSE WITHOUT IT

The Potential Role of RvD1 in Down-Modulation of the Immune Response of Leprosy

Amaral et al. revealed that sera levels of RvD1 in BT and LL leprosy patients were similar, but increased in comparison with the sera of healthy individuals (17). Interestingly, after MDT serum levels of RvD1 in BT and LL patients were reduced to those of healthy controls (17). These data indicated that RvD1 is being produced in response to inflammation and possibly also associated with the presence of the pathogen or pathogen products. However, induction of RvD1 production via *M. leprae* infection has not been investigated.

A comprehensive study to define the biological activity of the D-series resolvins (RvD1 and RvD2) and maresin-1 on the adaptive immune response demonstrated that these SPMs reduce the production of IFN-γ and IL-17 by Th1 and Th17 cells, respectively (26). Moreover, RvD1 was shown to promote the *de novo* generation of FoxP3$^+$ Treg cells, the expression of CTLA-4 (a surface marker of Treg cells) and IL-10 secretion. The similar levels of RvD1 in BT and LL patients, does not correlate well with this laboratory assessment of RvD1 activity, since BT patients present a strong Th1 and Th17 responses (3, 4, 6) and LL patients are characterized by T-cell anergy and increased frequency of Treg cells (4, 6). Nevertheless, it would be premature to conclude that RvD1 does not participate in the dichotomous immune responses of TT/BT and BL/LL patients. It is possible that the higher level of RvD1 down-modulates the Th1 immune response in TT/BT as well as BL/LL patients. Martins et al. demonstrated that peripheral mononuclear cells (PBMCs) from paucibacillary (TT/BT) leprosy patients possess a lower capacity to produce IFN-γ than healthy individuals exposed to *M. leprae* (3). Thus, the adaptive immune response in TT/BT individuals is still reduced as compared to healthy controls. Furthermore, it could be that RvD1 activity is related to the level of expression of its cognate receptors, GPR32 and ALX/FPR2. Thus, studies that assess the presence of these receptors in the T cells of TT/BT and BL/LL patients are required to fully understand the potential influence of RvD1 on the adaptive immune response across the spectrum of leprosy. Polymorphisms in the promoter region of the ALX/FPR2 gene resulting in a reduced expression of this receptor are known (100, 101). Thus, it would also be interesting to investigate whether polymorphisms exist between TT/BT and BL/LL patients in the promoter or functional regions of the GPR32 and ALX/FPR2 genes.

RvD1 Regulation of Macrophage Activity: A Possible Factor That Sustains Paucibacillary Infection

Besides the ability to reduce the activity of Th1 and Th17 cells, RvD1 also controls the activity of macrophages (102, 103). RvD1 induces efferocytosis in monocytes/macrophages (73), a process that engulfs apoptotic cells and is reported to play an important role in the clearance of *Mycobacterium tuberculosis* and *Mycobacterium avium* (104, 105). However, De Oliveira and colleagues indicated that this process might promote the persistence of *M. leprae* (106). Specifically, in the presence of *M. leprae*, efferocytosis alters the phenotype of the pro-inflammatory M1 macrophage toward anti-inflammatory M2 phenotype with increased the uptake and survival of *M. leprae*. Therefore, in paucibacillary patients, where apoptotic bodies are present in higher number (107, 108), efferocytosis may play an important role in the *in vivo* persistence of *M. leprae*. The increased levels of RvD1 in TT/BT patients could help drive this process (**Figure 3**).

Adding to the immunomodulatory activity of efferocytosis, it is recognized that *M. leprae* inhibits the capacity of macrophage to respond to IFN-γ stimulation (47) and impairs the production of pro-inflammatory cytokines (e.g., IL-6 and TNF-α) (109).

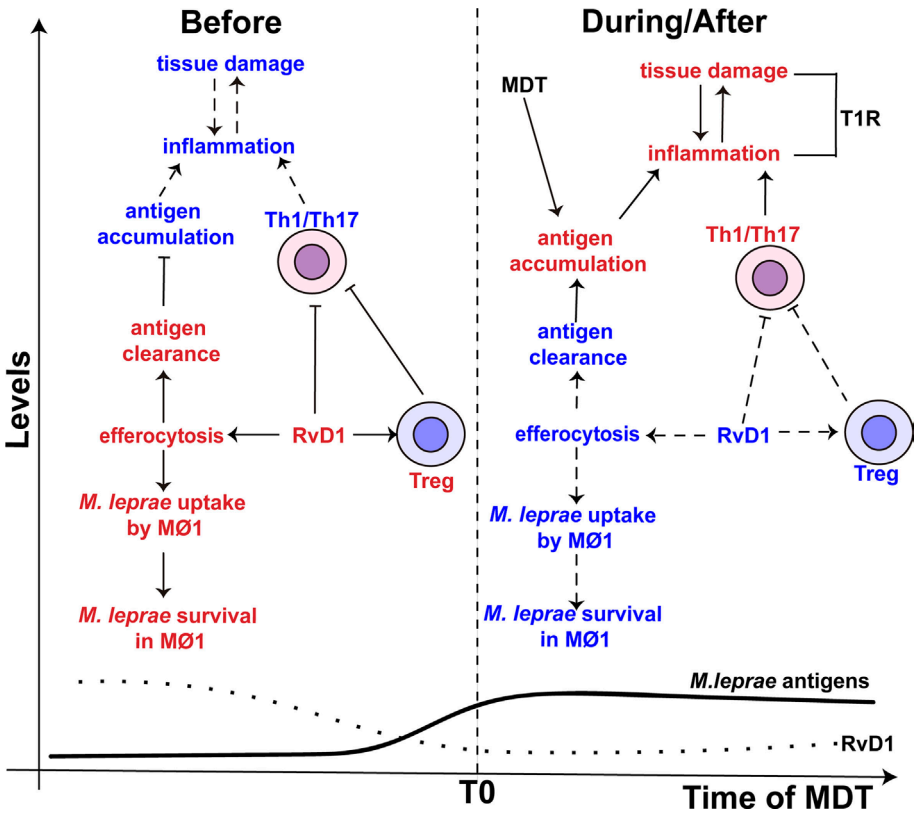

FIGURE 3 | The proposed role of resolvin D1 (RvD1) in leprosy. (Left side) The levels of RvD1 (dotted line) are higher before the start (T0) of multidrug therapy (MDT). The higher levels of RvD1 are hypothesized to increase the host's susceptibility to *M. leprae* infection. The increased levels of RvD1 prior to MDT could enhance the capacity of macrophages to engulf *M. leprae* antigens as well as the pathogen itself via efferocytosis. This would lead to antigen clearance, decreased antigen stimulation of T-helper type 1 (Th1) and T-helper type 17 (Th17) cells and favor the survival of *M. leprae*. Moreover, increased levels of RvD1 could directly inhibit Th1 and Th17 cells' response and promote the activity of T regulatory (Treg) cells. (Right side) After the start of MDT, the levels of RvD1 decrease (dotted line), while the abundance of *M. leprae* antigens increase (solid line) due to lysis and degradation of the bacilli, especially in multi-bacillary patients. The reduction of RvD1 could eliminate the suppression of the Th1 and Th17 responses, reduce the activation of Treg cells, and also decrease the ability of macrophages to promote efferocytosis. This impairment in efferocytosis would favor antigen accumulation. Thus, response to mycobacterial antigens by Th1 and Th17 cells would increase resulting in an immune-inflammatory response and potentially a T1R. The red color represents an intensification or increase in a process or abundance of a product, while the blue color symbolizes an attenuation of the process or product abundance. Arrows with solid lines indicate that a process related to the associated RvD1 level is favored, while an arrow with a hashed line indicates the process is not favored. (⊢) Represents inhibition of a process or activity. MΦ1 – M1, pro-inflammatory macrophages.

Macrophages infected with *M. leprae* have been found to preferentially prime Treg cells over Th1 or cytotoxic T cells (110). Thus, RvD1 may have an additive or synergistic effect on macrophage function that further reduces the innate responses against *M. leprae* and consequently allows the survival of the pathogen in leprosy patients with a robust Th1 and Th17 cells response (**Figure 3**). However, studies are required to determine whether RvD1 preferentially drives the response of *M. leprae*-infected macrophage, as well as enhancement of *M. leprae* uptake in the context of efferocytosis. While we would hypothesize that RvD1 would influence macrophage polarization in the context of *M. leprae* infection, the involvement of other lipid mediators in this process cannot be excluded.

The Reduction of RvD1 Levels in T1R: The Worse

T1R is a major complication in borderline leprosy patients (BT, BB, and BL) and occurs before, during and after MDT (111). The increased inflammation of T1R driven by Th1 and Th17 cells in skin lesions and/or nerves can result in permanent loss of nerve function (112, 113).

A higher bacillary load and MDT are factors associated with the development of T1R pathology (114–116). Thus, it has been hypothesized that the release of *M. leprae* antigens promoted by MDT drive an enhanced immune-inflammatory response, especially in multi-bacillary patients (116, 117). Interestingly, the levels of RvD1 in leprosy patients decrease after the conclusion of MDT (17). Thus, a reduction in circulating SPM may remove suppressive activity being placed on Th1/Th17 cells and contribute to susceptibility of developing T1R in the presence of *M. leprae* antigens (**Figure 3**). Recently, a metabolomics study of sera from leprosy patients with and without T1R, and that had not started MDT, confirmed that the level of RvD1 was significantly increased (9.01-fold) in non-T1R leprosy patients as compared to T1R leprosy patients and healthy controls (18). These findings indicate a direct correlation with reduced RvD1 levels and

destructive inflammation due to enhanced Th1/Th17 activity and revealed that reduced RvD1 production could occur during active disease.

As the balance of pro-inflammatory and pro-resolving lipid mediators are important in the development and control of inflammation, it is important to note that RvD1 also down-regulates the production of the pro-inflammatory lipid mediator LTB_4 (68). LTB_4 promotes chemotaxis of Th1 (32) and Th17 cells (33) and enhances the production of pro-inflammatory cytokines associated with Th1 responses (TNF-α and IFN-γ) (22). Although the concentration of LTB_4 in BT and LL patients are similar to healthy individuals (17), Silva and colleagues observed a significantly increased level of serum LTB_4 during T1R (18). Studies to define the mechanisms of RvD1 activity revealed that this SPM inhibits the translocation of 5-LO to the nucleus and this inhibits the synthesis of LTB_4 (68). This mechanism would explain why the levels of LTB_4 were not increased in leprosy patients without T1R, but with a reduction of RvD1, they become elevated in T1R patients. However, it does not explain why the levels of LTB_4 did not increase after MDT in leprosy patients without T1R since this treatment reduced RvD1 concentrations (17). It is possible that therapeutic elimination of infection reduces signals and stimuli leading to LTB_4 production, as well as those that drive RvD1 production.

In conclusion, although increased RvD1 levels may favor *M. leprae* infection by modulating the protective innate and adaptive immune responses (i.e., bad with it), at the same time, RvD1 is likely important to avoid exacerbated inflammation that may cause skin and nerve injuries. Once the levels of the RvD1 drop in a leprosy patient (e.g., because of MDT or other factors), we hypothesize that this increases susceptibility to pathogenic Th1 and Th17 responses against *M. leprae* antigens (i.e., worse without it).

THE BALANCE BETWEEN THE PRO-INFLAMMATORY LTB_4 AND THE SPECIALIZED PRO-RESOLVING LXA_4 IN LEPROSY

The Higher Levels of LXA_4 in Leprosy: A Possible Association with the Chronic Nature of *M. leprae* Infection

The study of Amaral et al. demonstrated that LXA_4 is increased in leprosy patients (17). However, the biological function of LXA_4 in *M. leprae* infection is not well understood, but has been studied in *M. tuberculosis* infection, another model of chronic infectious disease. In the murine model of tuberculosis, Bafica et al. showed that after 1 week of *M. tuberculosis* infection, LTB_4 and LXA_4 increase in abundance as compared to uninfected animals, but the levels of LTB_4 decrease after 10 days while those of LXA_4 persist during chronic *M. tuberculosis* infection (20). Interestingly, mice deficient for 5-LO (5-$lo^{-/-}$) did not produce LXA_4 increasing the resistance against *M. tuberculosis* due to higher production of Th1-derived cytokines (INF-γ and IL-12). Conversely, the 5-$lo^{-/-}$ mice treated with a LXA_4 analog reduce the levels of Th1 cytokines resulting in increased susceptibility to *M. tuberculosis* (20). These results indicate that LXA_4 has a more predominant effect than LTB_4 during *M. tuberculosis* infection and that a high LXA_4 favors the mycobacterial infection. Similar to the animal studies with *M. tuberculosis*, infection of humans by *M. leprae* and the presentation of leprosy, are associated with increased levels of LXA_4, but not LTB_4 (17). This likely reflects the capacity of an *M. leprae* infection to pass unnoticed for years (1–10 years), presumably due to a protective and non-pathogenic immune response. However, as observed for household contacts, a gradual increase in bacillary load and continuous exposure to antigen, down-modulates the immune response against *M. leprae* (3, 118). Thus, we hypothesize that the reduced capacity of the host to respond to *M. leprae*, even during an increase in the bacillary load, is exacerbated by a higher production of LXA_4. Once this SPM and RvD1 are produced in sufficient amounts they would inhibit the production of LTB_4 (68), and thus elevated levels of LXA_4, together with RvD1, might favor the chronic infection of *M. leprae*.

The Link between LXA_4/LTB_4 Ratios and the Expression of TNF-α in Leprosy

It is suggested that LTB_4 and LXA_4 modulate the expression or the effects of TNF-α, a pro-inflammatory cytokine involved with the resistance/susceptibility to leprosy (21, 22, 119). Moreover, an imbalance in the ratio of the pro-resolving LXA_4 to pro-inflammatory LTB_4 (LXA_4/LTB_4) is related with a poor control of the immune-inflammatory response in humans (120, 121). Collectively, metabolomics data produced with sera of leprosy patients indicate that the balance between LXA_4 and LTB_4 is altered (17, 18). However, the mechanisms by which altered ratios of LXA_4/LTB_4 affect the immunopathology of leprosy remain undefined.

Previous works from Tobin et al. demonstrated that the LXA_4/LTB_4 ratio was an important factor in susceptibility of zebrafish larvae to *Mycobacterium marinum*, due to the modulation of TNF-α expression (21, 88, 89). Specifically, shunting LTA_4 into LXA_4 synthesis resulted in an increase in the LXA_4/LTB_4 ratio and consequently a down-modulation of TNF-α expression (21, 88, 89). This culminated in a high bacterial burden, death of infected macrophages and increase in the severity of the disease. In contrast, accumulation of LTB_4 enhanced TNF-α expression and enabled macrophage control of infection, but an excess of TNF-α results in the necrosis of macrophages and a higher burden of infection (88, 89). Previous findings support a correlation between the levels of TNF-α and LXA_4/LTB_4 ratio in leprosy patients. Both paucibacillary and multi-bacillary leprosy patients exhibited similar levels of TNF-α, LTB_4 and LXA_4 (11, 17, 122). On the other hand, leprosy patients with T1R possess a lower LXA_4/LTB_4 ratio (18), which agrees with increased inflammation and higher levels of TNF-α observed in these patients (123). Thus, the balance between pro-inflammatory and pro-resolving lipid mediators is important to the outcome of infection.

Furthermore, support for the importance of a LXA_4/LTB_4 balance is provided through population genetics in humans (21). Vietnamese and Nepali individuals homozygous for a common promoter polymorphism at the human *LTA4H* locus display lower protection against tuberculosis and multi-bacillary leprosy, respectively. This polymorphism is associated with deficient (low activity alleles) or excessive (high activity alleles) expression of

the *LTA4H* gene. Conversely, heterozygous individuals displayed a moderated expression of *LTA4H* gene and consequently a more balanced production of LXA_4 and LTB_4, due to the presence of both a low-activity allele and a high-activity allele (21, 88). As a consequence, heterozygous *LTA4H* individuals exhibited better protection against mycobacteria infection.

The connection between LTA_4H and TNF-α is reciprocal, as TNF-α is able to modulate the expression of *LTA4H* (124–126). This suggests that the synthesis of TNF-α and the LXA_4/LTB_4 ratio could be regulated by a feedback loop generated by expression of *TNFA* and *LTA4H* (details in **Figure 4**). Interestingly, polymorphisms in the promoter region of the *TNFA* are associated with human susceptibility to leprosy (119, 127, 128).

Existing data strongly support the hypothesis that the LXA_4/LTB_4 ratio in leprosy disease is an important factor in regulation of TNF-α and hence the susceptibility or resistance to *M. leprae* infection. We hypothesize that an increase in the LXA_4/LTB_4 ratio leads to lower TNF-α secretion and reduced control of *M. leprae* replication (**Figure 4**). However, a decrease in LXA_4/LTB_4 ratio would promote higher *TNFA* expression and an intense inflammatory response as observed for leprosy patients with T1R.

A POSSIBLE LINK BETWEEN THE PRO/ANTI-INFLAMMATORY PGE_2 AND PGD_2 WITH IMMUNE PATHOLOGICAL EVENTS IN LEPROSY PATIENTS

PGE_2: A Potential Dual Role in *M. leprae* Infection

PGE_2 and PGD_2 are increased in LL patients (17), and previous studies indicate that foamy macrophages/Schwann cells, a classical hallmark of LL patients, are the main source of prostaglandins (129, 130). The higher levels of PGE_2 in LL patients (17) together with the lower levels in T1R patients (18) suggest that PGE_2 is related to the different clinical forms of leprosy. Indeed, this lipid mediator impairs the proliferation of T cells (39, 40) and inhibits the activation of macrophages by IFN-γ in *M. leprae* infection (47). Thus, levels of PGE_2, produced by foamy macrophages/Schwann cells, can contribute to the inhibition of Th1 responses against *M. leprae* in LL patients. This may also indicate that lower levels of PGE_2 in T1R patients favors the exacerbated acute responses of Th1 cells. Moreover, PGE_2 has the ability to augment the suppressive capacity of human $CD4^+CD25^+$ Treg cells and up-regulate the expression of transcription factor *FOXP3* (46). Garg and colleagues demonstrated that PGE_2, but not PGD_2, promotes the expansion of Treg cells during *M. tuberculosis* infection (45). Thus, the higher frequency of Treg cells, as well as the anergy of Th1 and Th17 cells in LL individuals, could be related with increased amounts of PGE_2 secreted by foamy macrophages/Schwann cells (**Figure 5**). Other mechanisms through which higher levels of PGE_2 might affect the differentiation of Th17 and Th1 cells in LL patients include, modulating the secretion of IL-23 by dendritic cells (**Figure 5**) (23) and impairment of IL-12 production by dendritic cells (19).

There is evidence that at the proper concentration and in the presence of a co-stimulatory signal, PGE_2 also stimulates Th1 response. Yao and colleagues showed that treatment of naive T cells with PGE_2 and antibody stimulation of CD28 induces the differentiation of Th1 cells (24, 44). It is well known that PGE_2, through interaction with EP2 and EP4, inhibits the differentiation of Th1 cells by increasing intracellular levels of cAMP (42, 43). However, with a concomitant stimulation of CD28, T cells are rescued from the inhibitory effects of cAMP and therefore

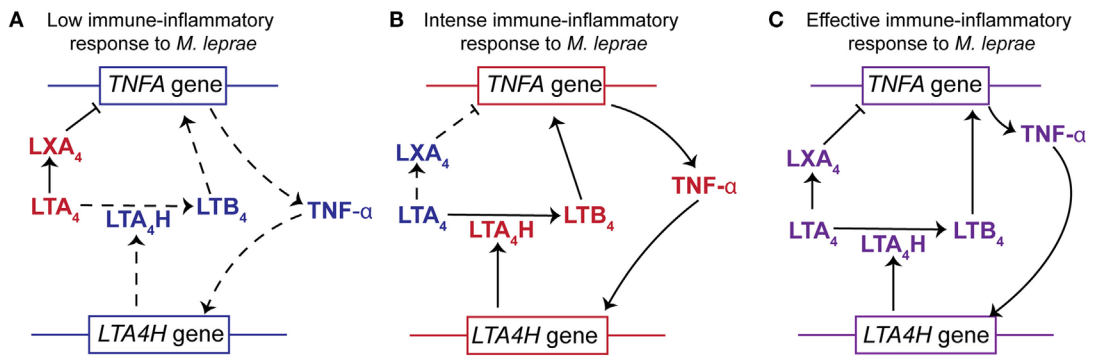

FIGURE 4 | The relationships between *LTA4H* gene polymorphisms, the LXA_4/LTB_4 ratios and TNF-α production to the outcome of *Mycobacterium leprae* infection. **(A)** Individuals homozygous for *LTA4H* locus with two low activity alleles display a higher concentration of LXA_4 than LTB_4 (high LXA_4/LTB_4 ratio). This would impair the production of TNF-α resulting in increased susceptibility to *M. leprae*. The higher levels of LXA_4 not only inhibit the expression of *TNFA* but also block the immune-inflammatory responses. In addition, the lower levels of TNF-α do not stimulate the expression of *LTA4H* and therefore do not increase the synthesis of LTB_4. **(B)** Subjects homozygous for *LTA4H* locus with two high activity alleles display a higher concentration of LTB_4 than LXA_4 (low LXA_4/LTB_4 ratio). The increased abundance of LTB_4 stimulates the expression of *TNFA* and production of TNF-α. Increased levels of TNF-α further enhance expression of *LTA4H*. Thus, an intense immune-inflammatory response to *M. leprae* would occur resulting in damage to the host tissue. **(C)** Individuals heterozygous for *LTA4H* locus, with a high and a low activity allele, synthesize a balanced amount of LXA_4 and LTB_4 (moderated LXA_4/LTB_4). This results in the production of TNF-α to levels that promote an effective immune-inflammatory response against *M. leprae* and promote a balance in the LXA_4/LTB_4 ratio. This balance in product abundance or gene expression is represented by the purple font. The red font represents an increased abundance of a product or increased gene expression, while the blue font symbolizes an attenuation of product abundance or gene expression. Arrows with solid lines indicate that the production of a lipid mediator or cytokine is favored, while an arrow with a hashed line indicates that the production is not favored. (⊢)Indicates that LXA_4 attenuates or impairs the expression of TNF-α.

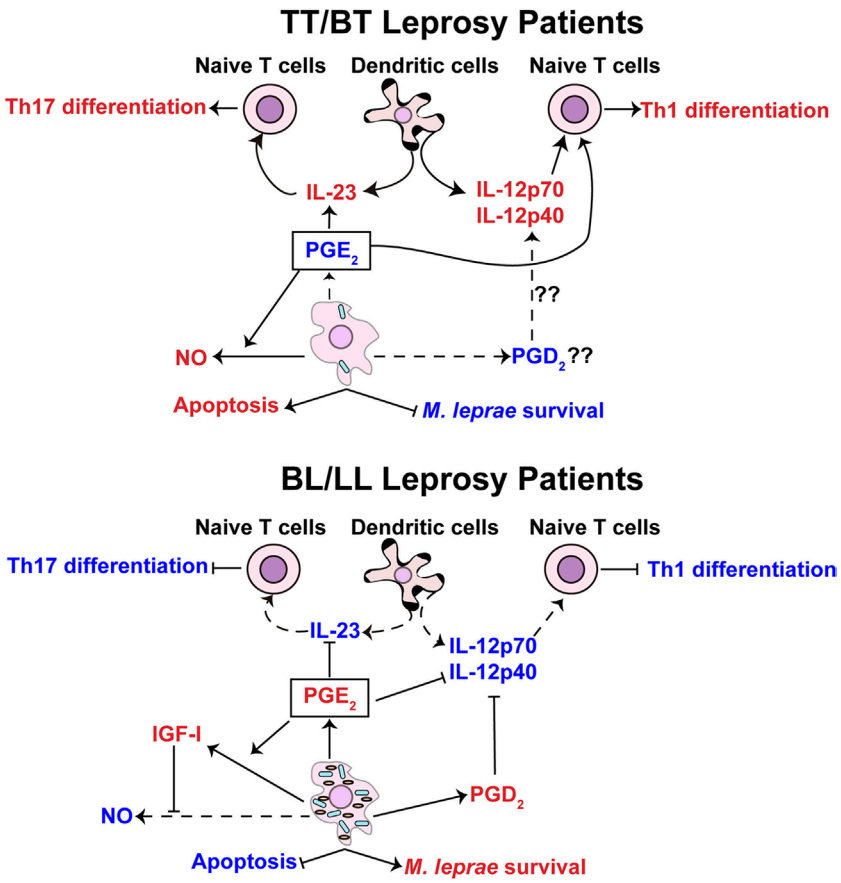

FIGURE 5 | Prostaglandin E_2 is hypothesized to exhibit different functions in pauci- and multi-bacillary leprosy patients. Tuberculoid (TT)/borderline tuberculoid (BT) leprosy patients (top panel) display a lower concentration of PGE_2 in comparison with borderline lepromatous (BL)/lepromatous leprosy (LL) patients (lower panel). The lower concentration of PGE_2 in TT/BT patients is hypothesized to facilitate the differentiation of T-helper type 17 (Th17) cells through upregulation of interleukin (IL)-23 cytokine production by dendritic cells. Findings from Yao et al. (44) provide evidence that small amounts of PGE_2 may favor the differentiation of T-helper type 1 (Th1) cells in TT/BT individuals. The levels of PGE_2 in TT/BT patients may also promote the production of nitric oxide (NO) in *M. leprae*-infected macrophages leading to the control of the bacterial load. In BL/LL patients *M. leprae*-infected foamy macrophages/Schwann cells produce a higher level of PGE_2 that is hypothesized to inhibit the differentiation of Th1 cells through impairment of the production of IL-12p70 by dendritic cells. The higher concentration of PGD_2, possibly secreted by foamy macrophages/Schwann cells from BL/LL patients, may also inhibit the production of IL-12p70. Additionally, the increased levels of PGE_2 could potentially inhibit the production of IL-23 in dendritic cells, thus blocking the differentiation of Th17 cells. Increased release of insulin-like growth factor I (IGF-I) stimulated via PGE_2 might potentially inhibit NO synthesis and apoptosis. The capacity of PGE_2 to prevent NO production and apoptosis favors the multiplication of *M. leprae*. The red color represents an intensification or increase in a process or abundance of a product, while the blue color symbolizes an attenuation of the process or product abundance. Arrows with solid lines indicate processes (production/secretion of cytokines, helper T-cell differentiation, apoptosis, and/or mycobacteria survival) that are favored or induced, while an arrow with a hashed line indicates processes that are not favored. (⊢) Represents inhibition of a process or activity.

differentiate to Th1 cells (24). Interestingly, *M. leprae* antigens are able to reduce the expression of B7-1 and CD28 molecules in PBMC cultures from healthy controls (131), and the levels of B7-1 and CD28 molecules in BL/LL patients, but not in BT patients, are reduced. Therefore, the higher levels of PGE_2 that leads to an increase in the intracellular levels of cAMP together with lower expression of CD28 could inhibit the differentiation of Th1 cells in LL patients. Conversely, BT patients that secrete basal levels of PGE_2 and express higher levels of CD28 would be expected to propagate and maintain a Th1 response. T1R patients also exhibit a basal level of PGE_2 (18). Hence, our hypothesis is that lower PGE_2 levels promote Th1 and Th17 cell activities in BT and T1R patients, but in LL patients, the higher concentration of this prostaglandin inhibits Th1 and Th17 responses (**Figure 5**). Together, these studies highlight the controversial role of PGE_2 in the human adaptive immune response and underscore the need for studies to determine other possible roles of PGE_2 in leprosy.

The Control of NO Production by PGE_2

The prostaglandin PGE_2 has been shown to also interfere with the control of cell death (48) and the production of NO by phagocytic cells (41). Studies using an experimental animal model of pulmonary tuberculosis demonstrated that at the early phase of *M. tuberculosis* infection, BALB/c mice produce lower amounts of PGE_2 and this promotes the expression of the inducible form of NO synthase (*iNOS*). In contrast, at later stage of

infection, higher amounts of PGE_2 are produced and inhibit the expression of *iNOS* (41). These assays support the idea that lower production of PGE_2 favors the bacterial control, and at higher concentrations, PGE_2 inhibits microbicidal mechanisms in the murine model. In line with these observations, skin lesions of BT leprosy patients exhibit a higher expression of *iNOS* than those of BL patients (11), and macrophages isolated from BT patients secrete higher concentrations of nitrite, a marker for iNOS activity, than macrophages derived from LL patients (132). Thus, we hypothesize that the lower levels of PGE_2 in BT patients (17) directly promote the microbicidal activities of phagocytic cells to control *M. leprae* replication as well as enhance the Th1 responses. Interestingly, the higher production of NO may cause nerve damage in BT patients as hypothesized in previous work (15). On the other hand, higher concentrations of PGE_2 secreted by foamy macrophages/Schwann cells would inhibit these same antimicrobial activities and thus favor multi-bacillary disease (**Figure 5**).

PGE_2 Might Differently Influence Apoptosis in Tuberculosis and Leprosy Patients

A potential mechanism by which PGE_2 would inhibit the production of NO in LL patients is through the induction of insulin-like growth factor I (IGF-I). PGE_2 induces the expression of IGF-I in murine macrophages (133) and osteoblasts (134, 135), and IGF-I inhibits the NOS2 pathway (136). A recent study has demonstrated that increased amounts of IGF-I are found in the skin lesions of LL patients and that IGF-I inhibits signaling cascades required for NO production (137). Therefore, it is possible that the elevated levels of PGE_2 could be linked to the inhibition of NO production via the induction of IGF-I in LL patients.

The production of IGF-I, possibly mediated by PGE_2, may also promote *M. leprae* survival by inhibition of apoptosis. Live *M. leprae* induces the production of IGF-I in Schwann cells and this was found to prevent apoptosis (138). The inhibition of apoptosis could be a significant advantage for *M. leprae* since this mechanism of cell death promotes the presentation of mycobacterial antigens to T cells (139). Thus, via an IGF-I network, PGE_2 may directly impact antigen presentation and favor *M. leprae* replication (**Figure 5**). However, a direct functional link between increased IGF-I and PGE_2 levels in LL individuals and apoptotic activity needs to be experimentally established.

It is interesting to highlight that the role of PGE_2 in *M. leprae* infection may greatly differ from the function of PGE_2 during *M. tuberculosis* infection. It appears that, during the early phase of infection, virulent *M. tuberculosis* (H37Rv) inhibits the synthesis of PGE_2, by inducing synthesis of LXA_4, to prevent apoptosis and consequently inhibit early T-cell activation and promote necrosis of macrophages (48, 49, 139, 140). In contrast, at the chronic stage, PGE_2 is highly produced (41), which could control the bacillary load by apoptosis. Furthermore, macrophages infected by the avirulent strain of *M. tuberculosis* (H37Ra) produced increased levels of PGE_2 (48), promoting the protection against mitochondrial inner membrane perturbation and induced plasma membrane repair, crucial processes to avoid necrosis and induce apoptosis (48, 49). Thus, PGE_2 might be crucial for the resistance against *M. tuberculosis* but promote susceptibility to *M. leprae*. These possible differences between *M. tuberculosis* and *M. leprae* infections could be partially related with different modulation of EP1-4 receptors by the two pathogens and should be explored in future studies.

PGD_2 in Leprosy: A Lipid Mediator Exploited by the Pathogen or a Host Response to Nerve Damage

Based on the several findings regarding PGD_2 and its effects on the modulation of T cells we suggest that PGD_2 production via foamy macrophages/Schwann cells promotes Th2 response in LL patients. It is well established that PGD_2 decreases the numbers of $CD4^+$ and $CD8^+$ T cells that produce IFN-γ and IL-2, through interactions with the DP1 receptor, while contributing to the Th2 responses with induction of IL-4, IL-5, and IL-13 by binding the CRTH2 receptor (60, 61). Besides a direct effect on T cells, PGD_2 modulates the T-cell response through dendritic cells and their production of IL-12 (19, 59). Braga et al. has revealed that monocyte-derived dendritic cells from LL patients produced less IL-12 (25), and although a direct association has not been made, the decreased IL-12 levels in LL patients could be driven by increased PGD_2 production and secretion by foamy macrophages/Schwann cells (**Figure 5**).

One observation that does not fit with the PGD_2 immune suppressing scenario in leprosy is that PGD_2 levels increase during a T1R (18). T1R is considered a delayed type hypersensitivity (DTH) reaction (141) and several works indicate that PGD_2, or its metabolite 15d-PGJ_2 (142), is highly produced during DTH to control the inflammatory activity in animal models (143). Thus, the increasing of PGD_2 in T1R patients may be a response by the host to control inflammation.

Individuals with acute inflammatory demyelinating polyneuropathy, an autoimmune disease that directly attack the peripheral nerve myelin (144), have increased levels of PGD synthase enzyme in their cerebrospinal fluid (145). In a murine model of spinal cord contusion injury, the levels of PGD synthase are also elevated (146). Interestingly, although the expression of PGD synthase was never determined, COX-2 is increased during T1R (147, 148). Thus, an increase in PGD_2 is not unexpected during T1R as these leprosy patients suffer the most severe nerve damage. PGD_2 is known to promote the myelination of neurons (55). In addition, mice that lack PGD synthase are unable to promote myelination of the neurons. These studies, as well as the fact that mast cells that are in close proximity to the peripheral nerve fibers in the tissue are the major producers of PGD_2, support the hypothesis that increased PGD_2 is a consequence of the T1R in leprosy and not a driver of the pathology.

Given the potentially varied activities of PGD_2 at different stages of leprosy, it is important to determine not only the source of this prostaglandin, foamy macrophages/Schwann cells versus mast cells, but also the receptors that bind PGD_2 during the different manifestations of leprosy and the cells that are expressing these receptors. Additionally, PGD_2 potentiates the formation of edema (56, 57), a factor that might contribute to the nerve damage in leprosy (149). Therefore, further studies are required to determine if PGD_2, through edema formation, can contribute to the pathology of leprosy lesions.

SUMMATION AND CONCLUSION

Through the multiple metabolomics studies performed with clinical samples from leprosy patients it is clear that alterations in the metabolism of lipid mediators derived from ω3 and ω6 PUFA occur with this disease. However, there is a lack of research that directly links these lipid mediators to the breadth of immune responses that occur across the clinical manifestations of leprosy. Detailed investigations to define enzymes and biochemical pathways for lipid mediator synthesis, along with elucidation of lipid mediator receptors and mechanisms by which lipid mediators influence both innate and adaptive immune responses, has nevertheless allowed the development of well supported hypothesis on the function of various lipid mediators in different manifestations of leprosy. A common theme that has emerged from existing studies is that several of the lipid mediators identified in the metabolomics studies of leprosy patients and discussed here (RvD1, LXA_4, PGE_2, and PGD_2) down-regulate the immune-inflammatory responses promoted by Th1 and Th17 cells and facilitate the activity and proliferation Treg cells. This would indicate that *M. leprae* might exploit the pro-resolving activities of lipid meditators to maintain a persistent infection. Nonetheless, some of these lipid mediators such as PGE_2 and PGD_2, as well as LTB_4 can influence the protective response against *M. leprae*. Another emerging theme is that alteration of the balance between pro-inflammatory and pro-resolving lipid mediators has the potential to dramatically skew the Th1/Th17 and Treg responses in leprosy. This same concept also applies to variations in the relative concentration of individual products such as PGE_2. Thus, a coordination of the dynamics of the lipid mediator response and that of the adaptive and innate immune systems seems to be a driving factor in the specific presentation of leprosy.

As existing and future data are interpreted to develop models of lipid mediator involvement in the pathology and immunology of leprosy, it is important to consider the complexity of lipid mediator metabolism, and that most lipid mediators can serve as ligands for multiple receptors. Additionally, the spatial and temporal aspects of lipid mediator metabolism and receptor expression, along with the complementary or opposing activities of multiple lipid mediators must be addressed to fully elucidate the role lipid mediators play in leprosy. Mathematical models, as performed for *M. tuberculosis* infection (150), may be important to elucidate the influence PUFA-derived lipid mediator complexity in disease outcomes that might occur in individuals infected with *M. leprae*. It is also important to highlight that lipid mediators not identified or targeted in previous metabolomics studies on leprosy, may also contribute to immuno-pathogenesis. Thus, further targeted metabolomics investigations supported by orthogonal approaches, such as transcriptomics and proteomics, are needed to elucidate the full complement lipid mediators involved in leprosy and define how systemic alterations in their levels modify the phenotype of innate and adaptive immune cells in different presentations of leprosy. Future research efforts will not only provide an understanding of the contribution of lipid mediators to chronic infectious diseases but also provide the basis for the development of new diagnostic/prognostic and treatment approaches to address leprosy as a public health problem.

AUTHOR CONTRIBUTIONS

CS and JB contributed to the review of published literature, development of the concepts, and design of the review article, as well as the writing and editing of the manuscript. CS is responsible for the design and concepts of the figures.

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Serum Levels of Migration Inhibitory Factor (MIF) and *In Situ* Expression of MIF and Its Receptor CD74 in Lepromatous Leprosy Patients: A Preliminary Report

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Leprosy is a chronic disease caused by *Mycobacterium leprae* that affects the skin and peripheral nerves. It may present as one of two distinct poles: the self-limiting tuberculoid leprosy and the highly infectious lepromatous leprosy (LL) characterized by *M. leprae*-specific absence of cellular immune response. The pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) enhance the bactericide activities of macrophages after interaction with its receptor, CD74. Importantly, MIF also possesses chemoattractant properties, and it is a key factor *in situ* for the activation of macrophages and in blood to promote leukocytes migration. MIF-mediated activation of macrophages is a key process for the elimination of pathogens such as *Mycobacterium tuberculosis*; however, its participation for the clearance of *M. leprae* is unclear. The aim of this study was to evaluate the serum levels of MIF as well as MIF and CD74 expression in skin lesions of LL and compare it with healthy skin (HSk) taken from subjects attending to dermatological consult. Samples of serum and skin biopsies were taken from 39 LL patients and compared with 36 serum samples of healthy subjects (HS) and 10 biopsies of HSk. Serum samples were analyzed by ELISA and skin biopsies by immunohistochemistry (IHC). IHC smears were observed in 12 100× microscopic fields, in which percentage of stained cells and staining intensity were evaluated. Both variables were used to calculate a semi-quantitative expression score that ranged from 0 to 3+. We found no differences in MIF levels between LL patients and HS in sera. In addition, MIF was observed in over 75% of cells with high intensity in the skin of patients and HSk. Although we found no differences in MIF expression between the groups, a CD74 score statistically higher was found in LL skin than HSk ($p < 0.001$); this was the result of a higher percentage of cells positive for CD74 ($p < 0.001$). As a conclusion, we found that CD74-positive cells are intensely

recruited to the skin with LL lesions. In this manner, MIF signaling may be enhanced in the skin of LL patients due to increased expression of its receptor, but further studies are required.

Keywords: leprosy, lepromatous leprosy, skin, serum cytokines, migration inhibitory factor, CD74

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*. The disease in patients may develop as one of two poles that differ clinically and immunologically between each other. Few lesions in the body and an active immune response mediated by cells characterize the tuberculoid leprosy (TT) pole; on the other hand, patients who develop lepromatous leprosy (LL) show numerous erythematous macules around the body coupled with *M. leprae*-specific anergy and disrupted immune response. The reasons for developing either pole are still unknown, but the environment and host genetics are probable factors that mediate such polarization (1). As a consequence, the ratio of LL to TT patients varies around the world, and it can be as high as 3:1 in countries like Mexico, where TT cases are scarce (2).

In LL patients, *M. leprae* proliferates in the skin and peripheral nerves (1). Histologically, skin lesions of LL patients are formed by diffuse infiltration of macrophages, lymphocytes, and plasma cells in the dermis, along with a dysfunctional epidermis. Anti-inflammatory cytokines, such as IL-10 and TGF-β, are highly expressed in these lesions when compared with TT lesions (3). As a consequence of the immunological imbalance, macrophages get infested by abundant bacilli and become foamy macrophages (4). These macrophages seem unable to kill *M. leprae* but serve as a reservoir instead and release additional anti-inflammatory mediators and reduce their production of key activator cytokines, namely TNF-α and IL-1β (5).

Macrophage migration inhibition factor (MIF) is a constitutively released cytokine that is able to subvert the anti-inflammatory activities of glucocorticoids and also initiates intense immune reactions by stimulating macrophages toward an inflammatory profile that involves productions of ROS, increases in the expression of TLR4, and diminishes cell susceptibility to apoptosis (6, 7). MIF participates not only in skin homeostasis by regulating the differentiation and proliferation of keratinocytes (8) but also in skin disorders by enhancing the activity of inflammatory macrophages (9). To exert its biological activities, MIF needs to interact with its receptor CD74, a molecule with a wide array of described functions in immune cells (10). In addition, CD74 may form a complex with chemokine receptors and lead cells to a process of MIF-mediated migration (11).

Only few studies analyze the expression of both MIF and CD74 in infectious diseases, even though MIF has proved to be an important mediator against several infections, especially those caused by *Mycobacterium tuberculosis* (12) and *Leishmania major* through a mechanism dependent on TNF-α and reactive nitrogen intermediates (13). In addition, the early stimulation of CD74 triggers the MAPK and PI3K pathways that lead to proliferation and recruitment of immune cells, thus starting the inflammation in injured tissues (7). However, the specific role of MIF/CD74 interaction in the context of skin diseases has been poorly studied. Due to its relevance in the inflammatory process, it is interesting to know whether the MIF/CD74 axis is implicated in the immunopathology of LL. We previously reported that the susceptibility to develop LL in Western Mexico is associated to the alleles of the STR-794 $CATT_{5-8}$ polymorphism of the *MIF* gene linked to higher expression of MIF (14). This paradoxical observation relates the inflammatory cytokine MIF with the anti-inflammatory LL pole and is suggestive of an important role for MIF in this spectrum of leprosy. Thus, we are interested to study the levels of MIF in the serum of LL patients as well as the expression of both MIF and CD74 in the skin lesions of LL patients.

MATERIALS AND METHODS

Subjects and Samples

Patients for this study were diagnosed by Dermatologists working in the Instituto Dermatológico de Jalisco "Dr. José Barba Rubio" according to their clinic, bacilloscopic, and histopathologic characteristics. Due to the low prevalence of TT in Mexico (2), only patients diagnosed as LL and borderline lepromatous in the absence of reactional episodes were included in the study. Patients who presented chronic, inflammatory, and dermatological conditions were excluded. Serum samples from patients were requested upon diagnosis confirmation. Healthy subjects (HS) of similar age and gender characteristics were asked for blood donation to compare MIF serum levels. In total, serum of 39 patients and 36 HS were considered for determination of MIF. The demographic and disease characteristics of participants are shown in **Table 1**.

TABLE 1 | Demographic and disease characteristics of participants.

	HS	LL
Age (years)	40.5	57.5
Gender		
Male (*n*, %)	16 (44.4)	20 (51.3)
Female (*n*, %)	20 (55.6)	19 (48.7)
Duration of disease (years)	14.3	
Baciloscopic index (*n*, %)		
0		1 (2.5)
1		4 (10.3)
2		2 (5.1)
3		5 (12.8)
4		9 (23.1)
5		9 (23.1)
6		9 (23.1)

Tissue samples of 39 patients were obtained for immunohistochemistry (IHC) assays from the biopsies taken for their diagnostics; in addition, 10 tissues of healthy skin (HSk) were obtained from the repertoire of the Institute. Samples were embedded in paraffin for preservation and cut into 3-µm sections for mounting onto precharged slides.

Serum Quantification of MIF

Migration inhibitory factor quantification was performed by the ELISA kit for "Human MIF Immunoassay" (Cat. No. DMF00B; R&D, MN, USA) following the manufacturer's indications. Briefly, samples were diluted 10-fold with the kit Calibrator Diluent and 50 µl of the mix were added to the microplate wells, followed by 2-h incubation. Wells were washed and the detection antibody was incubated for 2 h. Wells were washed and the color was developed by 30-min incubation with Substrate Solution in the dark. Finally, stop solution was added and optical density was measured at 450 nm and corrected at 570 nm. The concentration of MIF was calculated interpolating the optical density of samples with a multiparametric curve generated with the MIF standard included in the kit.

In Situ Characterization of MIF and CD74

We analyzed the expression of MIF and CD74 in skin biopsy samples. Slices of tissue samples were incubated at 60°C for 10 min and deparaffinized on xylene bath for 20 min. Samples were then rehydrated through a series solutions of decreasing concentrations of ethanol. Antigens were retrieved in a bath of sodium citrate solution 10 mM at 95°C for 10 min followed by cooling in a citrate cold solution. Non-specific binding to proteins was blocked by incubation with bovine fetal serum 10% by 30 min; endogenous peroxidase activity of samples was blocked by incubation with H_2O_2 3% solution by 30 min. Afterward, sections of each biopsy were incubated overnight at 4°C with one of the following primary antibodies: anti-MIF FL-115 (Cat# sc-20121 RRID:AB_648587) 2 µg/ml or anti-CD74 FL-296 (Cat# sc-20082 RRID:AB_2075501) from Santa Cruz Biotechnology, Inc., TX, USA. The detection of primary antibodies will be performed using the polymer conjugated to secondary antibodies from Dako EnVision™ + Dual Link System-HRP (Dako Agilent Technologies, Denmark). Finally, sections were counterstained with hematoxylin. The presence of antigens were analyzed in four random 40× fields using the semi-quantitative algorithm of Li et al., which took into account both the intensity of staining and the percentage of stained cells (15). Briefly, percentage of stained cells was transformed into histological index I (HI I) as follows: Neg (0+) ≤ 5% of stained cells; "+" (1+) = 6–25%, "++" (2+) = 26–50%, "+++" (3+) = 51–75%; and "++++" (4+) > 76%. Similarly, a second histological index (HI II) for staining intensity was calculated as: Neg (0+) = no staining, "+" (1+) = low intensity, "++" (2+) = moderate intensity, "+++" (3+) = high intensity. Finally, HI I and HI II were multiplied and ranked as an expression score of Neg if the product of (HI I)*(HI II) was between 0 and 1; "+" (1+) if the product was between 2 and 4; "++" (2+) if the product was between 5 and 8; and "+++" (3+) if the product was between 9 and 12.

Statistical Analysis

Qualitative variables of the study were expressed as frequencies ± SD and quantitative variables were expressed as mean values ± SD. Statistical analyses were performed on IBM SPSS Statistics ver. 20. Differences in MIF levels between groups, as well as differences of expression score, percentage of stained cells, and staining intensity for MIF and CD74 were analyzed using Mann–Whitney *U* test. In addition, we studied whether there exists correlation between the bacillary index of LL patients, the soluble levels of MIF as well as the *in situ* expression of MIF and CD74 using the non-parametric one-tailed Rho spearmen correlation coefficient. Statistical analysis resulting with p values <0.05 were considered as significant.

Ethical Consideration

The study was designed in agreement with the Declaration of Helsinki (16). All participants were informed about the goals of the study and provided written agreement. This study was approved by the ethical committee *Comité de Investigación y Bioseguridad del Centro Universitario de Ciencias de la Salud* of the University of Guadalajara (No. CI-02515).

RESULTS

Serum Levels of MIF

We quantified the serum levels of MIF in 39 LL patients (44.49 ± 20.21 ng/ml) and 36 HS (53.35 ± 34.13 ng/ml). However, we found no significant differences regarding the levels of MIF between groups. Also, we did not find significant correlation between the bacillary index and serum MIF levels ($r = 0.272$, $p = 0.154$).

MIF-Related Markers in skin

We analyzed the expression of MIF and CD74 in skin biopsies of 39 LL patients as well as 10 samples of HSk.

We found that MIF is highly expressed in the epithelia of both patients and HSk (**Figure 1**). There exist also several MIF^+ cells in the dermis in both groups. We observed that MIF is mainly expressed in the cytoplasm but it can also be found in some nuclei. We found no significant differences in the expression score between both groups (**Table 2**). Most of the participants presented 1+ or 2+ of MIF expression score in skin samples. To better understand this index, we analyzed the percentage and intensity index. We found that most of the LL skin samples could be classified as 1+ (37.2%) or 2+ (34.9%); similarly, most of HSk fell into 1+ (30%) and 2+ (40%) categories. Interestingly, the staining of MIF in cells was a little stronger in HSk than in LL samples, but no significant differences were found. In addition, MIF expression score was not correlated to the bacillary index of patients ($r = 0.190$, $p = 0.153$).

CD74 is expressed in the dermis, but its epidermal expression is scarce (**Figure 2**). Although it is expressed in the membrane of some cells, it is mostly expressed in the cytoplasm. Interestingly, we found that its expression score is significantly higher in LL skin than HSk ($p < 0.001$), since 0% of HSk could be classified as 2+ or 3+, whereas 32.6 and 23.3% of LL was classified as 2+ and 3+, respectively (**Table 3**). We analyzed whether this difference

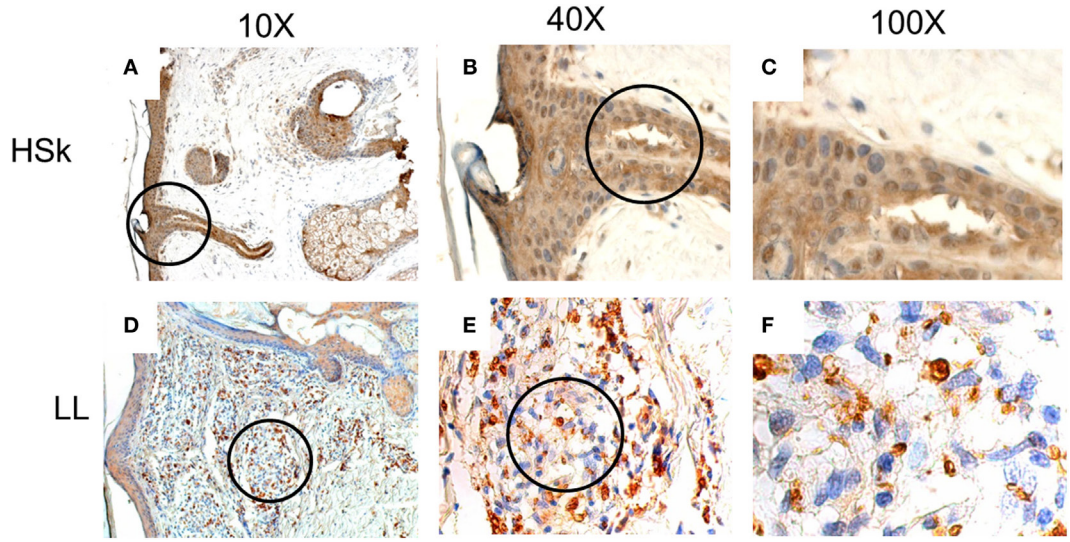

FIGURE 1 | *In situ* expression of migration inhibitory factor (MIF) in healthy skin (HSk) and lepromatous leprosy (LL) skin biopsy. Photographs of MIF-directed immunohistochemistry assays on HSk and skin lesions of LL patients on progressive magnifications are displayed. **(A–C)** represent the staining of MIF on HSk at 10×, 40×, and 100×, respectively. **(D–F)** show staining of MIF on LL skin at 10×, 40×, and 100×. The magnified fields are shown within circles. MIF expression is intense on epidermis and annexes of both HSk and LL. In dermis, its expression also seems to be constitutive. MIF is expressed mostly in cytoplasm and can also be found in some nuclei.

TABLE 2 | Ranking of lepromatous leprosy (LL) patients and healthy skin (HSk) according to their score of migration inhibitory factor (MIF) staining.

Score							
MIF	**Negative**	**+**	**++**	**+++**		**Total**	***p* = 0.990**
LL (*n* = 39)	16.3%	37.2%	34.9%	11.6%		100%	
HSk (*n* = 10)	20%	30%	40%	10%		100%	
Percentage of stained cells							
	Negative	**+**	**++**	**+++**	**++++**	**Total**	***p* = 0.550**
LL (*n* = 39)	14.0%	16.3%	32.5%	23.2%	14.0%	100%	
HSk (*n* = 10)	10%	10%	30%	40%	10%	100%	
Staining intensity							
	Negative	**+**	**++**	**+++**		**Total**	***p* = 0.499**
LL (*n* = 39)	11.6%	14.0%	44.2%	30.2%		100%	
HSk (*n* = 10)	0%	40%	40%	20%		100%	

Statistical analysis using two-sided Mann–Whitney U test, where p < 0.05 values were considered as significant.

was due to an increased infiltrate of $CD74^+$ cells, a higher staining intensity or both. We found that none of the HSk presented an infiltrate of over 1+ (equivalent to 25% of cells), whereas 27.9% LL skin samples presented 2+, 46.5% presented 3+, and 4.7% of LL samples presented 4+. However, the intensity of CD74 staining was not different between groups; notably, CD74 presented high intensity (3+) in up to 40% of HSk and 39.5% of LL. Finally, CD74 expression score was not correlated to the bacillary index of patients ($r = 0.040$, $p = 0.416$).

DISCUSSION

The immune response has been well characterized in the advanced forms of leprosy, but there are still several gaps where research is required. One of the main differences between the two poles is the presence of inflammatory cells and markers in TT, meanwhile, anti-inflammatory conditions are rather present in LL patients (3); however, it is not clear what mechanisms lead to such polarization. We previously reported that *MIF* polymorphisms in the promoter region are associated with LL in Western Mexico population (14). Thus, in this work, we investigated the serum levels of MIF in LL patients. Despite the genetic association, we found no differences in the serum levels of MIF between LL patients and HS. Recently, Bansal et al. measured MIF levels in leprosy patients and they also found that the MIF levels in serum are similar between healthy controls and patients presenting LL, borderline lepromatous, or borderline tuberculoid (17).

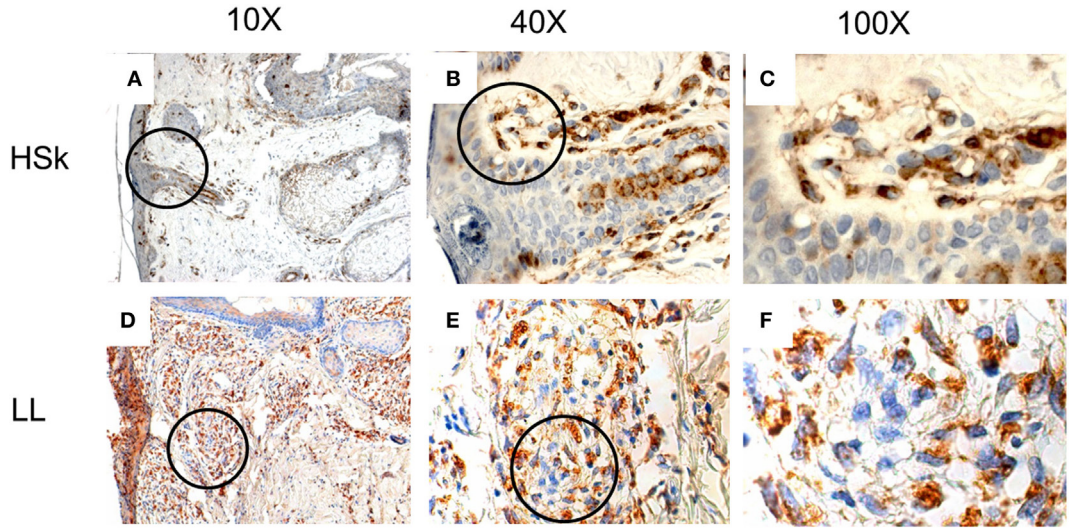

FIGURE 2 | *In situ* expression of CD74 in healthy skin (HSk) and lepromatous leprosy (LL) skin biopsy. Photographs of CD74-directed immunohistochemistry assays on HSk and skin lesions of LL patients on progressive magnifications are displayed. **(A–C)** represent the staining of CD74 on HSk at 10×, 40×, and 100×, respectively. **(D–F)** show staining of CD74 on LL skin at 10×, 40×, and 100×, respectively. The magnified fields are shown within circles. CD74 is scarcely expressed in epidermis and annexes. It is expressed in the dermis, where a major infiltrate can be observed in LL skin than HSk. Paradoxically, it seems to be expressed mostly in cytoplasm rather than the plasma membrane.

TABLE 3 | Ranking of lepromatous leprosy (LL) patients and healthy skin (HSk) according to their score of CD74 staining.

Score							
CD74	**Negative**	**+**	**++**	**+++**		**Total**	$p < 0.001$
LL ($n = 39$)	7.0%	37.1%	32.6%	23.3%		100%	
HSk ($n = 10$)	40%	60%	0%	0%		100%	
Percentage of stained cells							
	Negative	**+**	**++**	**+++**	**++++**	**Total**	$p < 0.000$
LL ($n = 39$)	4.7%	16.3%	27.9%	46.5%	4.7%	100%	
HSk ($n = 10$)	30%	70%	0%	0%	0%	100%	
Staining intensity							
	Negative	**+**	**++**	**+++**		**Total**	$p = 0.422$
LL ($n = 39$)	4.7%	11.6%	44.2%	39.5%		100%	
HSk ($n = 10$)	20%	20%	20%	40%		100%	

Statistical analysis using two-sided Mann–Whitney U test, where $p < 0.05$ values were considered as significant.

To obtain more information about MIF role in leprosy, we examined skin biopsies by IHC to inquire about its cellular and tissular localization. As in serum, we found that the expression of MIF is not different between skin lesions of LL patients and HSk, despite the elevated expression of IL-4, IL-10, and TGF-β reported in LL skin lesions (18). Therefore, we may postulate that anti-inflammatory mediators do not reduce the expression of MIF, although they may interfere with MIF capacity to enhance the bactericidal activities of macrophages, which is an intrinsic activity of MIF (6). Since MIF can be stored within the cytoplasm (19), our results most likely reflect the constitutive expression of MIF (20). Noteworthy, MIF presence is crucial for cell homeostasis due to its multiple activities. It may be secreted from the central nervous system to regulate cell proliferation (6), and it also promotes glucose intake by increasing the expression of GLUT4 (21). Nevertheless, MIF functions may vary according to other microenvironment components and the cell that it is targeting. For example, MIF expression is increased in inflammatory skin diseases such as alopecia areata and skin tumors (22, 23) and MIF orthologs are produced by *Leishmania* spp. to promote the survival on parasite-infected macrophages (24).

Since MIF expression is not different between LL skin and HSk, we measured CD74 expression to determinate if it is involved in leprosy lesions. Paradoxically, we found that its expression is significantly higher in LL skin than HSk. However, the elevated expression of CD74 does not correlate with the anti-inflammatory environment of LL lesions. In addition to being

MIF receptor, CD74 excerpts several functions that include regulation of vesicular transport, dendritic cells migration, and protection of proteins as a chaperone (11). Noteworthy, we found that CD74 is expressed mostly in the cytoplasm and that few cells actually expressed CD74 on the plasma membrane, suggesting that in LL, CD74 is not acting as a receptor for MIF, whereas its overexpression could contribute to the lack of response toward *M. leprae*. Arguably, the transport of antigens on MHC-II toward the plasma membrane of monocytes could be altered. Indeed, the expression of both MHC-I and MHC-II are reduced in dendritic cells infected with *M. leprae* in a dose-dependent manner (25). Moreover, Lee et al. have described that the expression of leukocyte Ig-like receptor A2 (LILRA2) is decreased in TT compared with LL, where it reduced the capacity of monocytes to present leprosy antigens on MHC-II to T cells, although antigen processing was not disrupted (26). Given that CD74 is a chaperone for MHC-II (27) and that it is involved in vesicle transport (11), cytoplasmic CD74 in LL could arrest the movement of antigens-loaded MHC-II toward the cell surface in an LILRA2-mediated mechanism.

Given that 80% of new cases of leprosy Mexico are multibacillary cases (2), our observations of MIF and CD74 are limited to LL patients without treatment. It is important to pursue paucibacillary cases to better detail the role of MIF in leprosy; in particular, the study of MIF in indeterminate leprosy results of particular interest due to the functions of MIF in innate immunity (6, 28). In addition, its participation in reactional episodes has been highlighted by Bansal et al., whose group found increased serum concentration of MIF in erythema nodosum leprosum (17). Further description of MIF and CD74, as well as the possibly involved signaling pathways, namely, PI3K, MAPK could yield valuable insight into leprosy immunopathology.

In summary, we have found that the expression of MIF in LL patients is similar to HS both in serum and in skin. However, the expression of CD74 is significantly increased in the skin lesions of LL patients, although its participation in the physiopathology leprosy remains unclear. Further studies in indeterminate leprosy and paucibacillary leprosy, as well as other infectious and inflammatory diseases of skin, are required to describe the participation of MIF/CD74 in the immune response against leprosy in the skin. In addition, their activities in the systemic response should also be explored. It is important to determine the molecules to which CD74 is binding to further understand the regulation of antigen presentation in the LL skin. In addition, the characterization of MIF^+ and $CD74^+$ cells could provide further insight into the immune microenvironment of skin lesions.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Comité de Investigación y Bioseguridad del Centro Universiario de Ciencias de la Salud of the University of Guadalajara (No. CI-02515) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Comité de Investigación y Bioseguridad del Centro Universitario de Ciencias de la Salud of the University of Guadalajara (No. CI-02515).

AUTHOR CONTRIBUTIONS

MM-G aided in ELISA assays and performed IHC identification of MIF and CD74; AA-N directed and designed the IHC assays; VD-R directed the ELISA assays; AG-O performed the ELISA assays; JM-R and AP-S performed the statistical analyses; MF-M is responsible for conception of the work and supervised the progress at all times. All authors participated in drafting, revising, and approval of the manuscript.

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Vaccines for Leprosy and Tuberculosis: Opportunities for Shared Research, Development, and Application

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Tuberculosis (TB) and leprosy still represent significant public health challenges, especially in low- and lower middle-income countries. Both poverty-related mycobacterial diseases require better tools to improve disease control. For leprosy, there has been an increased emphasis on developing tools for improved detection of infection and early diagnosis of disease. For TB, there has been a similar emphasis on such diagnostic tests, while increased research efforts have also focused on the development of new vaccines. Bacille Calmette–Guérin (BCG), the only available TB vaccine, provides insufficient and inconsistent protection to pulmonary TB in adults. The impact of BCG on leprosy, however, is significant, and the introduction of new TB vaccines that might replace BCG could, therefore, have serious impact also on leprosy. Given the similarities in antigenic makeup between the pathogens *Mycobacterium tuberculosis* (*Mtb*) and *M. leprae*, it is well possible, however, that new TB vaccines could cross-protect against leprosy. New TB subunit vaccines currently evaluated in human phase I and II studies indeed often contain antigens with homologs in *M. leprae*. In this review, we discuss pre-clinical studies and clinical trials of subunit or whole mycobacterial vaccines for TB and leprosy and reflect on the development of vaccines that could provide protection against both diseases. Furthermore, we provide the first preclinical evidence of such cross-protection by *Mtb* antigen 85B (Ag85B)-early secretory antigenic target (ESAT6) fusion recombinant proteins in *in vivo* mouse models of *Mtb* and *M. leprae* infection. We propose that preclinical integration and harmonization of TB and leprosy research should be considered and included in global strategies with respect to cross-protective vaccine research and development.

Keywords: antigen 85B, early secretory antigenic target, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, tuberculosis, leprosy, hybrid recombinant protein, vaccines

INTRODUCTION

Tuberculosis (TB) and leprosy are major infectious diseases that are caused by highly related mycobacterial pathogens, *Mycobacterium tuberculosis* (*Mtb*) and *M. leprae.* Although derived from the same mycobacterial ancestor (1), the target organs affected by these highly related mycobacteria (skin and nerves in leprosy; lungs and extrapulmonary lesions in TB) and the resulting

clinical symptoms, are strikingly different. Notwithstanding these differences, the two poverty-associated diseases also share important characteristics (2–4), including the important role of host cellular immunity in protection. In addition, both diseases display a wide spectrum of (immuno)-pathological features with characteristic granulomatous lesions that often result in chronic disease and require prolonged treatment with multidrug antibiotic therapies (5).

Although rarely lethal, leprosy is enormously feared for causing lifelong handicaps and deformities resulting from irreversible nerve damage. Leprosy is notable for its continued transmission, which results in a stable annual number of approximately 200,000 new cases (6). Moreover, predictions from mathematical modeling indicate that millions linger undetected (7).

Tuberculosis is a major threat due to its high morbidity and mortality, causing an estimated 10.4 million new cases and 1.8 million deaths in 2015 alone (8). This scenario is worsened by HIV co-infection as well as by the emergence of multi-, extensive-, and total-drug resistance (8). Though not as threatening as for TB, anti-microbial resistance also poses a risk for leprosy (9–13), which needs to be considered in post-exposure prophylactic (PEP) treatment strategies in leprosy endemic areas that aim to reduce transmission by administering a single dose of antibiotics to those at high risk of developing leprosy (14).

In order to combat both diseases, global strategies have been endorsed, promoting the implementation of new drugs to shorten lengthy chemotherapeutic regimens, including strategies to avoid occurrence of *de novo* antibiotic resistance (15). In addition, research is focusing on development of improved diagnostics for detection of infection and early stages of disease allowing prophylactic and timely treatment, respectively. In contrast to chemoprophylaxis, vaccines would be expected to give rise to active as well as long-term protection. Therefore, development of novel vaccines is an additional top priority to control TB and leprosy by preventing disease and transmission (6, 16, 17). To explore this further, we here review the current vaccine development pipelines for TB and leprosy focusing on shared features and antigenic components, as well as highlight potential differences and incompatibilities.

BACILLE CALMETTE–GUÉRIN (BCG), ONE VACCINE FITS ALL?

Mycobacterium bovis, BCG still is the only vaccine used against TB worldwide (18, 19). It is the first live-attenuated bacterial vaccine administered to newborns at or shortly after birth and has been applied in 172 countries (20, 21). In spite of its efficacy against severe TB in children, protection against TB in adolescents and adults is not sufficient to impact on disease and transmission. This urges for new, more efficient vaccines, and alternative strategies to replace or complement BCG (22–24).

Although being introduced and licensed for prevention of TB, BCG was soon recognized to protect partly also from leprosy (25–27). The efficacy of BCG vaccination against TB and leprosy has been evaluated in numerous clinical trials and observational studies. However, these studies also revealed inconsistent and sometimes even contradictory results. BCG's protective effects varied from 2 to 83% and from 58 to 74% in preventing pulmonary and extrapulmonary TB, respectively (28), while its efficacy against leprosy ranged from 26 to 41% in experimental studies to 61% in observational studies, with mild differences between the paucibacillary (62%) and multibacillary (76%) forms (25, 29–31). BCG vaccination does not seem to protect against the third most common mycobacterial disease, Buruli ulcer's disease, although a definite conclusion requires further well-designed prospective studies (32). Apart from its effect on mycobacterial diseases, BCG vaccination has been reported to have significant impact on unrelated diseases, probably through training of the innate immune system to respond more favorably to outer assaults (33, 34).

The remarkable differences in efficacy in various trials for TB and leprosy have been ascribed to several factors, including diversity in the genetic fingerprints of the mycobacterial pathogens in different geographic areas (35, 36), the various BCG strains used in the studies (37, 38), the immune, nutritional, and socioeconomic status of the vaccinees enrolled (39), the presence of helminths or viral coinfections (21, 40, 41), the background exposure to and induction of immunity by environmental mycobacteria, which might mask or block the effects of BCG (42), but the precise reasons for this remain largely unclear.

Our incomplete understanding of which components of the human immune system are responsible for either successful or inefficacious protection following BCG vaccination impedes the rational design of more effective vaccines (43). For instance, the limited efficacy of BCG in preventing local pulmonary TB disease compared to its effects on disseminated forms of TB is well documented, but remains unexplained (19). One hypothesis attributes this finding to its inability to induce durable and effective immune cells that home to the lung (19). Therefore, new routes of BCG administration, such as aerosol or intranasal immunization, are tested to initiate mucosal immunity and promote homing of immune cells to the lung mucosa (44, 45).

Another shortcoming of BCG is that its protective effects against TB as well as leprosy wanes over time, dropping to 14% efficacy after 10–20 years (46), indicating a suboptimal induction of long-term immune memory responses as discussed above (47, 48). Thus, BCG revaccination has been attempted in several countries. As a first attempt, a large trial in Malawi showed that BCG revaccination had limited impact on TB, while reducing the risk of leprosy with 50% (25, 49). Similarly, a large randomized controlled TB trial in Brazil showed that a second dose of BCG in adolescents did not confer better protection than a single dose given at birth (50). In contrast, for leprosy (30, 31), BCG revaccination is officially recommended in Brazil, since the 1970s for household contacts of leprosy patients as a boost to routine neonatal BCG vaccination. More recently, an extensive BCG revaccination trial of household contacts of leprosy patients in Brazil showed that the protection conferred by a booster BCG vaccination was 56% and was independent of previous BCG vaccination (29).

Notwithstanding, this lack of BCG boosting effects in TB and its beneficial effects on leprosy, BCG vaccination can also have less

favorable effects, such as increasing the numbers of paucibacillary leprosy cases within the first months after BCG immunization (51). This is thought to be due to excessive boosting of pre-existing *M. leprae*-specific T cells in those already frequently exposed to the bacterium (51, 52), or to hyperinflammatory innate immunity (53, 54). Both mechanisms could lead to pathogenic immunity, such as increased numbers of paucibacillary leprosy and leprosy reactions (55).

Based on the premise that BCG might overcome the phenotypic cellular immunological tolerance against *M. leprae* in multibacillary leprosy, BCG immunotherapy has been trialed in leprosy patients in Venezuela in the 1980s (56). These studies met with limited success, since complications of this therapy were the occasional occurrence of disseminated cutaneous BCG lesions and the induction of leprosy reactional episodes (57). In contrast, a small-sized clinical trial in India studied a combination of MDT and immunotherapy with BCG in newly diagnosed leprosy patients and found a significant reduction in duration of reactions, incidence of type 2 reactions as well as in time to achieve bacterial clearance (58).

In summary, BCG has significant protective efficacy against severe TB in children and against leprosy in adults, while BCG revaccination has added value in leprosy, but not in TB. Future changes in TB vaccination policies might, therefore, also affect leprosy control. To further analyze this issue, we review current vaccine development pipelines and policies for TB and leprosy, focusing on shared target product profiles and antigenic composition.

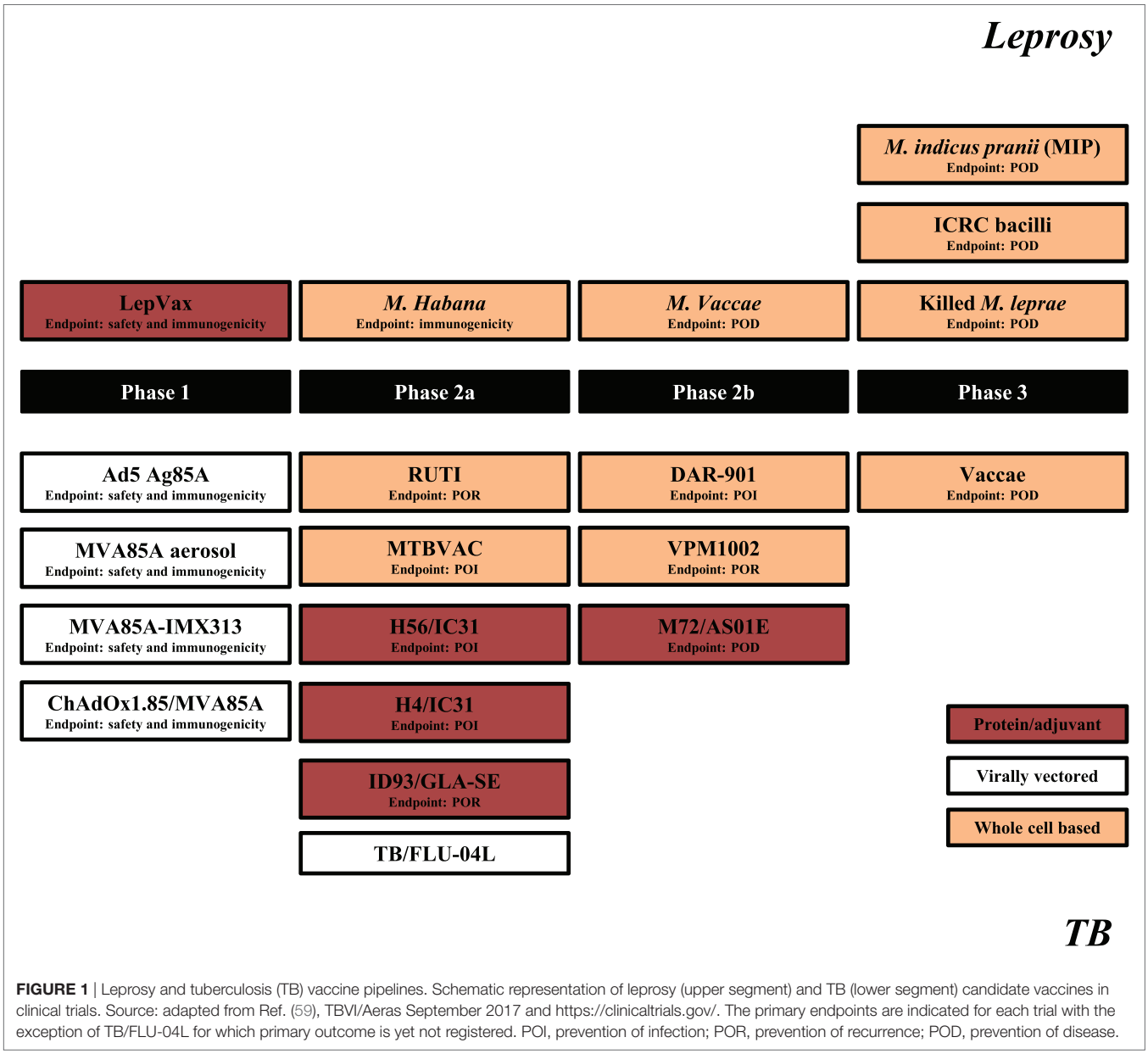

FIGURE 1 | Leprosy and tuberculosis (TB) vaccine pipelines. Schematic representation of leprosy (upper segment) and TB (lower segment) candidate vaccines in clinical trials. Source: adapted from Ref. (59), TBVI/Aeras September 2017 and https://clinicaltrials.gov/. The primary endpoints are indicated for each trial with the exception of TB/FLU-04L for which primary outcome is yet not registered. POI, prevention of infection; POR, prevention of recurrence; POD, prevention of disease.

VACCINES IN CLINICAL TRIALS: AT THE CROSSROAD BETWEEN LEPROSY AND TB

Although BCG vaccination trials in leprosy were executed decades ago, the current leprosy clinical vaccine pipeline is three times smaller than that of TB (**Figure 1**). This situation is relatively recent considering that in 2001 there were four candidate leprosy vaccines (being) tested in clinical trials vs. none against TB.

Vaccine Candidates

The leprosy vaccine pipeline employs both live (26, 60) and killed (26, 56, 61–63) whole cell mycobacterial vaccines as well as adjuvanted recombinant protein vaccines, such as LepVax (64), which have the advantage over BCG and other replicating live vaccines that they can be used safely also in immunocompromised individuals (65). LepVax comprises a hybrid recombinant protein, linking four *M. leprae* antigens: ML2531, ML2380, ML2055, and ML2028 (LEP-F1) (**Table 1**), formulated in a stable emulsion with a synthetic, TLR4 agonist (GLA-SE) as adjuvant which has recently finished pre-clinical testing (66). In line with the extent of the epidemic, the TB vaccine pipeline is much larger. This includes candidates using various delivery platforms, such as virally vectored vaccines (67–70), adjuvanted subunits vaccines (71–74), recombinant BCGs (75), genetically attenuated *Mtbs*, as well as heat killed whole mycobacterial cell-based vaccines (76–79) (**Figure 1**). Evidently, the TB subunit vaccine pipeline has focused on a limited number of candidate *Mtb* antigens, in particular: Ag85A, Ag85B, early secretory antigenic target (ESAT6), TB10.4, Rv1813, Rv2608, Rv3619–3620, Rv1196, and Rv0125 (**Table 1**).

Clinical Endpoints

Leprosy and TB vaccines have different target product profiles and clinical endpoints to be considered in efficacy trials, e.g., prevention of infection (POI), prevention of disease (POD), or prevention of recurrence (POR) (80). POD require extensive longitudinal studies due to the long incubation times (years) in TB and leprosy (years-decades), and the limited incidence rates in most populations studied. For these reasons, alternative clinical trial designs have been developed using alternative biologically relevant endpoints, such as prevention of recurrence (POR) in cured TB patients, which evaluate whether relapse rates can be reduced by post-therapy vaccination; or shortening of treatment trials, which evaluate whether treatment length can be reduced by complementary immunotherapy with TB vaccines during the last phase of TB treatment. For leprosy, vaccines could be positioned to help preventing nerve damage in patients, since this clinical endpoint has a much higher frequency in leprosy patients, requires a shorter follow-up period and is a highly relevant endpoint in leprosy. New clinical trial designs with alternative endpoints will be important to accelerate the clinical evaluation of new vaccines for TB and leprosy, and signals detected in such studies can be validated in larger studies against classical endpoints, such as POD and perhaps POI.

Clinical Trials

In most vaccination trials for leprosy, the protective effects of the tested new vaccine candidates were equivalent to that of BCG (81). Only in one study, vaccination with Indian Cancer Research Centre bacilli (an *M. lep*rae-related cultivable mycobacterium) and BCG plus killed *M. leprae* showed a twofold increased protection against leprosy compared to BCG alone (26). However,

TABLE 1 | Homology between tuberculosis (TB) vaccine components and *Mycobacterium leprae* proteins.

***Mycobacterium tuberculosis* proteins**		**Identity**		**Homology**		***M. leprae* orthologs**	**Vaccine candidate**	**References**
Rv number	**Gene name**	**Amino acid (aa) overlaps**	**%**	**aa overlaps**	**%**			
Rv3804c	Antigen 85A (Ag85A)	273/329	83%	296/329	90%	ML0097	Ad5 Ag85A; MVA85A aerosol; MVA85A-IMX313; ChAdOx1. 85/MVA85A; TB/FLU-04L	(64–67)
Rv1886c	Ag85B	269/324	84%	288/324	89%	ML2028	H56/IC31; H4/IC31; TB/FLU-04L; LepVax	(61)
Rv3875	Early secretory antigenic target	35/91	39%	61/91	68%	ML0049	H56/IC31	(68)
Rv2660	Rv2660						H56/IC31	(68)
Rv0288	TB10.4	68/96	71%	82/96	86%	ML2531	H4/IC31; LepVax	(61, 69)
Rv1813c	Rv1813c	nssf	nssf	nssf	nssf		ID93/GLA-SE	(70)
Rv2608	PPE42	65/156	42%	88/156	56%	PPE family[a]	ID93/GLA-SE	(70)
Rv3619c	EsxV	59/92	64%	74/92	80%	ML1056	ID93/GLA-SE	(70)
Rv3620c	EsxW	55/95	58%	73/95	76%	ML1055	ID93/GLA-SE	(70)
Rv1196	PPE18	173/419	41%	228/419	54%	ML1054[b]	M72/AS01E	(71)
Rv0125	PepA	250/358	70%	292/358	82%	ML2659	M72/AS01E	(71)
Rv1860	Apa	197/298	67%	218/298	74%	ML2055	LepVax	(61)
Rv0455c	Rv0455c	101/152	67%	113/152	75%	ML2380	LepVax	(61)

[a]*Accession number not known; nnsf, no significant similarity found.*
[b]*Pseudogene.*

M. indicus pranii (MIP) (also known as *Mycobacterium w.*) induced protective efficacy below that of BCG. Notwithstanding this result, MIP was evaluated also in a second, large-scale, double-blind trial with a 9-year follow-up (62). In this study, the protective efficacy of MIP in vaccinated household contacts after 3 years was the highest ever reported against leprosy (68%) for a vaccine other than BCG. However, its protective effect dropped considerably after 6 (60%) and 9 (28%) years of follow-up. Despite these conflicting results, MIP is currently being evaluated both as prophylactic and therapeutic vaccine against leprosy in two high endemic districts in India (82) in combination with a single dose of rifampicin (SDR). This design is reminiscent of a previous randomized vaccine field trial in which BCG as well as SDR was provided to leprosy contacts (83).

For TB, several vaccines and vaccine approaches are being pursued, with no new TB vaccine approved, yet for use, since the introduction of BCG in 1921. The results from the recent MVA85A vaccine phase 2b efficacy trial, the first new TB vaccine tested in an efficacy trial, since BCG, showed no improved protection in BCG-vaccinated South African infants (84), despite being highly immunogenic in adults (85). Several trials are ongoing (**Figure 1**), with the first outcomes to become available in 2018.

Correlates of Protection

Vaccine immunogenicity studies for both leprosy and TB vaccine candidates have mostly focused on their ability to induce type-1 cell-mediated immunity, particularly CD4+ Th cells releasing type 1 helper (Th1) cytokines. Indeed, Th1 immunity is widely considered to be key in controlling mycobacterial infections (86). HIV-induced CD4+ T cell deficiency, and genetic or acquired impairments in type 1 cytokine signaling (IL12-IFN-γ axis), all increase susceptibility to mycobacterial infection and progressive disease in humans and animal models (87–90). In leprosy, the presence of Th1 cytokines in lesions or in lepromin skin reactions has been related to better clinical prognosis and to localized rather than disseminating disease (91, 92). Furthermore, individuals that showed large local reactogenicity after intradermal BCG administration or lepromin injection are reported to have less risk for leprosy onset (93). Observation from a small Dutch cohort of BCG-vaccinated individuals showed that high skin inflammation responders had a larger amount of C-reactive protein in their sera than the low skin inflammation responders. In the same study, at 4, 8, and 12 weeks post-BCG vaccination, PBMCs of individuals with stronger local reactogenicity induced higher IFN-γ production

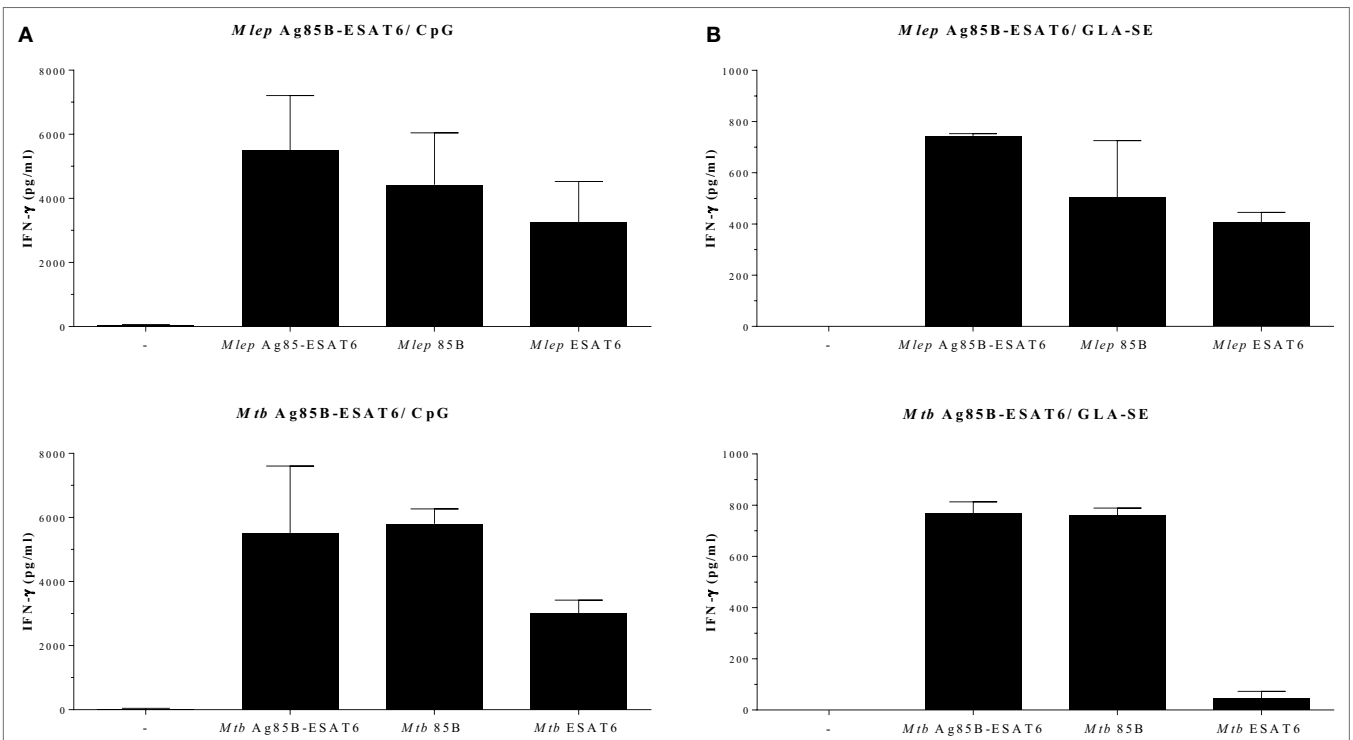

FIGURE 2 | IFN-γ secretion after Ag85-ESAT immunization. C57BL/6j and HLA-A2tg mice B6.Cg-Tg (117) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and housed under specific pathogen-free conditions. Recombinant proteins were overexpressed in *E. coli* BL21 (DE3) and purified to remove any traces of endotoxin as described in Ref. (116, 118). For the production of the antigen 85B (Ag85B)-early secretory antigenic target (ESAT6), hybrid recombinant hybrid protein, the Ag85B and ESAT6 genes were fused together by PCR with a linker coding for the amino acids NVA. C57BL/6j mice [**(A)**; 13–14 animals per group] and HLA-A2tg mice [**(B)**; 5 animals per group] were immunized three times subcutaneously with *Mycobacterium tuberculosis* (*Mtb)* Ag85-ESAT or *Mycobacterium leprae* Ag85-ESAT recombinant protein (25 μg) adjuvanted with GLA-SE [glucopyranosyl lipid adjuvant-stable emulsion (23) kindly provided by Infectious Disease Research Institute; Seattle, WA, USA; TLR4 agonist; 20 μg]; or CpG (ODN1826 5′-TCC ATG ACG TTC CTG ACG TT -3′; InvivoGen, San Diego, CA, USA; TLR9 agonist; 50 μg) (119). Splenocytes were harvested 4 weeks after final injections and restimulated *in vitro* with *Mtb* or *M. leprae* Ag85-ESAT hybrid recombinant proteins or the single Ag85B and ESAT6 recombinant proteins (all 10 μg/ml). IFN-γ secretion was analyzed by ELISA after 5 days. All mice were analyzed separately. Data shown indicate the mean and SE value of five mice per group.

after *in vitro* PPD stimulation than the one from the group with less local reaction to BCG (94). This suggests that skin reactogenicity after BCG vaccination causing local inflammation and systemic Th1 responses probably indicate protective immunity to mycobacteria. The failure of MVA85A against TB despite its induction of CD4+ Th1 immunity, the observation that BCG-specific CD4+ and CD8+ T-cell responses did not correlate with protection against TB disease in one study (95) together with the limited results achieved by current leprosy vaccines, clearly underline the need for a better understanding of the host mechanisms that are responsible for protection against both TB and leprosy. Several recent reports in animal models and humans have reported the involvement of other cell subsets in leprosy and TB (96, 97). Discovering these mechanisms may well prove to be a critical step for designing more effective vaccines.

Besides BCG, only MIP and killed *M. vaccae* have been clinically evaluated for both leprosy and TB, although in different trial designs and target populations. MIP has been tested for its putative therapeutic efficacy in tuberculous pericarditis (98) and as mentioned above for its protective efficacy against leprosy (26, 62). Killed *M. vaccae* has been assessed for its ability to prevent TB and leprosy disease in patients or contacts.

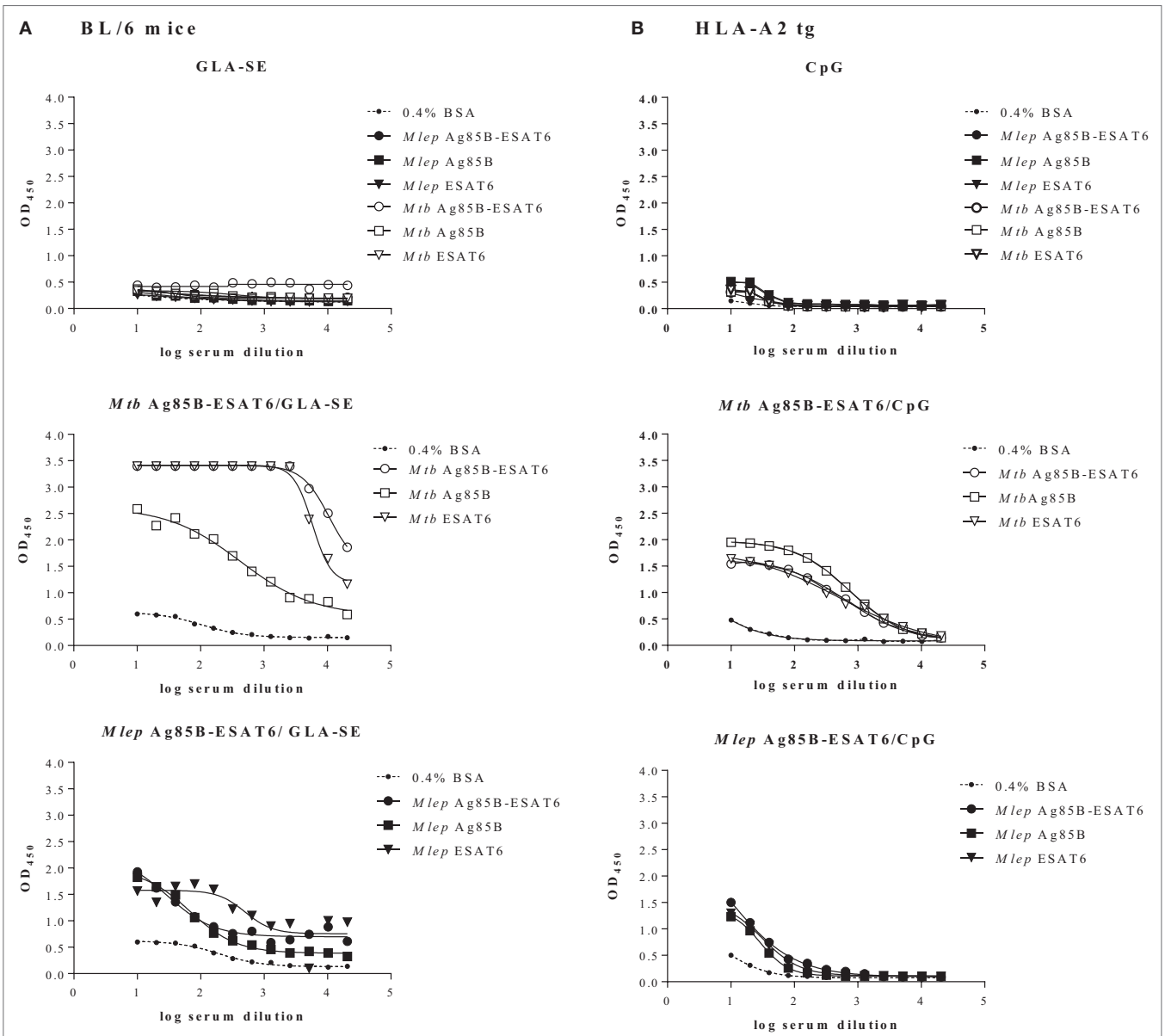

FIGURE 3 | Quantification of serum antibodies. Following immunization of C57BL/6j **(A)** and HLA-A2 tg **(B)** mice with adjuvant alone, *Mycobacterium tuberculosis (Mtb)* Ag85-ESAT or *Mycobacterium leprae* Ag85-ESAT recombinant protein in GLA-SE **(A)** or CpG **(B)**, antibody titers (OD_{450}) against *Mtb* Ag85-ESAT, *M. leprae* Ag85-ESAT, or *Mtb/M. leprae* Antigen 85B (Ag85B) and early secretory antigenic target (ESAT6) were determined by ELISA as described in Ref. (120). As a control coating with BSA (0.4% in PBS) was used. Sera from immunized mice were collected from cardiac blood 3 weeks after final immunization Serum dilutions are shown on the *x*-axis. Test groups included 3–5 mice. All mice were analyzed separately. Results are shown for one representative animal.

However, the administration routes (intramuscular vs. oral vs. intradermal injection of *M. vaccae*) and the eligibility criteria for the recruitment in the two trials (inclusion or not of individuals with BCG scar; HIV-positivity; anti-mycobacterial therapy) were quite diverse, impeding direct comparison of the impact of *M. vaccae* vaccination on both diseases (63, 78).

ONE SUBUNIT VACCINE FOR BOTH TB AND LEPROSY?

With the exception of *M. habana* (60, 99), the majority of vaccines evaluated for both leprosy and TB were initially designed as TB vaccines, and only evaluated at a later stage for their potential in leprosy. Since *M. leprae* has undergone massive gene reduction (100), not all *Mtb* antigens that are potential targets for TB vaccines have corresponding homologs in *M. leprae*. The first examples are ID83/GLA-SE and ID93/GLA-SE, two recombinant fusion proteins, formulated with the TLR4L-containing adjuvant GLA-SE, and consisting of three *Mtb* proteins: Rv1813, Rv2608, and Rv3620, with the further addition of Rv3619 in ID93. The amino acid (aa) sequences of Rv3619 and Rv3620 are 58 and 64% identical to the respective *M. leprae* proteins (ML1056 and ML1055, respectively) (**Table 1**). Likely due to these similarities, both *Mtb* hybrid recombinant proteins were also recognized by blood from paucibacillary leprosy patients, although latent *Mtb* infection could have explained these findings as well. Furthermore, when injected subcutaneously these vaccines reduced *M. leprae*-induced inflammation and bacterial growth in mouse models of leprosy (65), suggesting that TB subunit vaccines might have efficacy also against leprosy.

In a similar approach, we have investigated another TB subunit vaccine candidate, consisting of two major secreted *Mtb* proteins: *Mtb* ESAT6 and *Mtb* Ag85B, both present in short-term *Mtb* culture filtrates (101, 102). Ag85B is highly conserved among mycobacterial species, probably due to its critical role in cell wall synthesis as a mycolyltransferase (103). ESAT6 is a secreted virulence protein mainly restricted to the *Mtb* complex organisms (104). Both antigens have been extensively studied in the TB field over the past three decades and proved to be strongly recognized by CD4 Th1-cells of TB patients and latently TB infected (LTBI) individuals (105). Demonstrated to be immunodominant during *Mtb* infection, the two recombinant

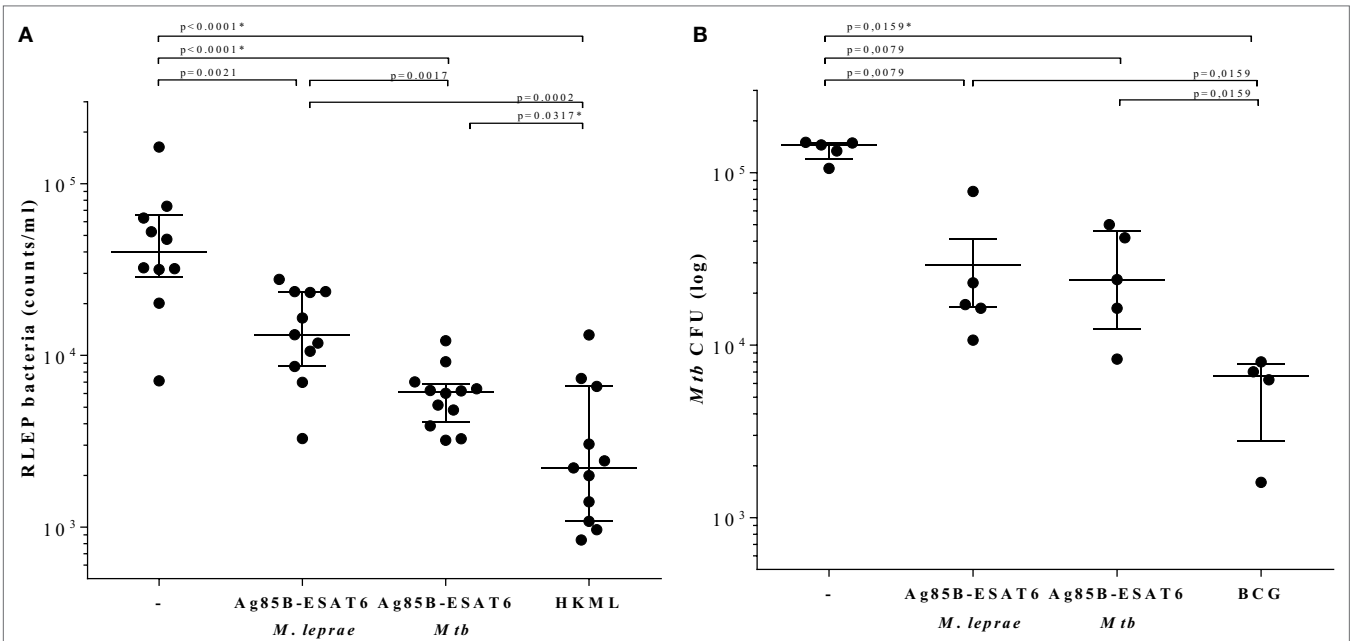

FIGURE 4 | Determination of bacterial burden. C57BL/6j mice were injected with 10^4 live *Mycobacterium leprae* (121) (viability: 11,000; in 40 µl PBS) in each hind foot pads 4 weeks after the final protein immunization. 7 months after *M. leprae* challenge, mouse footpads were harvested, and *M. leprae* were enumerated by RLEP PCR (122). HLA-A2tg mice were infected with live *Mycobacterium tuberculosis (Mtb)* strain H37Rv 6 weeks after the final protein immunization and 10 weeks after Bacille Calmette–Guérin (BCG) immunization (119). All animals included in the experiments were observed daily in order to ensure ethics requirements and to monitor any adverse effects possibly related to the vaccination or infection. **(A)** Bacteria were determined by the RLEP PCR from footpads from *M. leprae* infected C57BL/6j mice that had been immunized with GLA-SE adjuvant alone (–), *M. leprae* Ag85-ESAT/GLA-SE, *Mtb* Ag85-ESAT/GLA-SE, or heat killed *M. leprae* (HKML; 2×10^8 in 40 µl; viability: 6,400) as indicated on the *x*-axis. Each symbol represents one mouse. Calculated bacterial loads are expressed as RLEP counts on the *y*-axis. Horizontal lines indicate median values with interquartile range. **(B)** CFUs were determined in lung homogenates from *Mtb*-infected unimmunized (–) or *Mtb*-infected HLA-A2 tg mice that were immunized with BCG1331 (10^6 CFU), *M. leprae* Ag85-ESAT or *Mtb* Ag85-ESAT as indicated under the *x*-axis. Each symbol represents one mouse. Bacterial loads are expressed as log10 bacterial counts. Horizontal lines indicate median values with interquartile range. CFU of test and control groups were compared to the controls using the Mann–Whitney test and a $p < 0.01$ was considered significant. * marks differences that remained significant after multiple test correction using Kruskal–Wallis testing with Dunn's post-test.

proteins were fused into a recombinant hybrid protein, and adjuvanted with the Th1 inducing synthetic adjuvant IC31®. In several animal models, including mice, guinea pigs, and non-human primates, Ag85B-ESAT6/IC31 showed promising protective efficacy against TB disease (106, 107). Based on these results, the vaccine was progressed to human phase 1/2a trials (105, 108, 109). This work demonstrated the vaccine's safety and its remarkable ability to induce long-lasting Th1-type immune reactivity in healthy or HIV-negative, mycobacterially naive individuals, LTBI, and BCG-vaccinated volunteers (109–111) even 3 years after the second vaccination.

In view of several characteristics, Ag85B-ESAT6 is an interesting candidate for leprosy as well. *Mtb* ESAT6 and Ag85B share 68 and 89% aa overlaps (homology according to pre-computed Tuberculist Blastp) with *M. leprae* homologs, ML0049, and ML2028, respectively (**Table 1**). These proteins are widely recognized by antibodies of multibacillary leprosy patients (112, 113), as well as by IFN-γ secreting cells from paucibacillary leprosy patients (64). We previously demonstrated T-cell cross-reactivity between *Mtb* and *M. leprae* ESAT6 in leprosy and TB patients (114). Moreover, a previous study showed that Ag85B overexpression in BCG significantly increased BCG's protective efficacy against *M. leprae* (115). To further explore and compare the efficacy of Ag85B-ESAT6-based vaccines against TB and leprosy, we generated both *Mtb* Ag85B-ESAT6 and *M. leprae* Ag85B-ESAT6 and studied their *in vivo* efficacy in mouse models of *Mtb* and *M. leprae* infection.

Mtb and *M. leprae* Ag85B-ESAT6-Based Vaccines: A Comparative Evaluation

In order to evaluate the immunogenicity of *Mtb*-Ag85B-ESAT6 and *M. leprae*-Ag85B-ESAT6, both hybrid recombinant proteins were produced (116) and injected subcutaneously in wild-type C57BL/6j (BL/6) mice, as well as in C57BL/6j (BL/6) mice expressing an HLA-A*0201 transgene. The proteins were formulated with GLA-SE (TLR4 agonist) or CpG (TLR9 agonist), respectively, both of which have been reported to drive Th1-type responses. As expected, we detected high levels of IFN-γ released by splenocytes from immunized mice in response to *Mtb* Ag85B-ESAT6, *M. leprae* Ag85B-ESAT6, and their individual components (**Figure 2**). Total IgG, IgA, and IgM levels against *Mtb* Ag85B-ESAT6, *M. leprae* Ag85B-ESAT6, and the individual proteins were increased as well in both mouse strains following immunizations (**Figure 3**). Interestingly, the highest antibody titers were observed against *Mtb* Ag85B-ESAT6, regardless of whether *Mtb* Ag85B-ESAT6 or *M. leprae* Ag85B-ESAT6 had been used to immunize BL/6 mice (**Figure 3A**). Most importantly, both *Mtb* Ag85B-ESAT6/GLA-SE or *M. leprae* Ag85B-ESAT6/GLA-SE vaccines were capable of inducing host control of *Mtb* and *M. leprae* infection to a significant and comparable extent. Interestingly, *Mtb* Ag85B-ESAT6/GLA-SE controlled *M. leprae* infection significantly better than *M. leprae* Ag85B-ESAT6/GLA-SE (**Figure 4**). In summary, these results suggest that novel subunit vaccines designed for TB, such as *Mtb* Ag85B-ESAT6 could have efficacy against both TB and leprosy.

CONCLUDING REMARKS

Leprosy and TB are still major poverty-related health concerns. Leprosy is primarily endemic in geographic areas, where TB is also highly prevalent (115). To date, BCG has been used predominantly as a vaccine against TB, but it also contributes to the control of leprosy. However, due to its limited efficacy especially against pulmonary TB in adults, the main and contagious form of TB, novel vaccines are being developed to replace or boost BCG (**Figure 1**). Although these vaccines will likely also impact leprosy incidence, this issue is rarely considered, let alone studied in extensive trials.

There are two leprosy vaccine candidates, MIP in India (82) and LepVax (66), and the TB vaccine pipeline is much more advanced and diverse than the one for leprosy. Even though it is likely that a TB vaccine candidate will emerge, for none of the current TB candidate vaccines, the impact on leprosy is currently being taken into account.

Only two highly similar recombinant subunit TB vaccines, based on the same backbone design, have been tested for their potential use against leprosy (65). Here, we describe original data showing a second TB subunit candidate vaccine platform, based on Ag85B/ESAT6. Collectively, our data suggest that novel TB vaccine candidates can cross-protect against leprosy, providing support for integrating leprosy vaccine research with TB vaccine research (65, 81, 115). At the moment, the most advanced new TB vaccine candidates have been tested in India, Tanzania, China, South Africa, the first two of which have elevated incidences of leprosy. Thus far, none of these recent trials have included evaluation of impact on leprosy, unlike what was done decades ago for BCG(61). We contend that preclinical integration and harmonization of TB/leprosy discovery and development research would well be feasible with respect to the design of subunit vaccines, as we have in fact applied in our recent approach for vaccine antigen discovery (123). With respect to antigen selection algorithms, it is of interest to consider the extensive genomic reduction that *M. leprae* has undergone during evolution (100, 124), causing this *Mycobacterium* to become a highly specialized and obligate intracellular pathogen (125). Studying *M. leprae*'s successful minimalistic approach will reveal genetic and metabolic pathways that pathogenic mycobacteria need to survive in the host, and inspire drug and vaccine efforts to combat both diseases which have put such a heavy toll on humans for millennia.

ETHICS STATEMENT

The handling of mice was conducted in accordance with the regulations set forward by the animal care committee of the LUMC and in compliance with European Community Directive 86/609 for the care and use of laboratory animals. The experiments at NHDP were performed under a scientific protocol reviewed and approved by the NHDP Institutional Animal Care and Use Committee (Assurance #A3032-01) and were conducted in accordance with all state and federal laws in adherence with PHS policy and as outlined in The Guide for the Care and Use of Laboratory Animals, Eighth Edition.

AUTHOR CONTRIBUTIONS

Concept of the review: AG and TO. Designed and wrote the review: MC, TO, and AG. Figures and legends: SE, KF, NR, LW, and LA. Final approval of the version to be published: all authors.

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miRNome Expression Analysis Reveals New Players on Leprosy Immune Physiopathology

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Leprosy remains as a public health problem and its physiopathology is still not fully understood. MicroRNAs (miRNA) are small RNA non-coding that can interfere with mRNA to regulate gene expression. A few studies using DNA chip microarrays have explored the expression of miRNA in leprosy patients using a predetermined set of genes as targets, providing interesting findings regarding the regulation of immune genes. However, using a predetermined set of genes restricted the possibility of finding new miRNAs that might be involved in different mechanisms of disease. Thus, we examined the miRNome of tuberculoid (TT) and lepromatous (LL) patients using both blood and lesional biopsies from classical leprosy patients (LP) who visited the Dr. Marcello Candia Reference Unit in Sanitary Dermatology in the State of Pará and compared them with healthy subjects. Using a set of tools to correlate significantly differentially expressed miRNAs with their gene targets, we identified possible interactions and networks of miRNAs that might be involved in leprosy immunophysiopathology. Using this approach, we showed that the leprosy miRNA profile in blood is distinct from that in lesional skin as well as that four main groups of genes are the targets of leprosy miRNA: (1) recognition and phagocytosis, with activation of immune effector cells, where the immunosuppressant profile of LL and immunoresponsive profile of TT are clearly affected by miRNA expression; (2) apoptosis, with supportive data for an antiapoptotic leprosy profile based on *BCL2*, *MCL1*, and *CASP8* expression; (3) Schwann cells (SCs), demyelination and epithelial–mesenchymal transition (EMT), supporting a role for different developmental or differentiation gene families, such as Sox, Zeb, and Hox; and (4) loss of sensation and neuropathic pain, revealing that *RHOA*, *ROCK1*, *SIGMAR1*, and aquaporin-1 (*AQP1*) may be involved in the loss of sensation or leprosy pain, indicating possible new therapeutic targets. Additionally, *AQP1* may also be involved in skin dryness and loss of elasticity, which are well known signs of leprosy but with unrecognized physiopathology. In sum, miRNA expression reveals new aspects of leprosy immunophysiopathology, especially on the regulation of the immune system, apoptosis, SC demyelination, EMT, and neuropathic pain.

Keywords: leprosy, immunology, Schwann cells, apoptosis, neuropathic pain, microRNA, miRNome, epigenetics

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INTRODUCTION

Leprosy is an ancient disease caused by *Mycobacterium leprae*, an obligate intracellular pathogen that infects macrophages and Schwann cells (SCs), resulting in nerve and skin lesions with loss of sensation, the hallmark of the disease (1).

After contact with the bacilli, most people control *M. leprae* multiplication and will never develop leprosy (2). If the bacilli survive, the host may develop two stable polar forms of disease, the paucibacillary (PB) tuberculoid (TT) form or the multibacillary (MB) lepromatous (LL) form, besides the three borderline intermediate unstable forms, borderline TT, borderline borderline, and borderline LL. PB patients have a good cellular immune response (CIR) that may restrict bacillus proliferation, resulting in a few lesions that are usually limited to a specific part of the tegument and to a few nerve trunks. MB patients, on the other hand, have a poor CIR with an exacerbated humoral immune response that is not effective for controlling bacillus proliferation. Patients have many lesions disseminated through the body, including the skin and peripheral nerves (3).

The natural history of the disease results in disabilities. Demyelination caused by SC degeneration is one of the main events in leprosy physiopathology, together with exacerbations of the immune response, known as leprosy reactions. While patients evolve a loss of sensation on the skin, they may also have peripheral nerve neuropathic pain that can be exacerbated by the reactions and may last for many years, even after multidrug therapy (MDT) (4).

Genetic studies of portions of the genome that do not encode protein revealed one class of small non-coding RNAs [named microRNAs (miRNAs)] that are involved in posttranscriptional control of gene expression (5). Knowledge about the interaction between miRNA and leprosy is limited (6–12). A recent study demonstrated that a miRNA can influence the mechanism whereby the cell host can prevent bacillus growth and generate natural barriers against infection by *M. leprae* (9). Evidence has shown that miRNAs are able to modulate host antibacterial pathways during the infection process and influence the outcome of disease (9). Analysis of miRNAs that are differentially expressed in distinct poles of the disease could provide a better understanding of targets for an efficient immune response to prevent infection, as well as elucidate novel possible biomarkers for leprosy, for example, subclinical infection and one possible predictor of who will develop leprosy (13, 14).

Upon contact with *M. leprae*, the human immune system must recognize and process the bacteria to activate immune effector cells. During the interaction, the host cells may be induced to undergo apoptosis, hindering bacillus adaptation or maintaining its survival with attenuated microbicide capacity, to shelter the bacilli (15). Demyelination is a key event in leprosy, and SC are critical for myelin production and maintenance on peripheral nerves. Epithelial–mesenchymal transition (EMT) is a biological process in which specialized cells may undergo a phenotypic change to mesenchymal cells, with higher motility, greater resistance to apoptosis, induction of fibrosis, loss of markers for specialized cells, and the acquisition of new proliferation markers (16). Mechanisms responsible for the loss of sensation and neuropathic pain are poorly understood.

Our work presents the first leprosy miRNome from lesions and blood of LP. In addition to describing the miRNAs, we chose those with significant differential expression, searched for their target genes, and constructed possible pathways based on current knowledge of leprosy immune pathophysiology.

MATERIALS AND METHODS

Study Design and Participants

A total of 28 biological samples from leprosy patients (LP) before starting MDT treatment who attended the Dr. Marcello Candia Reference Unit in Sanitary Dermatology of the State of Pará (UREMC) on 2014, in Marituba, Pará, Brazil, and individuals without leprosy and with no other diseases [healthy subjects (HS)] were included in the present study, according to the following groups: (a) 17 tissue biopsies samples [11 from LP (6 LL and 5 TT) and 6 skin tissue from HS for controls] and (b) 11 peripheral blood samples [9 from LP (5 LL and 4 TT) and 2 from HS for controls]. **Table 1** describes gender, age, bacterial index, anti-PGL-I optical density, and disability grading of the 11 LP selected for the study.

This study adhered to the Declaration of Helsinki and was approved by the Institute of Health Sciences Research Ethics Committee at Universidade Federal do Pará, certified by CAAE 26765414.0.0000.0018. A written informed consent to publish was obtained from every individual who accepted to participate in this study. The small RNAseq number register is ERP105473 on European Nucleotide Archive database.

Total RNA Storage, Extraction, and Quantification

A flowchart (**Figure 1**) presents all the steps performed during miRNA Seq (extraction, library, sequencing data processing and analysis pipeline, target gene identification). The whole peripheral blood samples were collected into a Tempus Blood RNA Tube (Thermo Fisher Scientific, USA) and stored at −20°C until extraction. The skin tissue biopsy samples were collected in a propylene tube with 2 mL RNAlater (Thermo Fisher Scientific, USA) and stored in liquid nitrogen until use. Total RNA was extracted

TABLE 1 | Leprosy patients enrolled on the study: ID, gender, age, bacterial index, anti-PGL-I IgM, and disability grade.

ID[a]	Gender	Age	Bacterial index	Anti-PGL-I[b]	Disability grade
LL 1	M	30	3.25	2.023	0
LL 2	F	81	3.50	1.551	2
LL 3	M	72	5.75	2.145	1
LL 4	M	64	4.25	1.849	2
LL 5	M	51	5.00	1.158	1
LL 6	F	58	4.75	0.792	0
TT 1	M	40	0	0.041	0
TT 2	F	44	0	1.200	0
TT 3	M	37	0	NR	0
TT 4	M	20	0	0.184	0
TT 5	M	19	NR	0.022	0

[a]*The ID is composed of the clinical form followed by a sequential number.*
[b]*Optical density of ELISA.*
NR, not realized.

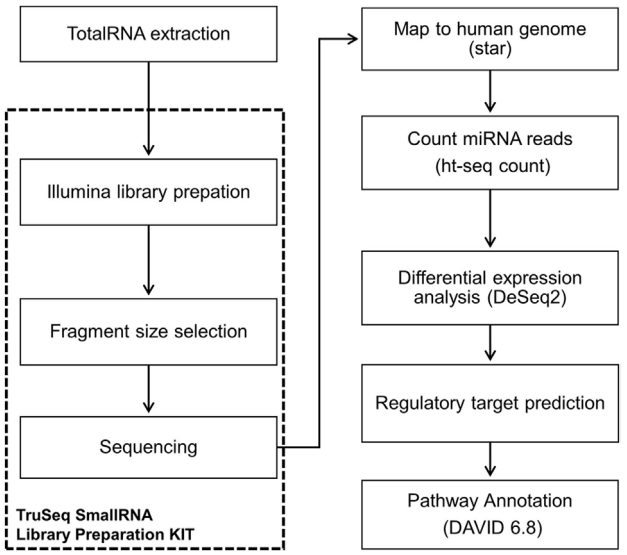

FIGURE 1 | Flowchart showing workflow of miRNome sequencing and analysis.

from the tissue sample using TRIzol reagent (Invitrogen, USA), and samples were eluted in DEPC water and stored in liquid nitrogen. Total RNA was extracted from blood samples using the MagMAX RNA Isolation Kit (Thermo Fisher Scientific, USA). Total RNA quantity and quality assessed were performed with a NanoDrop 1000 (Thermo Scientific, USA) and Agilent 2200 TapeStation (Agilent Technologies, USA).

Library Preparation and Next-Generation Sequencing (NGS)

The library was prepared using the TruSeq Small RNA Library Preparation Kit (Illumina, Inc., USA) according to the manufacturer's instructions, and all samples used for the library had an initial concentration of 1 μg/5 μL of total RNA. The library was validated and quantified with an Agilent 2200 TapeStation (Agilent Technologies, USA) platform and by real time PCR with the KAPA Library Quantification Kit (KAPA BIOSYSTEM, USA). The libraries were then diluted to a concentration of 4 nM and sequenced using the MiSeq Reagent Kit v3 150 cycle (Illumina, Inc., USA) on a MiSeq System (Illumina, Inc., USA). The tissue and blood samples were sequenced separately.

Sequencing Data Processing and Analysis-Small RNA-Seq Pipeline

The sequencing data were processed on an Illumina MiSeq reporter and extracted in FASTQ format. A pipeline of pre-processing using the Fastx_toolkit was applied for a low filter quality, trimers of extreme 3′ reads and contaminant removal. The pipeline was performed according the chronogram: (a) average Phred quality score (*Q*) greater than 30, (b) reads more than 17 nucleotides long, and (c) base calling error probabilities (*P*) greater than 80. Next, read alignment with the human genome (GRCh37) in combination with the miRNA data base (MirBase v.19) was performed using Spliced Transcripts Alignment to a Reference. The miRNA was scored with htseq-count toll, and the results were normalized and analyzed using the Bioconductor-*DESeq2* package with R statistical software. Thus, the following comparisons were conducted: (a) LP vs. HS; (b) TT leprosy vs. HS; (c) LL leprosy vs. HS; (d) TT vs. LL leprosy. Adjusted values of $p \leq 0.05$ and a log2 fold change >2 were considered statistically significant.

Target Gene Identification

The genes regulated by the differentially expressed miRNAs detected during the analysis were identified using four tools:(i) TargetCompare (http://lghm.ufpa.br/ferramentas-de-estudos/targetcompare/) (17); (ii) miRTarBase (http://mirtarbase.mbc.nctu.edu.tw); (iii) DIANA miRPath v.3 (18); and (iv) TargetScan (19). We selected only genes regulated by two or more miRNAs with strong experimental evidences support, such as those confirmed by western blotting, reporter assay or qPCR.

Selected targets were further investigated using the pathway enrichment tool DAVID 6.8 (20) that provides a comprehensive set of functional annotation tools and searches in BioCarta and KEGG pathway maps to help investigators to understand biological meaning behind a large list of genes. The pathway enrichment analysis was performed separately for the following groups: (i) HS vs. LP downregulated miRNAs in tissue; (ii) HS vs. LL downregulated miRNAs in tissue; (iii) HS vs. LL upregulated miRNAs in tissue; and (iv) HS vs. LP downregulated miRNAs in blood.

RESULTS

This study evaluated two types of leprosy samples, skin biopsies and blood by distinct NGS. For the two sample types, the differential expression profiles of the miRNAs were analyzed to identify possible leprosy biomarkers to assist our understanding of epigenetic control mechanisms of the immune response, apoptosis, SC demyelination, EMT, and neuropathic pain.

miRNA Sequencing and Differential Expression Profiles of Tissue Samples

This sequencing yielded 4 million reads. After the process pipeline, more than 96% of the reads were aligned with the human genome, and the miRNA count was performed using *htseq-count* (miRNA count ≥ 10), with an average of 36,745 reads per sample and 656 miRNAs expressed in at least one sample.

A heatmap was constructed using the RPKM (Reads per Kilobases per Million) expression for all differentially expression miRNA (**Figure 2**). The analysis identified the RPKM z-score of 67 differentially expressed miRNAs, 43 downregulated and 24 upregulated (**Table 2**; Data Sheet S1 in Supplementary Material) in skin biopsies among HS, LL, and TT. miRNAs (rows) were hierarchically clustered according their expression, and organized according to the three groups, HS, LL, and TT (columns). A hierarchical clustering of the data illustrates how those markers were able to distinguish HS from leprosy patients in general, and LL from TT poles.

Figure 3 shows the 39 simultaneous differentially expressed miRNAs, for at least two of the three comparisons conducted

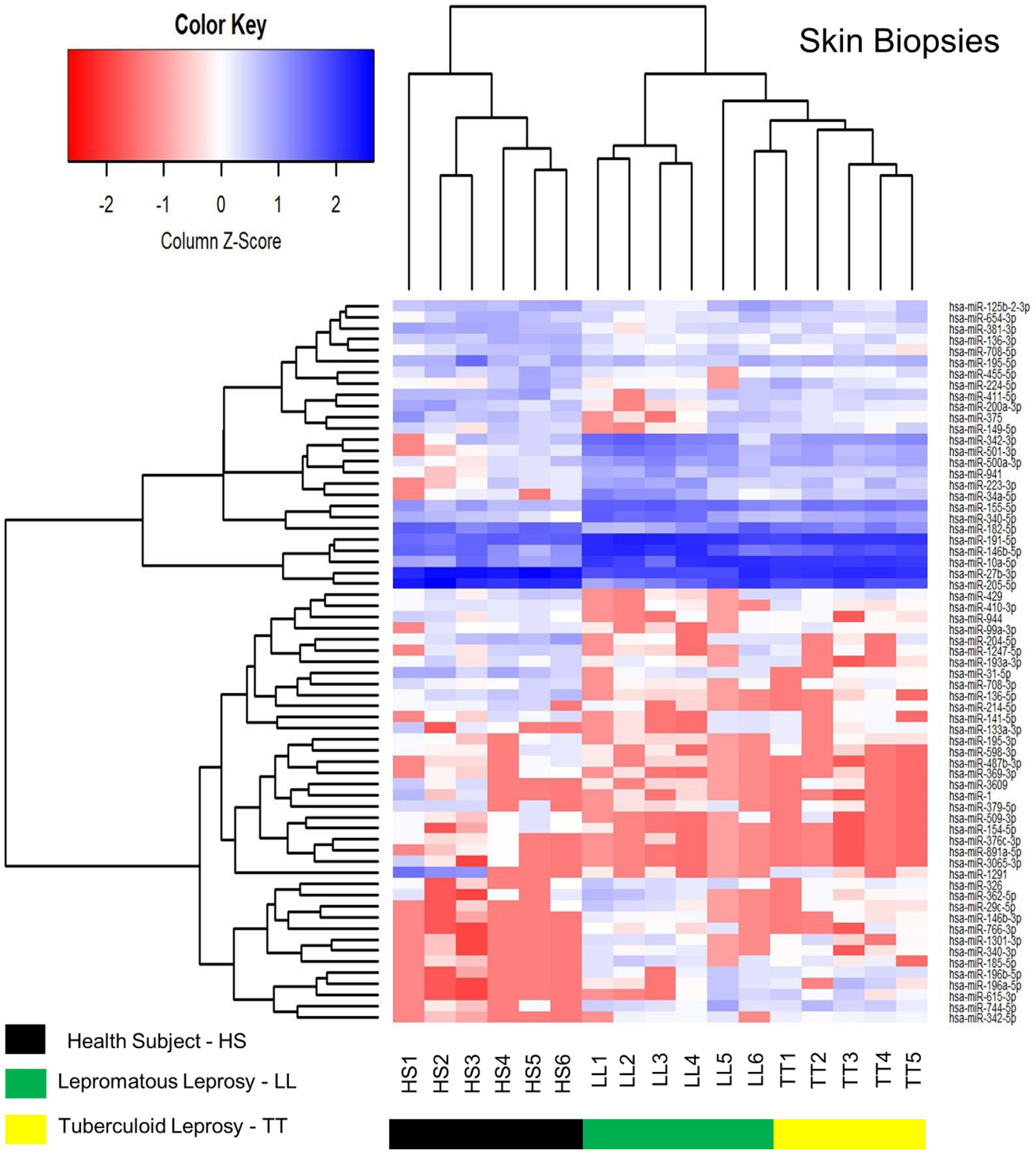

FIGURE 2 | Heatmap of skin biopsies for the differentially expressed microRNAs (miRNAs) among health subjects (HS), lepromatous (LL) leprosy, and tuberculoid (TT) leprosy. It represents the RPKM *z*-score of 67 differentially expressed miRNAs. miRNAs (rows) were hierarchically clustered according their expression in the three different samples (HS, LL, and TT). The clustering was able to distinguish HS from leprosy patients, as well as LL from TT.

as described in **Table 2** (HS vs. LP, HS vs. TT, and HS vs. LL), organized by their fold change showing 24 downregulated and 15 upregulated miRNAs.

The comparison of extreme poles of leprosy, TT and LL, revealed five differentially expressed miRNAs, of which three were down-regulated (*hsa-miR-340-5p, hsa-miR-34a-5p, hsa-miR-362-5p*)

and two were upregulated (*hsa-miR-429, hsa-miR-200a-3p*). The *hsa-miR-362-5p* appeared only when TT and LL were compared, but not when LP were compared to HS. The volcano plot shows the only five miRNAs differentially expressed (**Figure 4**).

TABLE 2 | Number of miRNAs that were differentially expressed in LP (TT and LL) compared with HS in skin biopsy samples.

Analysis	miRNAs	miRNAs downregulated	miRNAs upregulated
LP vs. HS	43	26	17
TT vs. HS	14	7	7
LL vs. HS	60	41	19

LP, leprosy patients; TT, tuberculoid; LL, lepromatous; HS, health subjects.

miRNA Sequencing and Differential Expression Profiles of Whole Blood Samples

Sequencing yielded 6 million reads. After the process pipeline, more than 95% of the reads were aligned with the human genome, and the miRNAs were counted using *htseq-count* (miRNA count ≥ 10), with an average of 371,325 reads per sample and 527 miRNAs expressed in at least one sample.

The differential expression analysis of blood miRNAs was conducted similarly to that applied for the tissue and revealed a total of 10 differentially expressed miRNAs, with nine downregulated (*hsa-let-7f-5p, hsa-miR-126-3p, hsa-miR-126-5p, hsa-miR-144-5p, hsa-miR-15a-5p, hsa-miR-20a-5p, hsa-miR-26b-5p,*

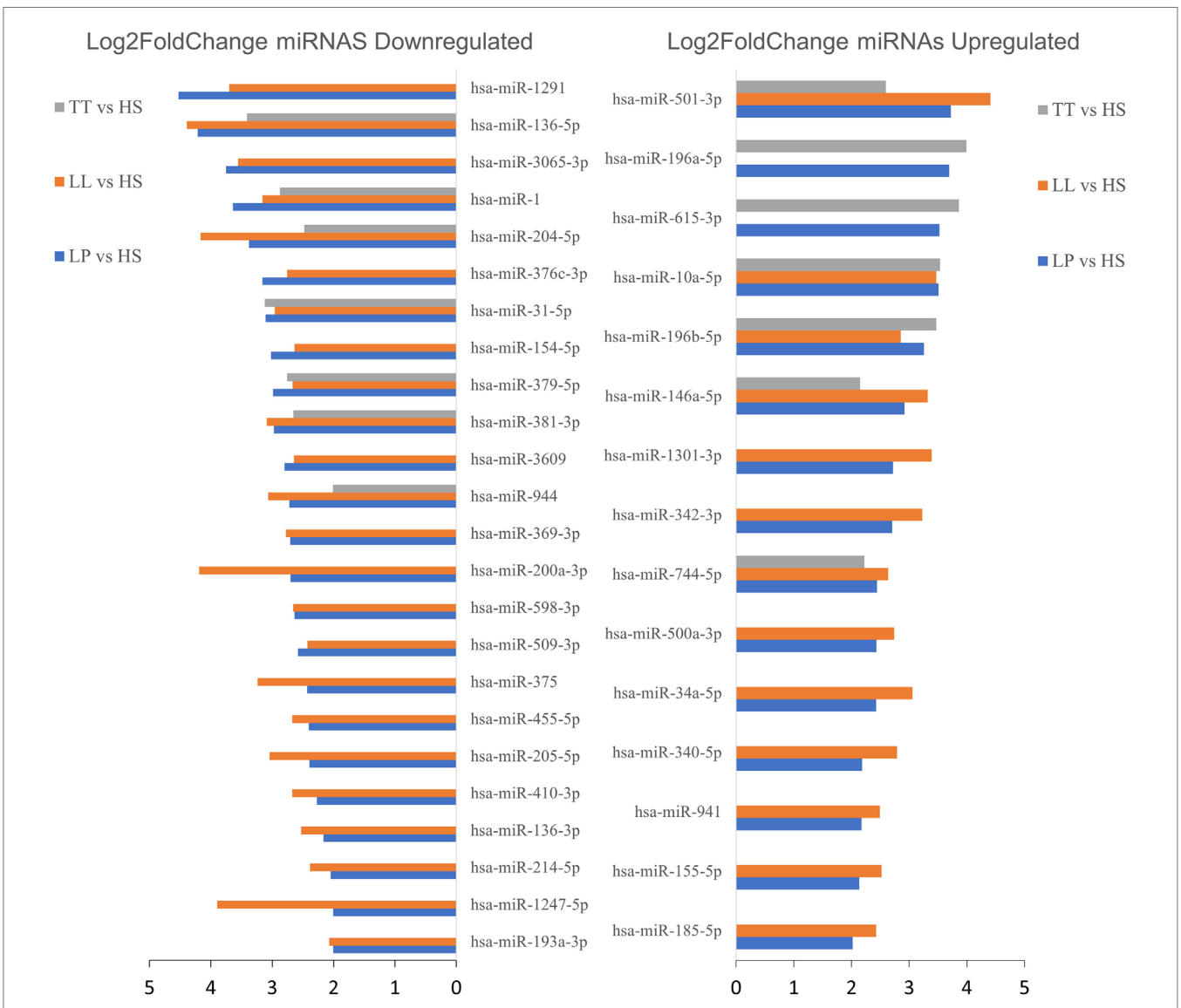

FIGURE 3 | Upregulated or downregulated microRNAs (miRNAs) in at least two comparisons [tuberculoid (TT) vs. healthy subjects (HS), lepromatous (LL) vs. HS, or leprosy patients (LP) vs. HS] on skin biopsies samples. Among the 67 differentially expressed miRNAs (|log2 fold change| > 2 and adjusted *p*-value < 0.05), here we highlight 39 miRNAs differentially expressed between HS versus LP and HS versus LL or TT patients, each indicated by blue, orange, and gray bars, respectively. The barplot represents each miRNA (*y*-axis) absolute log2 fold change (*x*-axis) for each comparison and separated in up- and downregulated miRNA regarding HS.

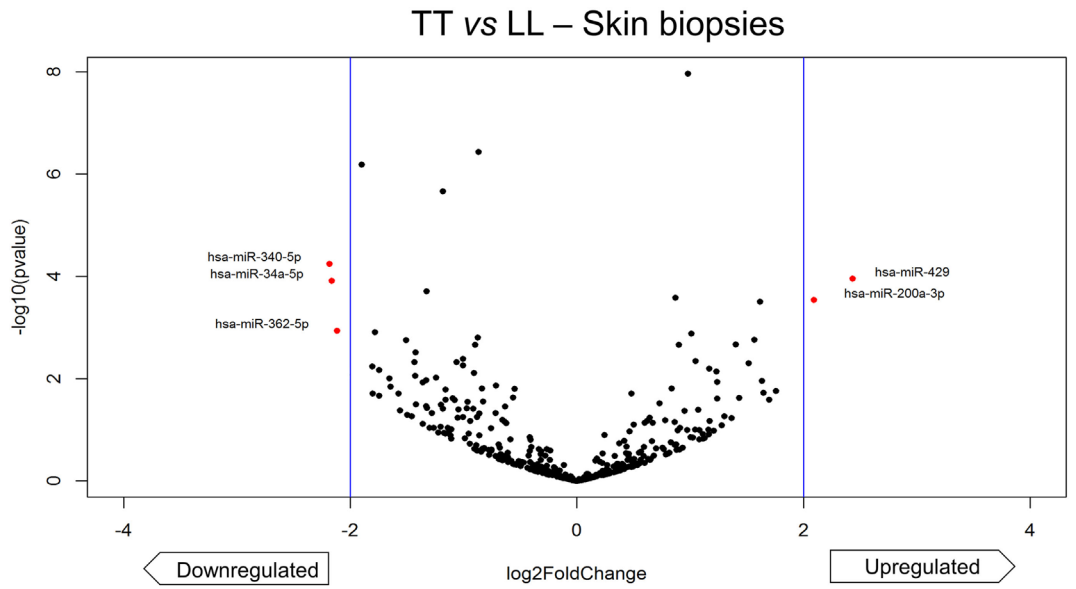

FIGURE 4 | Volcano plot of differentially expressed microRNAs (miRNAs) of skin biopsies between tuberculoid (TT) leprosy and lepromatous (LL) leprosy. The plot represents each miRNA differential expression analysis result where the y-axis is $-\log_{10}$ *p*-value of the comparison and the *x*-axis is $\log_2$ fold-change (*x*-axis) regarding LL. Differentially expressed miRNAs were considered only if the analysis showed |log2 fold-change| > 2 (indicated by the blue vertical lines) and adjusted *p*-value < 0.05. We found five differentially expressed miRNA (highlighted in red), two upregulated (*hsa-miR-429, hsa-miR-200a-3p*) and three downregulated (*hsa-miR-340-5p, hsa-miR-34a-5p, hsa-miR-362-5p*).

hsa-miR-106b-5p, hsa-miR-16-5p) and one upregulated (*hsa-miR-1291*) (**Table 3**; Data Sheet S2 in Supplementary Material). From the differentially expressed miRNAs identified, a heatmap was constructed using RPKM expression and two clusters were observed, with standards of expression able to differentiate LP of HS (**Figure 5**), although comparisons between TT vs. LL showed no differentially expressed miRNAs.

Target Gene Identification

Using the differentially expressed miRNAs from either blood or skin lesions, we investigated the genes regulated by them separately for up and down regulated miRNAs. The miRNAs and their targets are described in **Tables 4**–**8**, as follow: (i) HS vs. LP downregulated (**Table 4**) and upregulated (**Table 5**) miRNAs in skin biopsies; (ii) HS vs. LL downregulated (**Table 6**) and upregulated (**Table 7**) miRNAs in skin biopsies; and (iii) HS vs. LP downregulated miRNAs in blood (**Table 8**).

TABLE 3 | Number of miRNAs that were differentially expressed in LP (TT and LL) compared with HS in blood.

Analysis	miRNAs	miRNAs downregulated	miRNAs upregulated
LP vs. HS	7	7	0
TT vs. HS	5	5	0
LL vs. HS	4	3	1

LP, leprosy patients; TT, tuberculoid leprosy; LL, lepromatous leprosy; HS, health subjects.

DISCUSSION

Recognition, Engulfment, and Activation of Immune Effector Cells

The metalloproteinase ADAM9 and the integrin ITGA5 are two transmembrane proteins involved in mycobacteria invasion of macrophages (21, 22) and endothelial cells (23). Phagocytosis of *M. leprae* may be stimulated by IGF1R (24), LRP1 (25), and PIK3CA (26). In blood, with the exception of *hsa-miR1291*, all miRNAs were downregulated when LPs were compared with HS, indicating that the phagocytosis of *M. leprae* in LP blood was not inhibited. Furthermore, miRNAs that control *IGF1R* gene were upregulated in lesional tissue of LL patients in comparison to HS (**Figure 6**), which together with the decrease in *IGF1* gene in LL patients (27) may result in inhibition of the microbicidal function of macrophages against *M. leprae* (15) in tissue.

VAC14 is induced to control vacuolation in macrophages (28), while APP produces the oxidative burst (29) and PIK3CA stimulates *M. leprae* phagocytosis (26). Several miRNAs control the expression of *VAC14* and *PIK3CA* genes in lesional tissue and *APP* gene in blood, all of which were downregulated in LP in comparison to HS (**Figure 6**). When LL patients were compared to HS, only miRNAs that control *VAC14* gene were downregulated, indicating that *VAC14* gene expression was necessary for vacuole formation in LL patients.

Protein kinase C epsilon encoded by the *PRKCE* gene was also found to have downregulated miRNAs in LP. PRKCE is coupled to TLR-4, which is responsible for *M. leprae* recognition (30). Upon phosphorylation, two pathways may be activated: *IRF3* gene upregulation may result in the production of IFN-β and IL-10, especially in LL patients (31), or NFκB upregulation, with

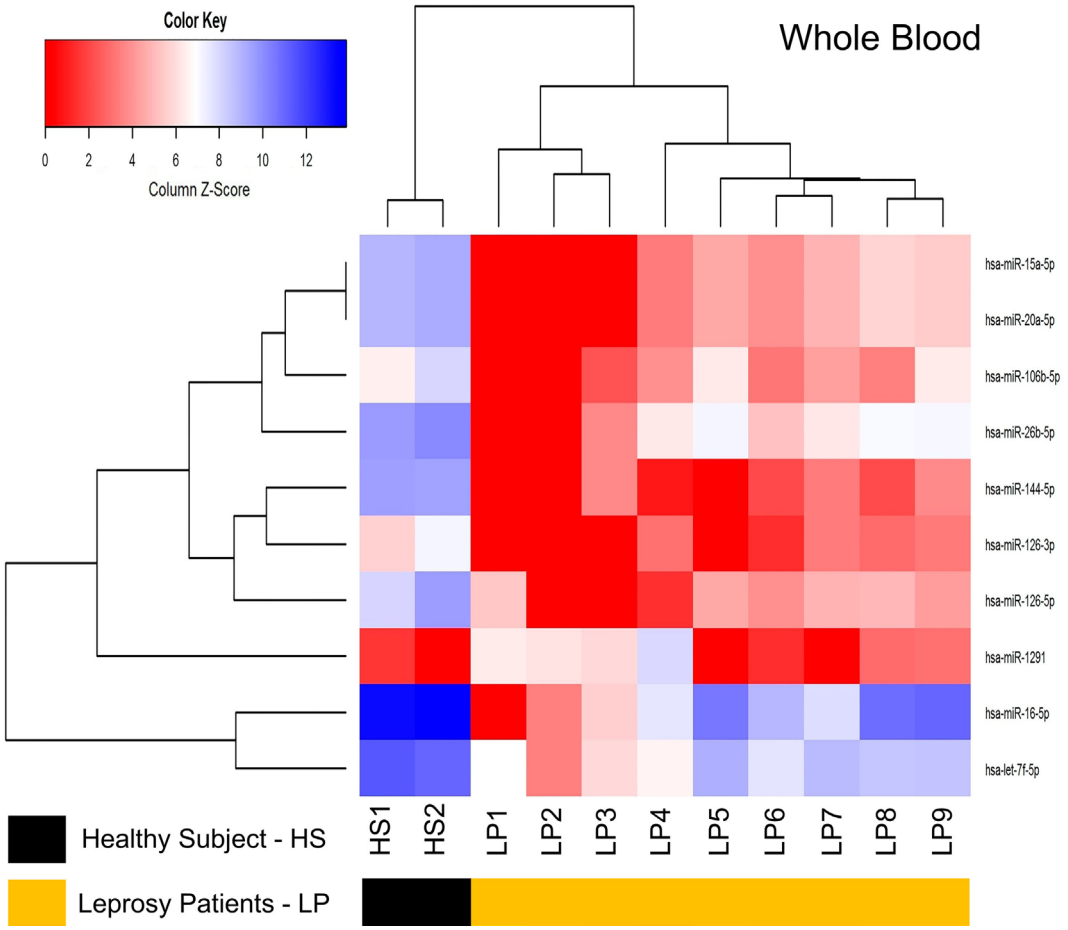

FIGURE 5 | Heatmap of whole blood for the differentially expressed microRNAs (miRNAs) between healthy subjects (HS) and leprosy patients (LP). It represents the RPKM *z*-score of 10 differentially expressed miRNAs. miRNAs (rows) were hierarchically clustered according their expression on HS or LP (columns). The clustering was able to distinguish LP from HS.

the production of proinflammatory cytokines, such as TNF-α and IL-6, by NFκB activation (30). IFN-γ and TNF-α may disrupt the TGF-β pathway by SMAD7 activation followed by TGFBR1 downregulation (32).

Leprosy patients miRNAs control *TGFBR1*, *SMAD7* gene and the zinc finger transcription factor *KLF11* gene, which regulates *SMAD7* expression. LL patients showed a downregulation of miRNAs that control *KLF11* gene expression, which may result in *SMAD7* gene inhibition and an increase in *TGFBR1* gene, with more TGF-β capture contributing to the immunosuppressive profile of LL. SMAD7 also stimulates CHUK, which inhibits the NFKB1 and COX2 inflammatory pathway. Interestingly, the central inflammatory player, NFKB1, was also found to be regulated by miRNAs in LL patient lesions (**Figure 6**). It has been previously demonstrated that TGF-β secretion is augmented in LL patients (33) and is secreted by CD4+ CD25+ FOXP3+ T regulatory cells (34), while TGF-β receptors are also increased in lesions of LL patients (35). Additionally, miRNAs that regulated cytoplasmic protein ERBIN were downregulated in LL patient lesions, indicating that ERBIN may also regulate *TGFBR1* gene pathway expression (**Figure 6**).

NOTCH1/2 have different functions in immune regulation, but overall seem to stimulate the immune system participating in the differentiation of naïve T cells (36) and modulating inflammation (37). An important regulator of M1 macrophage differentiation and the Th1 T cell profile in leprosy, the transmembrane protein NOTCH1 (38) was also found to be regulated by LP miRNAs in lesional tissue of LL patients. Additionally, *NOTCH1 gene*, *hsa-miR-34a-5p* also control *NOTCH2* and *JAG1* gene in LL lesions (**Figures 6** and **7**). NOCTH1 is known to be activated by JAG1 on endothelial cells, regulating the differentiation of M1 macrophages (38) in PB leprosy. Both NOTCH1 and NOTCH2 are expressed on Th0 cells and are related to Th17 differentiation (39). Furthermore, NOTCH1/2 are expressed on activated Th1 cells and are critical to the protective response against *Leishmania major* infection by the production of IFN-γ (40), which is also important for leprosy protection by JAG1 stimulation (38).

Activation of TLR4, IL15R, IL1R1, and IL1A is important for antimicrobial activity, a key function for infection control. LL patients were found to have upregulated miRNAs for all those genes in lesional skin (**Figures 6** and **7**). TLR4 and IL15R converge to CYP27B1, which converts 25-hydroxyvitamin D (25D) to the

TABLE 4 | List of the genes targeted by two or more differentially expressed miRNAs among the 26 downregulated miRNAs in LP vs. HS skin biopsies.

Target gene[a]	MicroRNA	Number of miRNAs
BCL2	*hsa-miR-136-5p, hsa-miR-204-5p, hsa-miR-375, hsa-miR-205-5p*	4
ERBB2	*hsa-miR-375, hsa-miR-205-5p, hsa-miR-193a-3p*	3
MET	*hsa-miR-1-3p, hsa-miR-31-5p, hsa-miR-410-3p*	3
ABCC1	*hsa-miR-1291, hsa-miR-1-3p*	2
ARID1A	*hsa-miR-1-3p, hsa-miR-31-5p*	2
BDNF	*hsa-miR-204-5p, hsa-miR-1-3p*	2
CDC42	*hsa-miR-204-5p, hsa-miR-375*	2
DDX5	*hsa-miR-1-3p, hsa-miR-205-5p*	2
ETS1	*hsa-miR-1-3p, hsa-miR-31-5p*	2
GRB2	*hsa-miR-200a-3p, hsa-miR-376c-3p*	2
IGF1R	*hsa-miR-375, hsa-miR-376c-3p*	2
IL11	*hsa-miR-204-5p, hsa-miR-379-5p*	2
ITGA5	*hsa-miR-205-5p, hsa-miR-31-5p*	2
LRP1	*hsa-miR-1-3p, hsa-miR-205-5p*	2
MTDH	*hsa-miR-136-5p, hsa-miR-375*	2
PIK3CA	*hsa-miR-375, hsa-miR-1-3p*	2
PRKCE	*hsa-miR-1-3p, hsa-miR-31-5p*	2
PTEN	*hsa-miR-200a-3p, hsa-miR-205-5p*	2
RHOA	*hsa-miR-375, hsa-miR-31-5p*	2
SIGMAR1	*hsa-miR-1-3p, hsa-miR-205-5p*	2
SMAD4	*hsa-miR-204-5p, hsa-miR-205-5p*	2
SNAI2	*hsa-miR-204-5p, hsa-miR-1-3p*	2
SOX9	*hsa-miR-1247-5p, hsa-miR-1-3p*	2
SP1	*hsa-miR-375, hsa-miR-1-3p*	2
SRC	*hsa-miR-205-5p, hsa-miR-31-5p*	2
SRF	*hsa-miR-200a-3p, hsa-miR-1-3p*	2
TGFBR1	*hsa-miR-204-5p, hsa-miR-376c-3p*	2
TP53	*hsa-miR-200a-3p, hsa-miR-375*	2
WASF3	*hsa-miR-200a-3p, hsa-miR-31-5p*	2
YAP1	*hsa-miR-200a-3p, hsa-miR-375*	2
YWHAZ	*hsa-miR-375, hsa-miR-1-3p*	2
YY1	*hsa-miR-205-5p, hsa-miR-31-5p*	2
ZEB1	*hsa-miR-200a-3p, hsa-miR-205-5p*	2
ZEB2	*hsa-miR-200a-3p, hsa-miR-205-5p*	2

[a]*Target gene with strong evidence only.*
LP, leprosy patients; HS, health subjects.

TABLE 5 | List of the genes that were potentially targeted by two or more differentially expressed miRNAs among the 17 upregulated miRNAs in LP vs. HS skin biopsies.

Target gene[a]	MicroRNA	Number of miRNAs
MYC	*hsa-miR-34a-5p, hsa-miR-155-5p, hsa-miR-744-5p*	3
RHOA	*hsa-miR-340-5p, hsa-miR-185-5p, hsa-miR-155-5p*	3
AR	*hsa-miR-34a-5p, hsa-miR-185-5p*	2
BACH1	*hsa-miR-155-5p, hsa-miR-196a-5p*	2
BMP7	*hsa-miR-34a-5p, hsa-miR-342-3p*	2
CCND1	*hsa-miR-34a-5p, hsa-miR-155-5p*	2
CDK6	*hsa-miR-34a-5p, hsa-miR-185-5p*	2
CEBPB	*hsa-miR-34a-5p, hsa-miR-155-5p*	2
CSF1R	*hsa-miR-34a-5p, hsa-miR-155-5p*	2
DNMT1	*hsa-miR-185-5p, hsa-miR-342-3p*	2
FADD	*hsa-miR-155-5p, hsa-miR-146a-5p*	2
FAS	*hsa-miR-196b-5p, hsa-miR-146a-5p*	2
HMGA1	*hsa-miR-185-5p, hsa-miR-196a-5p*	2
HNF4A	*hsa-miR-34a-5p, hsa-miR-766-3p*	2
HOXB7	*hsa-miR-196b-5p, hsa-miR-196a-5p*	2
HOXB8	*hsa-miR-196b-5p, hsa-miR-196a-5p*	2
HOXC8	*hsa-miR-196b-5p, hsa-miR-196a-5p*	2
ICAM1	*hsa-miR-155-5p, hsa-miR-146a-5p*	2
IL8	*hsa-miR-155-5p, hsa-miR-146a-5p*	2
KRAS	*hsa-miR-340-5p, hsa-miR-155-5p*	2
L1CAM	*hsa-miR-34a-5p, hsa-miR-146a-5p*	2
MECP2	*hsa-miR-340-5p, hsa-miR-155-5p*	2
MEIS1	*hsa-miR-155-5p, hsa-miR-196b-5p*	2
MET	*hsa-miR-340-5p, hsa-miR-34a-5p*	2
MTA2	*hsa-miR-34a-5p, hsa-miR-146a-5p*	2
MYB	*hsa-miR-34a-5p, hsa-miR-155-5p*	2
RAC1	*hsa-miR-155-5p, hsa-miR-146a-5p*	2
RDX	*hsa-miR-196b-5p, hsa-miR-196a-5p*	2
ROCK1	*hsa-miR-340-5p, hsa-miR-146a-5p*	2
SMAD2	*hsa-miR-155-5p, hsa-miR-146a-5p*	2
SMAD4	*hsa-miR-155-5p, hsa-miR-146a-5p*	2
SOX2	*hsa-miR-340-5p, hsa-miR-34a-5p*	2
SPI1	*hsa-miR-34a-5p, hsa-miR-155-5p*	2
SREBF1	*hsa-miR-185-5p, hsa-miR-342-3p*	2
SREBF2	*hsa-miR-185-5p, hsa-miR-342-3p*	2
VEGFA	*hsa-miR-34a-5p, hsa-miR-185-5p*	2

[a]*Target gene with strong evidence only.*
LP, leprosy patients; HS, health subjects.

active hormone 1,25 dihydroxyvitamin D (1,25D) and links to the vitamin D receptor, resulting in the expression of antimicrobial peptides (9, 41). *IL1R1* and *IL1A*, and *TLR2* gene were found to have upregulated miRNAs for all LP, which are also involved in pathways culminating in antimicrobial activity (9). Although *has-mir-21* seems to be critical for the control of the *TLR4*, *IL15R*, *IL1R1*, and *IL1A* gene expression in a cell culture-based systems, using our human approach, other miRNAs seemed to be more relevant; however, additional studies are necessary for validation.

TNFAIP3 (A20), which is produced by macrophages infected by *M. tuberculosis*, has been recently described as a new NFκB blocker (42). We found that *hsa-miR-125b* is downregulated only in LL, and *hsa-let-7f-5p* is downregulated in LP (**Figure 7**). Both miRNAs inhibit *A20* gene expression, leading to an increase in NFκB production. According to our LP miRNA profile, *hsa-miR-125b* and *hsa-let-7f-5p* expression are decreased and therefore do not block *A20* gene, resulting in NFκB abrogation. This phenomenon may drive macrophages toward a M2 profile, with more *TGFB1* (43), *IL6* (44), and *IL10* gene (45) production (all with downregulated miRNAs, **Figure 7**) that may stimulate Th2 cells to produce more IL-4 (44). Upon ligation, the IL-4 receptor activates STAT6, stimulating the transcription of *miR-1301*, *miR-342*, and *miR155*, which supports M2 by activating *BCL2* gene and promotes Th2 activation (46). All three miRNAs were found to be upregulated in LL patients, confirming their importance in driving LP toward a Th2 profile.

In addition, *IL10 gene*, with miRNA downregulated only in LL patients, *IL13* gene miRNAs were downregulated in the blood of all LP. Together with lesional *IL11*, *ETS1* and *CDC42*, *IL10*, and *IL13* gene induce the differentiation of B cells from plasma cells (47–50). The interaction of CD40 with CD40L results in IL-12 production, which is impaired in LL patients (51). In addition to ICAM1, which has been demonstrated to be inhibited in LL (52), *CD40L gene* miRNAs were upregulated in all LP, and the control of *IL2*, *IL1A*, *IL1R*, *TLR4*, and *NFKB1* gene miRNAs were upregulated in LL (**Figure 7**), These phenomena may lead to increased *IL4*, *IL10*, and *TGFB1* production, blocking *IFNG gene* expression (53–56) and resulting in the impaired CIR observed in LL patients.

TABLE 6 | List of the genes that were potentially targeted by two or more differentially expressed miRNAs among the 34 specific downregulated miRNAs in LL vs. HS skin biopsies.

Target gene[a]	MicroRNA	Number of miRNAs
BCL2	*hsa-miR-375, hsa-miR-205-5p, hsa-miR-429, hsa-miR-182-5p, hsa-miR-195-5p, hsa-miR-708-5p, hsa-miR-224-5p*	7
CDC42	*hsa-miR-375, hsa-miR-133a-3p, hsa-miR-195-5p, hsa-miR-224-5p*	4
IGF1R	*hsa-miR-375, hsa-miR-376c-3p, hsa-miR-133a-3p, hsa-miR-125b-2-3p*	4
PTEN	*hsa-miR-200a-3p, hsa-miR-205-5p, hsa-miR-429, hsa-miR-182-5p*	4
ZEB2	*hsa-miR-200a-3p, hsa-miR-205-5p, hsa-miR-429, hsa-miR-708-5p*	4
ERBB2	*hsa-miR-375, hsa-miR-205-5p, hsa-miR-193a-3p*	3
EZH2	*hsa-miR-200a-3p, hsa-miR-429, hsa-miR-708-5p*	3
MYB	*hsa-miR-200a-3p, hsa-miR-429, hsa-miR-195-5p*	3
SMAD4	*hsa-miR-205-5p, hsa-miR-182-5p, hsa-miR-224-5p*	3
SP1	*hsa-miR-375, hsa-miR-133a-3p, hsa-miR-149-5p*	3
VEGFA	*hsa-miR-205-5p, hsa-miR-133a-3p, hsa-miR-195-5p*	3
ZEB1	*hsa-miR-200a-3p, hsa-miR-205-5p, hsa-miR-429*	3
AKT1	*hsa-miR-199a-3p, hsa-miR-708-5p*	2
BAP1	*hsa-miR-200a-3p, hsa-miR-429*	2
BIRC5	*hsa-miR-195-5p, hsa-miR-708-5p*	2
CAB39	*hsa-miR-375, hsa-miR-195-5p*	2
CCND1	*hsa-miR-195-5p, hsa-miR-708-5p*	2
CCND2	*hsa-miR-154-5p, hsa-miR-182-5p*	2
CD44	*hsa-miR-199a-3p, hsa-miR-708-5p*	2
CDK6	*hsa-miR-200a-3p, hsa-miR-195-5p*	2
CDKN1A	*hsa-miR-182-5p, hsa-miR-654-3p*	2
DICER1	*hsa-miR-154-5p, hsa-miR-195-5p*	2
DNMT1	*hsa-miR-200a-3p, hsa-miR-429*	2
ELMO2	*hsa-miR-200a-3p, hsa-miR-429*	2
ERBB2IP	*hsa-miR-200a-3p, hsa-miR-429*	2
GRB2	*hsa-miR-200a-3p, hsa-miR-376c-3p*	2
HOXB5	*hsa-miR-200a-3p, hsa-miR-429*	2
KLF11	*hsa-miR-200a-3p, hsa-miR-429*	2
KLHL20	*hsa-miR-200a-3p, hsa-miR-429*	2
MAPK14	*hsa-miR-200a-3p, hsa-miR-199a-3p*	2
MCL1	*hsa-miR-193a-3p, hsa-miR-133a-3p*	2
MET	*hsa-miR-410-3p, hsa-miR-199a-3p*	2
PARP1	*hsa-miR-375, hsa-miR-708-5p*	2
PHLPP1	*hsa-miR-375, hsa-miR-224-5p*	2
PTPRD	*hsa-miR-200a-3p, hsa-miR-429*	2
RASSF2	*hsa-miR-200a-3p, hsa-miR-429*	2
RIN2	*hsa-miR-200a-3p, hsa-miR-429*	2
SEPT7	*hsa-miR-200a-3p, hsa-miR-429*	2
SHC1	*hsa-miR-200a-3p, hsa-miR-429*	2
TCF7L1	*hsa-miR-200a-3p, hsa-miR-429*	2
TP53	*hsa-miR-200a-3p, hsa-miR-375*	2
VAC14	*hsa-miR-200a-3p, hsa-miR-429*	2
WASF3	*hsa-miR-200a-3p, hsa-miR-429*	2
WDR37	*hsa-miR-200a-3p, hsa-miR-429*	2
YAP1	*hsa-miR-200a-3p, hsa-miR-375*	2
ZFPM2	*hsa-miR-200a-3p, hsa-miR-429*	2

[a]*Target gene with strong evidence only.*
LL, lepromatous leprosy; HS, health subjects.

TABLE 7 | List of genes potentially targeted by two or more differentially expressed miRNAs among the 14 specific upregulated miRNAs LL vs. HS skin biopsies.

Target gene[a]	MicroRNA	Number of miRNAs
CCND1	*hsa-miR-34a-5p, hsa-miR-155-5p*	3
CDK6	*hsa-miR-34a-5p, hsa-miR-185-5p, hsa-miR-191-5p*	3
CEBPB	*hsa-miR-34a-5p, hsa-miR-155-5p, hsa-miR-191-5p*	3
RHOA	*hsa-miR-340-5p, hsa-miR-185-5p, hsa-miR-155-5p*	3
AR	*hsa-miR-34a-5p, hsa-miR-185-5p*	2
BMP7	*hsa-miR-34a-5p, hsa-miR-342-3p*	2
CSF1R	*hsa-miR-34a-5p, hsa-miR-155-5p*	2
DNMT1	*hsa-miR-185-5p, hsa-miR-342-3p*	2
E2F1	*hsa-miR-34a-5p, hsa-miR-223-3p*	2
FOXO3	*hsa-miR-155-5p, hsa-miR-223-3p*	2
IGF1R	*hsa-miR-185-5p, hsa-miR-223-3p*	2
KIT	*hsa-miR-34a-5p, hsa-miR-146b-5p*	2
KRAS	*hsa-miR-340-5p, hsa-miR-155-5p*	2
MDM4	*hsa-miR-34a-5p, hsa-miR-191-5p*	2
MECP2	*hsa-miR-340-5p, hsa-miR-155-5p*	2
MET	*hsa-miR-340-5p, hsa-miR-34a-5p*	2
MMP16	*hsa-miR-155-5p, hsa-miR-146b-5p*	2
MYB	*hsa-miR-34a-5p, hsa-miR-155-5p*	2
MYC	*hsa-miR-34a-5p, hsa-miR-155-5p*	2
NOTCH1	*hsa-miR-34a-5p, hsa-miR-326*	2
NOTCH2	*hsa-miR-34a-5p, hsa-miR-326*	2
PDGFRA	*hsa-miR-34a-5p, hsa-miR-146b-5p*	2
SCARB1	*hsa-miR-185-5p, hsa-miR-223-3p*	2
SOX2	*hsa-miR-340-5p, hsa-miR-34a-5p*	2
SPI1	*hsa-miR-34a-5p, hsa-miR-155-5p*	2
SREBF1	*hsa-miR-185-5p, hsa-miR-342-3p*	2
SREBF2	*hsa-miR-185-5p, hsa-miR-342-3p*	2
VEGFA	*hsa-miR-34a-5p, hsa-miR-185-5p*	2

[a]*Target gene with strong evidence only.*
LL, lepromatous leprosy; HS, health subjects.

MicroRNAs targeting the $CD8^+$ T cell differentiation gene *ZEB2* gene and activation genes *CADM1* and *CRTAM* gene were found to be downregulated in LP patients (**Figure 7**). The adhesion molecule CADM1, which is expressed on dendritic cells (DCs), induces a $CD8^+$ cytotoxic profile upon ligation to CRTAM T-cells, with the release of IL2 and IFNG (57, 58). Although *IFNG* gene miRNAs were downregulated, *IL2 gene* miRNAs were upregulated in LL patients, suggesting a posttranscriptional blocking of *IL2* released from CD8+ T cells in LP.

Considering Th1, Th2, and Th17, we detected miRNAs controlling all three axes in LP. On the Th1 axis, although *IL12A* and *IFNG* gene miRNAs were downregulated in blood, *IL2 gene*, a key cytokine for Th1 proliferation (59), together with *IL1A*, *IL1R1*, *IL15R*, *TLR4*, and *NFKB gene*, were all found to be regulated by miRNAs in the lesional skin of LL patients. For Th2, *IL6*, and *IL10 gene*, together with the miRNAs *hsa-miR-125b*, *miR-1301*, *miR-342*, and *miR-155*, were all regulated by miRNAs, maintaining a suppressive profile in LL patients. Th17 differentiation is influenced by different factors. We found that *SHC1 gene*, which activates *STAT3 gene*, and *IL6* gene that can induce Th17 differentiation in association with *TGFB1gene* (60), both had downregulated miRNAs in LL patients, together with upregulation of the miRNA *hsa-miR-326*, which was described as a Th17 inductor. Although Th17 is known to produce IL8 (61), we found miRNAs controlling the expression of this chemokine in lesions of LL patients (**Figure 7**), corroborating the absence of this chemokine in polymorphonuclear cells of LL patient unstimulated blood (62).

TABLE 8 | List of genes that were potentially targeted by two or more differentially expressed miRNAs among the nine downregulated miRNAs in LP vs. HS blood.

Target gene[a]	MicroRNA	Number of miRNAs
CCND1	*hsa-let-7f-5p, hsa-miR-15a-5p, hsa-miR-20a-5p, hsa-miR-106b-5p, hsa-miR-16-5p*	5
BCL2	*hsa-miR-126-3p, hsa-miR-15a-5p, hsa-miR-20a-5p, hsa-miR-16-5p*	4
PURA	*hsa-miR-15a-5p, hsa-miR-16-5p, hsa-miR-106b-5p, hsa-miR-20a-5p*	4
APP	*hsa-miR-15a-5p, hsa-miR-20a-5p, hsa-miR-106b-5p*	3
CCND2	*hsa-miR-15a-5p, hsa-miR-20a-5p, hsa-miR-106b-5p*	3
CCNE1	*hsa-miR-15a-5p, hsa-miR-26b-5p, hsa-miR-16-5p*	3
PTEN	*hsa-miR-20a-5p, hsa-miR-106b-5p, hsa-miR-26b-5p*	3
RB1	*hsa-miR-20a-5p, hsa-miR-106b-5p, hsa-miR-26b-5p*	3
WEE1	*hsa-miR-20a-5p, hsa-miR-16-5p, hsa-miR-106b-5p*	3
ADAM9	*hsa-miR-126-3p, hsa-miR-126-5p*	2
AKT3	*hsa-miR-15a-5p, hsa-miR-16-5p*	2
BMI1	*hsa-miR-15a-5p, hsa-miR-16-5p*	2
BRCA1	*hsa-miR-15a-5p, hsa-miR-16-5p*	2
CADM1	*hsa-miR-15a-5p, hsa-miR-16-5p*	2
CDK6	*hsa-miR-26b-5p, hsa-miR-16-5p*	2
CDKN1A	*hsa-miR-20a-5p, hsa-miR-106b-5p*	2
CHORDC1	*hsa-miR-26b-5p, hsa-miR-16-5p*	2
CHUK	*hsa-miR-15a-5p, hsa-miR-16-5p*	2
E2F1	*hsa-miR-20a-5p, hsa-miR-106b-5p*	2
E2F3	*hsa-miR-106b-5p, hsa-miR-20a-5p*	2
HMGA1	*hsa-miR-15a-5p, hsa-miR-16-5p*	2
HMGA2	*hsa-miR-16-5p, hsa-miR-15a-5p*	2
IFNG	*hsa-miR-16-5p, hsa-miR-15a-5p*	2
IGF1R	*hsa-miR-16-5p, hsa-miR-26b-5p*	2
PTGS2	*hsa-miR-26b-5p, hsa-miR-16-5p*	2
RBL1	*hsa-miR-106b-5p, hsa-miR-20a-5p*	2
RBL2	*hsa-miR-20a-5p, hsa-miR-106b-5p*	2
STAT3	*hsa-miR-20a-5p, hsa-miR-106b-5p*	2
SMAD7	*hsa-miR-20a-5p, hsa-miR-106b-5p*	2
VEGFA	*hsa-miR-106b-5p, hsa-miR-16-5p*	2

[a]*Target gene with strong evidence only.*
LP, leprosy patients; HS, health subjects.

Apoptosis

The role of apoptosis during *M. leprae* infection is not clear, and different research settings have demonstrated both anti- (24, 63) and proapoptotic (64, 65) features, in addition to possible differences depending on the clinical form of leprosy (66). BCL2 has been shown to be highly expressed in LL patients (66), and *BCL2* and *MCL1 gene* are induced by *M. leprae* on monocytes (63), while CASP8 activity in LL is decreased (67). We found several miRNAs controlling apoptosis pathways in LP. In addition to the downregulation of all miRNAs acting directly on the antiapoptotic gene *BCL2* and its family member *MCL1*, especially in LL lesions, the proapoptotic *gene CASP8* inducers *MYC*, *FAS,* and *FADD gene* were found to have upregulated miRNAs in LP, while the *FASLG* inhibitor *GRB2* (68) presented downregulated miRNAs in LL patients (**Figure 8**).

AKT1 and PIK3CA kinases appeared to be central molecules involved in the apoptosis pathway related to *M. leprae*. In contrast, *PIK3CA* miRNAs were downregulated in all forms of leprosy in comparison to HS, and *AKT1* miRNAs was more prominent in LL patient lesions (**Figure 8**). Interestingly, PHLPP1, a serine-threonine family member that has never described in leprosy and that controls AKT1 (69), was found to have downregulated miRNAs in LL lesions, indicating that *PHLPP1 gene* may have a role in apoptosis control in LP. Furthermore, AKT1 blocks the expression of the proapoptotic *genes YAP1* and *FOX03*, while stimulate the antiapoptotic *gene MDM4*. YAP1 has a proapoptotic function after DNA damage of tumor cells (70), and FOXO3 blocks BCL2 (71). In addition to being inhibited by AKT1, *FOXO3 gene* was found to have upregulated miRNAs in all LP cases (**Figure 8**). Upon *FOXO3* blockade, *RBL1/2* are transcribed and indirectly block *TP53* expression by blocking *E2F1* (72). MDM4, P53 regulator is also stimulated by AKT1. miRNAs found in our miRNome regulate *FOXO3* and *E2F1* in all forms of leprosy, while miRNAs for *YAP1*, *MDM4*, and *TP53* were found exclusively in LL. Taken together, these findings show that the influence of LP miRNAs of those pathways in leprosy may result in an antiapoptotic profile.

Considering the miRNA profile controlling cell receptors, in addition to *FAS gene*, we found five other genes related to apoptosis control by miRNA in *M. leprae* infection: *TGFBR1*, involved in TGF-β signaling, a cytokine known to induce tolerance (73, 74) with a suppressive potential of Tregs in LL patients (75); *ITGA5*, an α-integrin linked to *M. tuberculosis* infection of macrophages (22); *VEGFR2*, known to participate in *M. tuberculosis* dissemination by triggering angiogenesis (76), while its ligand, *VEGFA*, has been demonstrated to be expressed in leprosy lesions (77); *PDGFRA*, which was shown to be upregulated in SC 27 days after *M. leprae* infection (78), and its ligand *PDGF,* a potential marker for erythema nodosum leprosum (79); and *IGF1R*, the receptor of *IGF1*, which inhibits macrophage and SC apoptosis upon *M. leprae* infection, in turn stimulating the production and secretion of *IGF1* (15).

In contrast to downregulated miRNAs controlling *PIK3CA*, *AKT1*, *BCL2*, and *MCL1*, miRNAs controlling the expression of the proapoptotic genes *MYC*, *E2F1*, and *FOXO3* were all found to be upregulated in lesional tissue. Additionally, miRNAs for *FAS* and *FADD*, members of the *CASP8* proapoptotic pathway, were also found to be upregulated (**Figure 8**). Taken together, concerning miRNA regulation, our data suggest an antiapoptotic profile for leprosy in general, driven by *BCL2*, *MCL1*, and *CASP8*.

SCs, Demyelination, and EMT

Demyelination is a pathologic process that destroys the myelin sheath and involves multiple factors, including inflammatory responses or infections (80). LP demyelination is the ultimate consequence of leprosy neuritis, and LL patients exhibit myelinated and non-myelinated SC infected by *M. leprae* (81).

Upon invasion, *M. leprae* stimulates *ERBB2* independently of *ERBB3* (82), resulting in *ERK1/2* activation, which leads to peripheral nerves demyelination (83). *ERBB2* miRNAs were downregulated in LL patients (**Figure 9**), indicating a possible role for the *SOX2* and *JUN* pathway in demyelination and EMT (78). However, *SOX2* miRNAs were found to be upregulated in all LP, while *ZEB1/2* miRNAs were downregulated in LL patients (**Figure 9**), indicating that ZEB1/2 may regulate SOX2 (84)

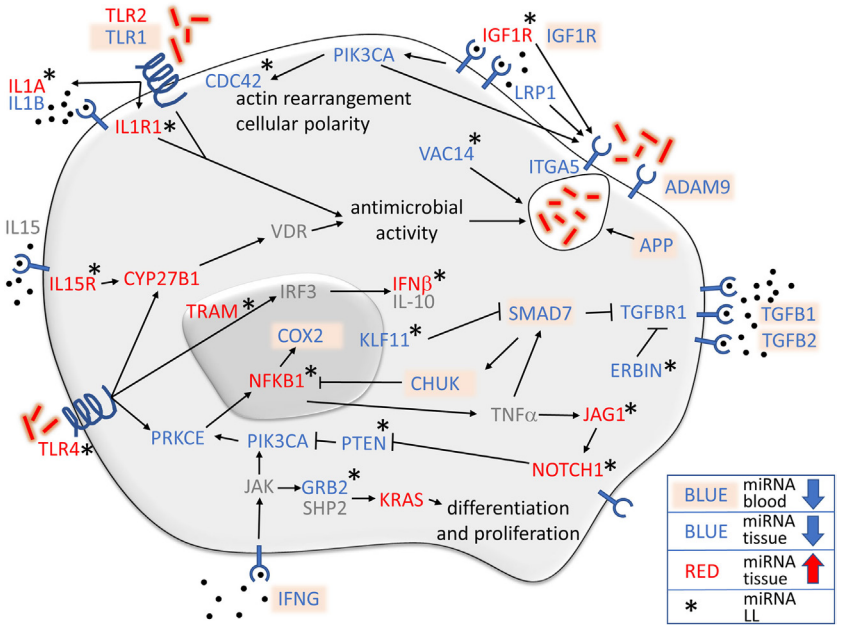

FIGURE 6 | Overview of the relationship of monocyte immunology to microRNAs (miRNAs) regulating genes in leprosy lesions and blood. Most of the genes controlled by the miRNAs were found in lesional tissue, shown either in blue (downregulated miRNAs) or in red (upregulated miRNAs). Blood miRNAs are marked in light orange, and those that were not found are shown in gray. Asterisks indicate genes with miRNAs that were differentially expressed only when lepromatous (LL) was compared with healthy subject. Upregulated miRNAs controlling *IL1A*, *IL1R1*, *IGF1R*, *NOTCH1*, *JAG1*, *IL15R*, *TLR4*, *CYP27B1*, *TRAM*, *NFKB1*, and *IFNB*, and downregulated miRNAs regulating the expression of *CDC42*, *VAC14*, *ERBIN*, *KLF11*, *PTEN*, and *GRB2*, all in LL patients, demonstrated an immunosuppressive phenotype controlling actin rearrangement and cellular polarity, including phagosome formation, vitamin D antimicrobial activity, cellular differentiation and proliferation, and diverse pathways to stimulate TGF-β-related genes while suppressing NFκB inflammatory functions.

expression to inhibit demyelination and EMT. Although *ZEB1* expression increases after *M. leprae* infection or after TGF-β stimulation (85), while ZEB2 is essential after nerve injury by allowing remyelination and functional recovery (86), they may be regulated by ERBIN, which blocks the TGFBR1 pathway (87), and the downregulated miRNAs were observed only in LL patients (**Figure 9**).

Schwann cell lesions induce the antiapoptotic molecule *AKT1* and the mitogen *MET*, both of which are regulated by miRNAs in LL patients. AKT1 signaling, one of the most important pathways involved in myelination (88), may be activated in injured peripheral nerves (89). We found that miRNAs for *AKT1* were downregulated in LL patients, indicating a possible role for *AKT1* in LP myelination (**Figure 9**). In parallel, MET, an important inducer of neural injury (90) and the *KRAS*, *RAF1*, and *MAPK14 genes* involved in EMT pathways (91) were found to have miRNAs in LL patient lesions. In contrast, *MAPK14* and *RAF1 genes* had downregulated miRNAs, and *MET* and *KRAS genes* had miRNAs upregulated, indicating a strict control of the first steps of the pathway. *MET* and *KRAS* also regulate cell motility through RAC1 and CDC42, which are important for actin rearrangement and cellular polarity (92).

Very few works have described the effects of *HOX* and *SNAI genes* on leprosy pathophysiology. It has been demonstrated that upon *M. leprae* infection, SC may switch off differentiation genes, such as *SOX10* and *p75^{ntr}*, while switching on EMT genes, especially the *HOX* family (78) and *CD44*, which are considered a marker for EMT in SC (78), with downregulated miRNAs in our LP skin lesion samples. Our study did not find miRNAs regulating *p75^{ntr}*, which is compatible with the switching off demonstrated in the previous work; however, we detected upregulated miRNAs for *SOX10* only in LL, downregulated miRNAs for *SNAI2* and *HOXB5*, with the latter only in LL patient lesions, and upregulated miRNAs for *HOXB7*, *HOXB8*, and *HOXC8* in all LP (**Figure 9**). Interestingly, the only genes that were detected in the earlier *in vitro* work and that for which we also detected miRNAs were *SNAI2* and *HOXB8*. miRNAs for *HOXB5*, *HOXB7*, and *HOXC8* were found in our work, but their expression was not detected previously.

HOXB5 gene is a marker for long-term hematopoietic stem cells (93) and it affects the differentiation of the vascular endothelium development from precursor cells (94). It is known that endothelial cells are important for the entry and maintenance of *M. leprae* in nerves (95) and that vasculitis may be observed in leprosy reactions, with endothelial proliferation in Lucio's phenomena (1). *HOXB7* is associated with EMT in breast cancer cells *via* the canonical TGF-β pathway (96), while *HOXC8* mutant mice present motoneuron abnormalities with analog molecular defects compared with mutant mice for retinaldehyde dehydrogenase 2 synthesizing enzyme (97), which is responsible for retinoic acid synthesis, with atrophy of the distal projections of the ramus profundus of the radialis nerve that supply the extensor muscles of the forepaw, resulting in forepaw neuromuscular defects. Vitamin A levels in LP have been shown

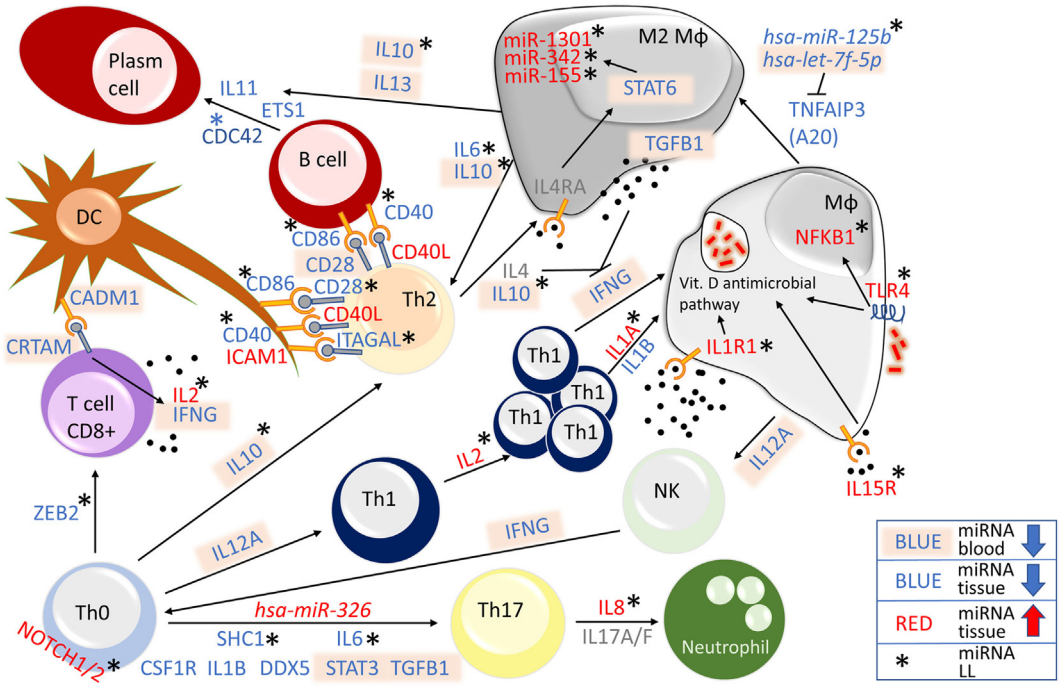

FIGURE 7 | Overview of microRNA (miRNA)-regulated genes related to the immune system in leprosy lesions and blood. Genes regulated by differentially expressed miRNAs were found in blood (all downregulated miRNAs are shown in blue with text marked in light orange) or lesional tissue (red upregulated, blue downregulated). Differentially expressed genes regulating miRNAs (shown in gray) were not found. Lepromatous (LL)—asterisk—upregulated miRNAs for *NOTCH1* and *NOTCH2*, *IL2*, *IL8*, *IL1A*, *IL1R1*, *IL15R*, *TLR4*, and *NFKB1*, and downregulated miRNAs for *ZEB2*, *SHC1*, *IL6*, *IL10*, *CDC42*, *ITAGAL*, *CD40*, and *CD86*, all in LL patients, indicate an immunosuppressive phenotype. Additionally, corroboration of the expression of *hsa-miR-326*, which was upregulated in leprosy patient (LP), *hsa-let-7f-5p*, which was downregulated in LP, *hsa-miR-1301*, *hsa-miR-342*, and *hsa-miR-155*, all of which were upregulated in LL, and *hsa-miR-125b*, which was downregulated in LL, drove the immune system of LL patients toward an immunosuppressive stage.

to be low in comparison to healthy controls, and much lower in LL patients (98).

SNAI2 and *HOXB8* have been demonstrated to be upregulated in SC infected with *M. leprae in vitro* (78). SNAI2 is activated by the canonical TGF-β pathway (99), inhibiting CDH1 (E-cadherin) and resulting in cellular EMT (100). We found that upregulated miRNAs controlled *SMAD2* and *SMAD3* expression in LP. *SMAD3* miRNAs were significantly upregulated only in LL patients, possibly participating in the control of SC-EMT (**Figure 9**). *HOXB8* null mutants show altered sensory responses in mice, probably due to a smaller number of neurons and neural disorganization (101), indicating that *HOXB8* expression levels may be related to sensory alterations in LP.

Loss of Sensation and Neuropathic Pain in Leprosy

ROCK1 is a Rho-associated protein kinase that is present in different signaling pathways in neurons (102), is known to regulate SC myelination (103), and may be activated by *RHOA gene* (104), for which we found downregulated miRNAs in LL lesions. *ROCK1 gene* stimulated by TGFBR1 induced EMT of SC *via* the MAPK14 pathway, which leads to SOX9 activation and SC EMT associated with the blockade of SOX10 (**Figure 9**), a recognized inducer of cell differentiation (105). We observed upregulated miRNAs for *SOX10 gene* in LL lesions, indicating that it may be expressed in LL patients. Myelin-associated inhibitors upon ligation with $p75^{ntr}$ activate *RHOA*, resulting in demyelination through *ROCK1 genes* (106), while *ROCK1* stimulated by *BDNF* through $p75^{ntr}$ and *RHOA* may lead to inflammation and pain (107).

LPAR1 signaling is required to initiate neuropathic pain after nerve injury. Mice lacking *LPAR1 gene* do not present signs of neuropathic pain, and inhibition of *RHOA* and *ROCK1* also prevent neuropathic pain (108). We found that miRNAs for *LPAR1* were downregulated in LL patients in both blood and lesions, indicating that the receptor may be available for ligation in LP. *RHOA* miRNAs were also downregulated in LL patients, while *ROCK1* was upregulated in all LP, indicating an attempt to control EMT, demyelination and pain.

The miRNA *hsa-miR-1291* was the only differentially expressed miRNA in both skin tissue and blood samples. It was predicted to regulate the aquaporin-1 (*AQP1*) gene (109), which influences the hydration, elasticity and glycerol permeability of skin (110). In LL lesions, frequent overexpression of lipid metabolism genes (111) indicates that *M. leprae* uses host lipids for growth and virulence. Therefore, downregulation of *hsa-miR-1291* in skin lesions could modulate *AQP1* expression and increase glycerol permeability to promote fatty acid metabolism. Altered *AQP1*

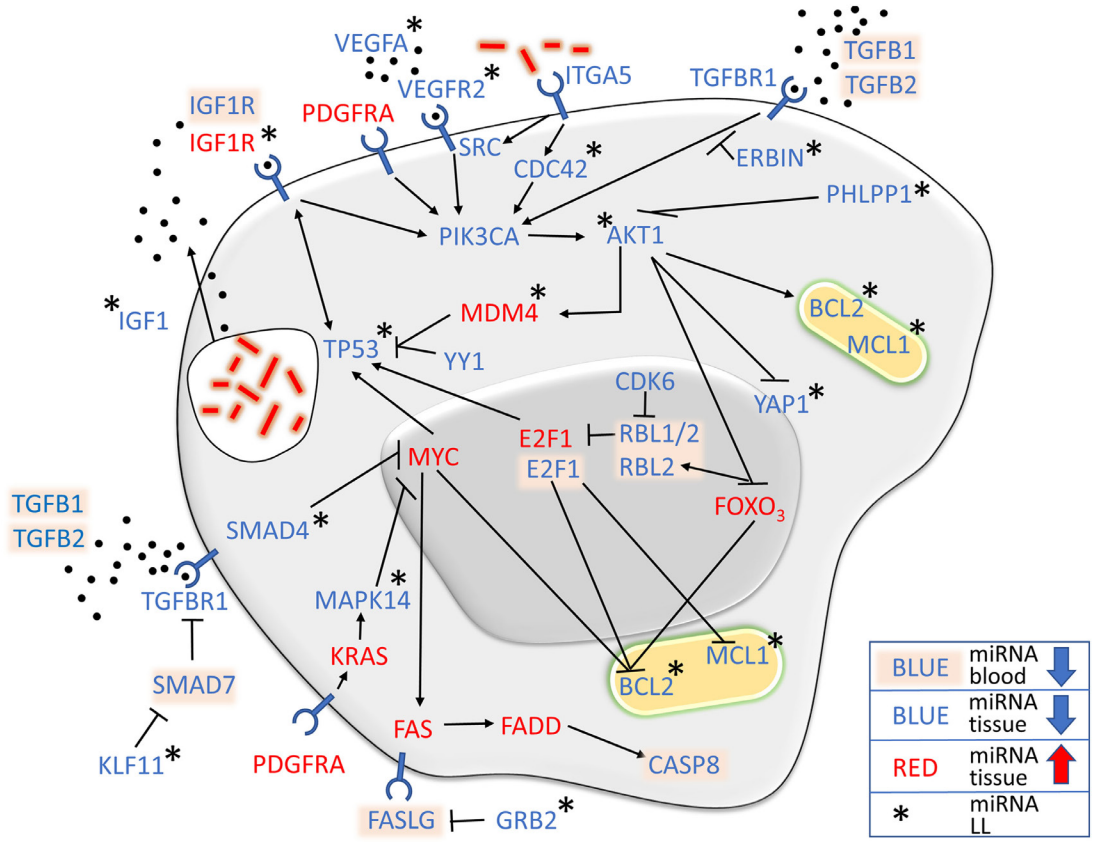

FIGURE 8 | MicroRNA (miRNA) expression strictly controls apoptosis-related pathways. In addition to the antiapoptotic genes *BCL2* and *MCL1*, which were found predominantly in lepromatous (LL) lesions, other genes had downregulated miRNAs in pathways that stimulate their expression, such as *PIK3CA* and *AKT1*, while their suppressors, such as *MYC* and *FOXO3*, had upregulated miRNAs, suggesting an antiapoptotic profile of leprosy patient, especially LL.

expression may improve our comprehension of some well-known clinical issues related to leprosy, such as the dryness found in skin lesions. Moreover, *MEF2C*, a transcription factor for *AQP1* (112), was found to have upregulated miRNAs in the skin lesions of LL patients, which could result in an absence of *AQP1* transcription in LL patients, contributing to loss of sensation.

Aquaporins may be key molecules in leprosy pathophysiology. *AQP1* knockout mice have impaired pain sensation (113), and human trigeminal neurons that mediate head nociception and innervate the oral mucosa express *AQP1*, indicating an involvement in sensory transduction (114). Peripheral nerve system expression of *AQP1* has been seldom investigated, but it has been shown in the sciatic nerve (115) and in Ruffini mechanoreceptors (116). *AQP4* is expressed in the olfactory epithelium (117) and in retinal glia (118), and it is the target of anti-AQP4 antibodies in autoimmune neuromyelitis optica (119). Loss of sensation is the hallmark of leprosy, but there is no definite mechanism explaining this phenomenon.

AQP1 participates in the mechanism of thermic and chemical pain, likely controlling neuronal ionic nociceptive homeostasis (113). Membrane depolarization activates *ATP1A1*, which regulates sodium potassium channels, and *TRPV1*, which is responsible for calcium influx into the cell. Calcium acts on calcitonin gene-related peptide (CGRP), which together with substance P (SP) results in pain (113). In addition to CGRP, calcium also stimulates *SIGMAR1* (120), for which we detected downregulated miRNAs in LP lesional skin (**Figure 9**). *SIGMAR1* acts as a chaperone for *IP3R* to maintain calcium signaling from the endoplasmic reticulum to mitochondria, and it has been implicated in pain (121). In contrast, *SIGMAR1* agonists potentiate pain, antagonists potentiate analgesia (122), and neuropathic pain was strongly attenuated in *SIGMAR1* knockout mice (123). Interestingly, *AQP1* silencing in tumor cells abrogates the expression of *RHOA* and *TGFB1/2* (124), indicating a possible mechanism to maintain EMT, demyelination, inflammation and pain through *AQP1*, *SIGMAR1*, *RHOA*, and *ROCK1* in leprosy.

Taken together, our data suggest an important role for miRNA expression in leprosy immunophysiopathology, especially the regulation of different parameters of the immune system, apoptosis, SC demyelination, EMT, and neuropathic pain. The epigenetic control of the genes expressed in leprosy lesions and blood by miRNAs may provide new insights into the different facets of leprosy, from *M. leprae*–host cell interactions to new therapeutic targets.

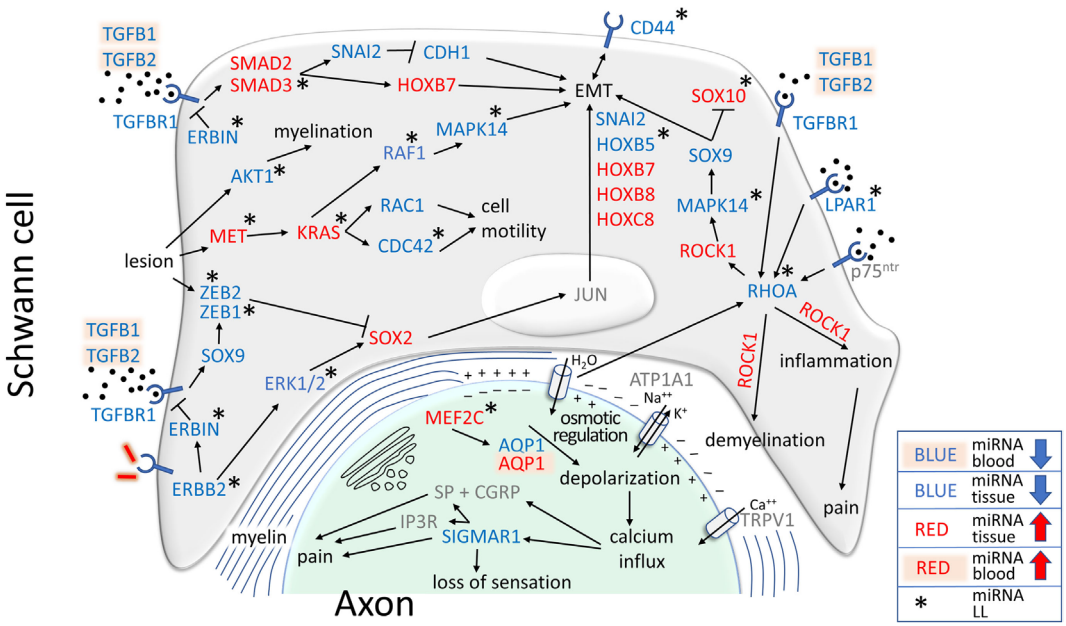

FIGURE 9 | MicroRNAs (miRNAs) related to the epigenetic control of genes involved in demyelination, epithelial–mesenchymal transition (EMT), inflammation, pain, and loss of sensation were differentially expressed in leprosy patient (LP). Different pathways, including canonical and non-canonical pathways of TGF-β, may drive cell toward EMT, with a Schwann cell (SC) undifferentiated phenotype consisting of the absence of $p75^{ntr}$, in gray, *SOX10*, with upregulated miRNAs in lepromatous (LL) and blockade by the *SOX9* pathway, and the presence of *CD44*, which had downregulated miRNAs in LL. *RHOA* miRNAs were downregulated in LL, stimulating *ROCK1* to drive EMT, inflammation and pain, which was controlled by upregulated miRNAs in all LP. *AQP1*, the only gene with upregulated miRNAs in blood in LP and downregulation in lesional skin, is one of the genes responsible for osmotic regulation. In lesions, downregulated miRNA for *AQP1* indicated that *AQP1* might not be expressed in the nerve, which was consistent with the upregulation of miRNAs for the *MEF2C AQP1* transcription factor, possibly resulting in depolarization and calcium influx stimulating *SIGMAR1*, which also had downregulated miRNAs. The low expression level of *SIGMAR1* may result in loss of sensation, but its overexpression may result in pain. Depolarization may stimulate *RHOA* to maintain a cycle of demyelination, inflammation, loss of sensation, and pain.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Brazilian National Ethics Committee (CONEP) guidelines, approved by Pará Federal University Ethics Committee number CAAE 26765414.0.0000.0018, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Pará Federal University Ethics Committee.

AUTHOR CONTRIBUTIONS

CS, PP, JB, MS, SS, and AR designed research; CS, RB, AG, AM, and MF enrolled patients, performed, and registered clinical diagnosis; CS, PP, TS, AS, FM, AV, SS, and AS performed research; CS, PP, RB, AG, AM, TS, AS, FM, AV, LG, JS, SS, and AS analyzed the data; CS, PP, RB, AG, AM, JS, SS, and AS wrote the article; CS, PP, RB, AG, AM, TS, AS, FM, AV, LG, JB, MS, MF, JS, SS, and AS agree with manuscript results and conclusions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00463/full#supplementary-material.

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REVIEW
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Innate Immune Responses in Leprosy

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Leprosy is an infectious disease that may present different clinical forms depending on host immune response to *Mycobacterium leprae*. Several studies have clarified the role of various T cell populations in leprosy; however, recent evidences suggest that local innate immune mechanisms are key determinants in driving the disease to its different clinical manifestations. Leprosy is an ideal model to study the immunoregulatory role of innate immune molecules and its interaction with nervous system, which can affect homeostasis and contribute to the development of inflammatory episodes during the course of the disease. Macrophages, dendritic cells, neutrophils, and keratinocytes are the major cell populations studied and the comprehension of the complex networking created by cytokine release, lipid and iron metabolism, as well as antimicrobial effector pathways might provide data that will help in the development of new strategies for leprosy management.

Keywords: leprosy, innate immune responses, skin, *Mycobacterium leprae*, autophagy, toll-like receptors, inflammasomes

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TRANSMISSION OF LEPROSY

Leprosy is a chronic granulomatous disease, which affects dermis and peripheral nerves and also can involve the eye, the mucosa of the upper respiratory tract, muscle, bone, and testes, caused by the intracellular pathogen *Mycobacterium leprae* (1, 2).

Early diagnosis of leprosy is a prerequisite for effective therapy and rehabilitation. According to Ridley (3) the earliest lesion in leprosy is an intraepidermal lymphocytic infiltration. Although the transmission pathways of *M. leprae* are not fully understood (4), there are several results that suggest that *M. leprae* is mainly dispersed by the nose, not the skin. The protective mucosal innate mechanism in the respiratory tract may contribute to low infectivity of *M. leprae* after exposition. The release of bacilli by multibacillary patients supports a respiratory transmission (5). Viable bacilli have been found for at least 2 days in discharged nasal secretion (6). The hypothesis of respiratory transmission is validated by studies that demonstrated that adhesins present in *M. leprae* surface, like heparin-binding hemagglutinin and histone-like protein may attach in alveolar and nasal epithelial cells and both cell types are capable of sustaining bacterial survival (7, 8). In addition, a previous study demonstrated that mce1a gene is found in *M. leprae* genome and that mce1a product is associated with *M. leprae* entry into respiratory epithelial cells (9).

HISTOPATHOLOGICAL FEATURES IN LEPROSY

The association of the histopathologic aspects and the immune state of the patient has made it the basis of the all leprosy classification and has helped to understanding the immunologic background of this disease and its transmission.

The histopathology of the nose demonstrates that the majority of all bacilli are present mainly in macrophages, as observed in lepromatous skin and other tissues. Bacilli were also seen inside monocytes, Schwann cells, polymorphs and columnar and goblet cells of the pseudostratified epithelium, secretory gland, and ducts (10).

Ridley and Jopling (11) classification establishes that the disease may present different clinical forms that may vary accordingly to histopathological findings and the immune status of the host. Tuberculoid or paucibacillary leprosy is characterized by cell-mediated immune responses to mycobacterial antigens and low infection whereas lepromatous or multibacillary leprosy is characterized by humoral immune response and high bacillary load. The different degree of cellular immune response to *M. leprae* is responsible for different types of granulomatous reaction. Analysis of skin lesion cells demonstrated that epithelioid cells are usually seen in paucibacillary patients [tuberculoid (TT) and borderline tuberculoid], whereas foamy macrophages are found in multibacillary cases [borderline lepromatous (BL) and lepromatous lepromatous (LL)]. Macrophages may present a granular eosinophilic cytoplasm with large numbers of bacilli in early and active lesions. In older lesions, on the other hand, cells are highly vacuolated and the cytoplasm has a foamy appearance (1). Recent studies have demonstrated that the macrophages in lepromatous skin cells are positive for ADRP, suggesting that their foamy aspect may be derived from lipid bodies accumulation induced by *M. leprae* (12, 13).

Two types of leprosy reactions may occur in leprosy patients. Reversal reaction is an acute inflammatory episode in skin and nerves that occurs because of an increase or emergence of cellular immunity against *M. leprae* antigens in lower or previously non-responder patients and may occur in patients of the whole clinical spectrum, except the tuberculoid, TT form (14). In addition, neuritis is frequently associated with reversal reaction episodes. Erythema nodosum leprosum (ENL) occurs in approximately 50% of patients from lepromatous pole due to a complex interaction between innate and cellular immunity poorly understood. Reversal reaction lesions show activated epithelioid macrophages, organized or not as granuloma (15, 16). The hallmark of ENL is an infiltrate of neutrophils in the profound dermis and hypodermis, frequently accompanied by macrophages (17–20). However, neutrophils are not always present (21–23) and skin fragments collected after 72 h demonstrate the presence of lymphocytes, plasma cells, and mast cells (24).

The pathogenesis of nerve destruction varies accordingly the clinical form of the disease (25); although the understanding of mechanisms associated with nerve damage and regeneration in leprosy-associated neuropathy are not fully understood (26). In the pure neural leprosy, bacilli are rarely detected despite the clinical neurological impairment. In multibacillary cases, which show macrophages in considerable numbers within the nerve, bacilli are in greater numbers, often as large bundles or globi. Ultrastructural analyses demonstrate that BL and LL foamy macrophages and vacuolated Schwann cells contain numerous electrondense structures considered as deteriorated and fragmented *M. leprae*. The dense materials are also found in the cytoplasm of vascular endothelial cells. In lepromatous cells both Schwann and endothelial cells frequently harbor *M. leprae* (25). The nerves are progressively destroyed and replaced by fibrous tissue, in both paucibacillary and multibacillary cases (27).

The peripheral nerve damage in leprosy often results in sensory and motor dysfunctions that lead to permanent deformities and/or disabilities (28). Innate immune and inflammatory genes were modulated by *M. leprae* during early infection (29). Therefore, the understanding of the innate immune pathways in the local of infection is crucial for the development of new strategies to control leprosy and its reactional episodes (**Table 1**).

INNATE IMMUNE CELLS IN LEPROSY

The use of monoclonal antibodies to label specific membrane antigens is one of the most used tools to identify the presence and the frequency of different cell populations in tissue. Several studies demonstrated an enormous diversity in cell phenotypes present in different tissues. The proportions of each cell type amongst the total population of non-lymphoid mononuclear cells are different in the various leprosy infiltrates (58). In addition, the characterization of different cell phenotypes in dermis and epidermis has been shown by many studies (16, 59–61).

Despite the existence of predominant macrophage phenotypes well described in literature, between the polar forms of leprosy, it is widely recognized that some terminologies are simplistic and cells like macrophages may present a broad spectrum of differentiation states, continuously regulated by a myriad of signals from the microenvironment (62, 63). In conjunction of Th1–Th2 dichotomy, macrophages have been classified in M1 and M2. Stimulation with proinflammatory cytokines as interferon (IFN)-γ activate M1 macrophages, characterized by enhanced antimicrobial, inflammatory, and antigen-presenting properties, whereas cytokines like interleukin (IL)-4 and IL-13 activate M2 macrophages, which portray anti-inflammatory actions, being associated with tissue repair and fibrosis (62, 63).

TABLE 1 | Innate immunity-modulating strategies and possible therapeutic targets.

Targets	Therapeutic strategies	Reference
TLR2	Vaccine	(30, 31)
	Vaccine adjuvant	(32)
TLR4	Vaccine	(33, 34)
	Vaccine adjuvant	(35, 36)
	Adjuvant immunotherapy	(37, 38)
TLR9	Vaccine adjuvant	(39, 40)
NOD1	Immunostimulant therapy	(41, 42)
NOD2	Immunotherapy	(43)
	Vaccine adjuvant	(44)
Bcl-2	Induction of apoptosis	(45, 46)
TNF	Inhibition of TNF cytokine effects	(47, 48)
Autophagy	Vaccine	(49)
	Pathogen replication control	(50)
	Restriction of mycobacteria growth	(51–57)

Our previous study has demonstrated that in skin cells from lepromatous patients that developed reversal reaction there is a coexistence of M1 and M2 populations in the midst of the inflammatory environment, together with a wide diversity of DC subsets (15, 64). The hallmark of the reversal reaction has been broadly accepted as the appearance of immature and loose epithelioid granulomas, which differ from the typical mature epithelioid granuloma seen in the TT forms. The epithelioid cell is described as a non-phagocyte of unknown ontogeny with high secretory capacity that could be a differentiation state of skin macrophage populations (65, 66). Facchetti et al. (67) described a cell type they called plasmocytoid monocytes (PM) and suggested, based on ultrastructure and immunohistochemical data, that they are the precursors of the epithelioid cells (68). These cells' phenotypic profile includes DCs and macrophage markers, being identified as a $CD3^-$, $CD11c^-$, $CD14^-$, $CD20^-$, $CD36^+$, $CD56^-$, $CD68^+$, $CD123^+$, $BDCA2^+$ population (69). Since PMs produce high levels of type I IFN and express CD123, they are also thought to be a previous immature state of the plasmocytoid DC (pDC) (67).

Although efforts to identify cell markers and inflammatory mediators *in situ* the immunopathogenesis of leprosy is not fully understand. The high heterogeneity and the existence of mixed cell phenotypes in different timepoints of infection that are influenced by the mediators produced in tissue microenvironment together with the inexistence of antibodies highly specific to clearly differentiate human cells contribute to the difficulty of establish a precise role of each cell type in leprosy immunopathogenesis.

Macrophages

Macrophages have been identified as key players in the pathogenesis of leprosy. It has been demonstrated that during an inflammatory response, bone marrow derived monocytes enter the tissue in large numbers and take part in the defense against the pathogens. In a very elegant study, Kibbie et al. (70) demonstrated that unstimulated endothelial cells trigger monocytes to become M2 macrophages and that IFN-γ activates endothelial cells to induce monocyte to differentiate into M1 macrophages by a mechanism regulated by Jagged 1 (JAG1), a protein localized in the vascular endothelium. It is known that tissue macrophage populations have a mixed embryonic and postnatal bone marrow origin, but the exact mechanisms of differentiation and activation is not understood. There are a lot of evidences that a significant percentage of tissue macrophages are independent from blood monocytes and different phenotypes or functions are the result of different macrophages origin (71). Therefore, it is not possible to differentiate resident-tissue macrophages and recruited monocyte-macrophages once they coexist in a common environment (63).

The heterogeneity of tissue resident macrophages during homeostasis and inflammation shows that macrophages cannot be correctly classified as M1 or M2 when in a specific tissue. Although too simplistic, this nomenclature has been used in order to establish the pivotal role of macrophages in the establishment of the different forms of the disease. Each clinical presentation in leprosy is associated with a different macrophagic population in host tissue. Macrophages can present a proinflammatory M1 phenotype in which vitamin D-dependent antimicrobial pathway predominates, as observed in the paucibacillary lesions and in the onset of reversal reaction (72, 73); through to anti-inflammatory M2 phenotype in which there is an upregulation of phagocytic pathways as found in lepromatous skin tissues (72–75).

Immunohistochemistry analysis demonstrated a high expression of Galectin-3 on macrophages found in skin lesions of lepromatous patients; in contrast, it is almost undetectable in tuberculoid lesions. The increase of Galectin-3 in lepromatous cells contributes for reduced T cell activation in these patients (76).

de Sousa et al. (74) have demonstrated that the understanding of the role of cytokines, arginase 1, and costimulatory molecules in macrophages may contribute for the comprehension of innate immunity function in the establishment of the polar forms of leprosy. In addition, Teles et al. demonstrated that in macrophages present in lepromatous skin cells there is an upregulation of IL-27 (77), a paradoxal cytokine that may activate IFN-β and IL-10 that contribute for the blockade of antimicrobial pathways (78).

Although the predominance of specific cell markers of M1 or M2 in the different clinical forms of leprosy, there is a continuum of phenotypes between these ranges with some cells sharing phenotypes of both M1 and M2 macrophages. Lepromatous macrophages, while predominantly expressing M2 markers like CD163, indoleamine 2,3 dioxygenase (IDO), arginase, and SRA-I (16, 61, 79–81), have some M1 characteristics like increased iron storage and a diminished expression of the iron exporter Ferroportin (Fpn-1) as well, which indicates that augmented iron deposits may favor *M. leprae* survival inside the foamy macrophages (79) (**Figure 1**).

Besides iron, *M. leprae* incorporates cholesterol and converts it to cholestenone; however, it does not use cholesterol as a nutritional source (82), although cholesterol colocalizes to *M. leprae*-containing phagosomes, and the blockade of cholesterol decreases the bacterial survival (83). Previous studies have demonstrated that *M. leprae* induces lipid body biogenesis and cholesterol accumulation in host cells (84). In lepromatous lesions, host-derived oxidized phospholipids were detected in macrophages, and one specific oxidized phospholipid, 1-palmitoyl-2-(5, 6-epoxyisoprostane E2)-sn-glycero-3-phosphorylcholine accumulate in macrophages infected with live mycobacteria (85). Normal HDL, a scavenger of oxidized phospholipids, may revert the inhibition of innate immune responses caused by mycobacterial infection. However, this effect was not observed when they have used HDL from lepromatous patients (85).

Dendritic Cells

In skin, DCs are located in the epidermis, as Langerhans cells and in the dermis, as dermal DCs (59). Langerhans cells in leprosy skin lesions express CD1a and langerin. These cells efficiently present antigens to T cells as part of the host response to *M. leprae* (86).

Previous reports have demonstrated that Langerhans cells are dendritic cells; however, recent findings that evaluate the

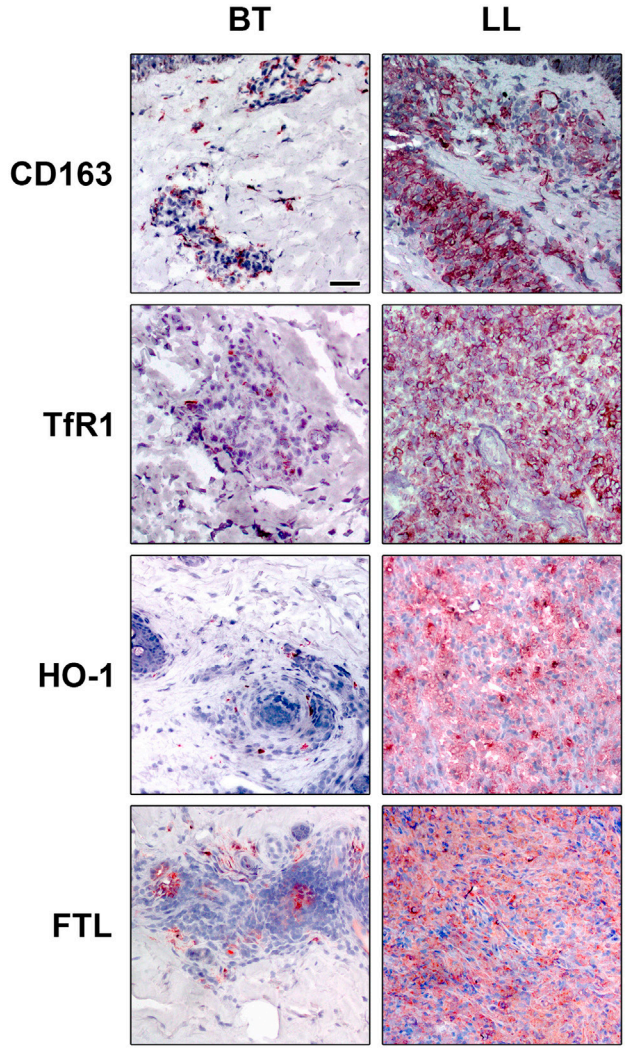

FIGURE 1 | Iron-related proteins are differentially regulated in leprosy clinical forms. Lepromatous skin lesions [lepromatous lepromatous (LL)] present a higher expression of the scavenger receptor of hemoglobin–haptoglobin complex, CD163 (upper panels), transferrin receptor 1 (TfR1, mid upper panels), the enzyme that catalyzes heme, heme-oxygenase 1 (HO-1, mid lower panels), and of the iron storage protein ferritin [ferritin light chain (FTL) lower panels]. The protein expression of CD163, TfR1, HO-1, and FTL was evaluated by immunohistochemistry. Images are representative of five independent samples from each group. Scale bars: 50 mm.

transcriptional profile have suggested that Langerhans cells may be considered resident macrophages (87, 88). Since several published studies classified these cells as dermal dendritic cells, we maintain this definition in this review.

CD1a$^+$ cells are associated with the outcome of reactional episodes in leprosy (89). CD1a is expressed in CD123$^+$ cells located in the dermis from both lepromatous and reversal reactional patients (15). Quantitative analysis showed a clear predominance of dendritic cells in tuberculoid leprosy (80, 89–91), whereas lesions from patients with the lepromatous pole of the disease show weak induction of CD1 proteins (89, 90). This weak expression of CD1 in lepromatous lesions is not result to a primary defect of the CD1 system itself because CD1a, CD1b, and CD1c could be induced to similar levels in both tuberculoid and lepromatous monocytes. Therefore, local factors at the site of infection may be responsible for the blockade of CD1 expression in lepromatous cells (90).

In lesions from tuberculoid leprosy patients, dendritic cells were linked with matrix metalloproteinase (MMP)-12 and contribute to granuloma formation (75). Previous studies have demonstrated that IDO-1 expression in myeloid dendritic cells and macrophages are part of the immune response associated with granuloma formation and may be associated with the granulomatous reactions in the skin (92). Our previous study has demonstrated that in lepromatous lesions IDO$^+$ cells with a dendritic-like morphology are detected on the dermis and in some endothelial cells (16). The characterization of IDO$^+$ cell phenotype demonstrates that almost all cells constituting the lepromatous dermal infiltrate are positive for HLA-DR, CD11c, CD86, and CD68. In tuberculoid lesion a few cells are positive for IDO and CD11c$^+$ and CD86$^+$ cells are detected in the center of the granuloma probably corresponding to epithelioid macrophages (16). In lepromatous patients that develop reversal reaction an increase in IDO gene expression is observed (15). The morphological changes in the reversal reactions skin lesions are accompanied by phenotypic heterogeneity of myelomonocytic populations. The epithelioid cells exhibit both DC and macrophage markers, hinting at the complexity of this cell type. These cells found in the reversal reaction granuloma are CD68$^+$, CD83$^+$, CD206$^+$, CD209$^+$, CD1b$^+$, CD11c$^+$, and CD123$^+$, but did not express CD163. Double-immunofluorescence data also show that these cells express BDCA2 and BDCA4, suggesting that define pDC populations.

Mycobacterium leprae components trigger CD209 on DCs to induce IL-10 production in lepromatous cells (93). In addition, CD209 may function as a receptor of entry for *M. leprae* in host cells (94). The dendritic cells phagocytose *M. leprae* and express antigens derived from the bacteria, such as phenolic glycolipid 1 (PGL-1). Hashimoto et al. (95) demonstrated that *M. leprae* infection decreases the capacity of DCs in inducing T-cell responses by a mechanism that involves PGL-1, since the blockade of PGL-1 in the surface of DCs enhanced CD4(+)- and CD8(+)-T-cell responses. Other studies have also demonstrated that PGL-1 impairs dendritic cells maturation and activation, thereby facilitating *M. leprae* survival (96, 97).

Keratinocytes

The response of the epidermis to *M. leprae* infection can be shown by the different aspects seen along the spectrum as well as during reactional states. The epidermis plays an important role in the local inflammatory response detected in leprosy. Keratinocytes expressing ICAM-1 are found in lesions from leprosy patients that present strong cellular immune response against *M. leprae* (tuberculoid, reversal reaction), but not in lepromatous lesions (98). PCR analysis demonstrated the expression of inflammatory cytokines TNF, IL-6, and IL-12 besides high expression of ICAM-1 in the epidermis of reactional leprosy lesions (99).

Keratinocytes are more susceptible to *M. leprae* infection than dendritic cells that spontaneously present higher concentrations of the antimicrobial peptide cathelicidin (100). Previous study demonstrated an up-regulation of human beta-defensins 2 and 3 (hBD2 and hBD3) in keratinocytes stimulated with *M. leprae*, which is reverted by corticosteroids. In addition, they have demonstrated that corticosteroid treatment of patients with reversal reactions causes a suppression of hBD2 and hBD3 in skin biopsies, as measured by qPCR (101).

The role of keratinocytes during the reactional episodes needs to be better evaluated since besides their possible role in reversal reaction, these cells may be involved in the pathogenesis of ENL. It was demonstrated that Thalidomide therapy down-regulates the expression of ICAM-1 and HLA-DR antigens in keratinocytes (102).

HLA-DR$^+$ keratinocytes could present *M. leprae* antigens to well-defined CD4$^+$ cells (103). However, increased keratinocyte expression did not represent a control of bacillary load since recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) administered intradermically or by subcutaneous route leads to enhanced keratinocyte growth but the bacillary numbers remain unchanged (104). In tuberculoid skin lesion cells keratinocytes are the major producer of CXCL-10 but not in lepromatous cells (105), probably because it is necessary IFN-γ produced by T cells to induce this chemokine.

Schwann Cells

Mycobacterium leprae may cause peripheral neuropathy. *M. leprae* is able to overcome a succession of physical barriers—epineurium, perineurium and endoneurium—until it reaches the Schwann cell, taking advantage of the difficulty of immune cells to access these impervious barriers (106–108). *M. leprae* may infect both myelinating and non-myelinating Schwann cells in patients with leprosy, but *M. leprae* preferentially infects the non-myelinating Schwann cells (109). There is not a consensus if the neural damage is a result of *M. leprae* entry inside cells or it occurs because of the inflammatory infiltrate.

Masaki et al. (110) demonstrated that *M. leprae* may generate dedifferentiated Schwann cells by causing initial demyelination to establish infection, colonize the cells, and subsequently reprogram them to a progenitor/stem cell-like cells (pSLCs) stage to spread the infection. In addition to downregulating Schwann cell lineage transcripts and reactivating developmental genes, *M. leprae* induce a large numbers of immune-related genes comprising mostly innate immunity from the very early stage of Schwann cell infection and peaking in their expression when Schwann cells have changed their cell identity to pSLCs (29). A previous study demonstrated that *M. leprae* could modulate Schwann cell glucose metabolism to increase the generation of the reduction capacity and free-radical control (111), but the impact of these regulation in nerve damage needs to be more clarified.

Schwann cells in skin lesions from leprosy patients express TLR2 (112, 113). In nerve biopsies from patients with neuritis, it was identified TNF, TNF receptors and TNF-converting enzyme in Schwann cells (26). It was speculated that *M. leprae* ligands induce Schwann cell death by a pathway that involves both TLR2 and TNF. It is possible that the pro inflammatory cytokines may contribute for Schwann cell apoptosis after cell interaction with *M. leprae*, which is associated with the pathogenesis of nerve damage (112, 113).

Analysis of nerves of pure neuritic patients demonstrated that MMP-2, MMP-9, and TNF mRNA production is highly induced in the AFB(−) lesions in relation to AFB(+) neuritic leprosy and non-leprosy control group (114), whereas CCL2 and CXCL10 chemokines are not determinant for the establishment of AFB(+) or AFB(−) in advanced stages of leprosy nerve lesions. CCL2 expression is associated with macrophage recruitment and fibrosis (115).

Recent findings have demonstrated that nerve damage begins in the early stages of the disease and may be more strongly related to response of innate immunity. In this context, the complement system has been placed with relevant role. This system is part of the innate immunity against bacterial pathogens through the formation of Membrane Attack Complex (MAC), but can lead to an inflammatory process followed by tissue injury if activated uncontrollably. Histopathological studies demonstrated MAC deposition on cutaneous sensory nerves (116) and on damaged nerves of lepromatous patients. However, the same was not found for tuberculoid patients (117).

Advancing in studies related to the complement system as a trigger for nerve damage, a pathogen-associated molecular pattern (PAMP), the glycolipid lipoarabinomannan (LAM), a component of the mycobacteria cell wall, has been investigated as the starting mechanism for activation of this pathway. It has been shown *in vitro* that this PAMP activates the Schwann cell by the formation of opsonin C3 and MAC (118). In nerve biopsies of leprosy patients, in turn, the LAM and MAC antigen deposition was found. MAC and LAM colocalizes on axons suggesting a relation between LAM in complement activation and nerve damage (117). In a mouse nerve lesion model, the interaction of LAM with the nerve was observed, activating the pathway of the complement system (117).

Recent evidences suggest that axon demyelination occurs in function of the interaction of PGL-1 with myelinating glia and their infection. According to Madigan et al. (119) demyelination and axonal damage are initiated by infected macrophages that patrol axons. PGL-1 induces nitric oxide synthase in infected macrophages that results in damaged axons by injuring their mitochondria and inducing demyelination (119).

Neutrophils

Little attention has been given to the function of the neutrophils in leprosy. It was previously demonstrated that both circulating neutrophils and monocytes are loaded with intracellular *M. leprae* without obvious inflammatory phenomena (120, 121). We reported that neutrophils isolated from lepromatous leprosy patients with or without ENL release TNF and IL-8 after stimulation with *M. leprae* (122). Moreover, the apoptotic rate of ENL neutrophils is higher as compared to lepromatous patients and healthy volunteers (122).

Microarrays analyses comparing skin lesions of lepromatous patients and patients with ENL revealed the up-regulation of cell movement genes, including E-selectin and its ligands, key

molecules that mediate neutrophil recruitment to inflammatory sites (123). According to these results "granulocyte adhesion and diapedesis" were identified by Dupnik et al. (124) as one of the top canonical pathways characterizing ENL. Moreover, neutrophil and endothelial cell gene networks were identified in ENL samples as part of the vasculitis that results in tissue injury (75).

Recently, we reported that during ENL, but not in RR, circulating neutrophils express CD64 on cell surface, while nonreactional leprosy or healthy volunteers have lower levels of CD64 expression. CD64 expression on circulating neutrophils and in ENL lesion is down modulated after thalidomide treatment. Moreover, the severity of ENL is associated with high levels of CD64 expression, also pointed as an early biomarker for ENL (20). Increased CD64 expression *in vivo* has been associated with an increase in neutrophil function and adhesion to the endothelium (125–128).

Elevated levels of TNF and other proinflammatory cytokines have been associated with episodes of ENL, while suppression of TNF leads to clinical improvement (102, 129). We reported evidence that pentraxin-3 (PTX3), originally described as a protein induced by primary inflammatory signals, such as TNF and IL-1β, is released systemically and at the site of ENL lesions (130). We also demonstrated that there is a positive correlation between PTX3 serum levels and CD64 surface expression on circulating neutrophils. Moreover, we showed that the majority of neutrophils (MPO^+ cells) presented throughout the ENL lesion express PTX3 (130). Additionally, thalidomide treatment of ENL downregulated PTX3 levels. Interestingly, PTX3 serum levels were higher in lepromatous patients without reaction that developed ENL, persisting after the onset. In contrast, lepromatous patients that developed reversal reaction had lower levels of PTX3 prior and during the inflammatory episode. Those data indicate that high levels of PTX3 may be associated with ENL occurrence and point to PTX3 as a potential ENL biomarker able to differentiate from a reversal reaction episode. Belone et al. (131) previously reported the PTX3 mRNA is exclusively expressed in ENL lesions.

MANIPULATION OF INNATE IMMUNITY BY *M. leprae*

To survive within the host cell, mycobacteria must escape intracellular mycobactericidal mechanisms.

The activation of innate immunity may occur after the interaction of PAMPs, which are conserved microbial structures, with their respective pattern recognition receptors (PRRs), present in host cells. PRRs are also able to recognize endogenous molecules from damaged cells, known as damage-associated molecular patterns (DAMPs), resulting in several chronic inflammatory and autoimmune diseases. After the interaction of PAMPs and/or DAMPs with PRRs, the release of intracellular signals leads to the induction of important genes transcription for cellular activation or induction of phagocytosis. Different PRRs are expressed in the same cell, which makes it able to recognize several classes of microorganisms and different endogenous molecules. The PRRs described so far are C-type lectin receptors, Nod-like receptors (NLRs), RIG-1-like receptors, and toll-like receptors (TLRs) (132–134).

Complement activation, apoptosis, and autophagy are other innate mechanisms modulated by the mycobacteria. The understanding of the mechanisms and pathways used by mycobacteria to manipulate the innate immunity may contribute for the development of new strategies of diagnostic and control of the disease.

Toll-Like Receptors

Several studies indicate that the recognition of mycobacteria by TLRs represents an essential step in generating an immune response capable of protecting the infection.

Different molecules that constitute *M. leprae* have been characterized as ligands and potent stimulators of TLRs, mainly involving TLR2. Killed *M. leprae* is able to mediate TLR2/1 heterodimers and TLR2 homodimers cell activation, indicating the presence of triacylated lipoproteins in the bacterium (135). In fact, a genome-wide scan of *M. leprae* identified 31 lipoproteins with potential to act as ligands of TLR2/1 heterodimer (135). As *M. leprae* cannot be grown *in vitro*, the purification of proteins from the few bacteria in armadillos becomes very difficult. Therefore, Krutzik et al. (135) used synthetic lipopeptides to show that the 19 and 33-kDa lipoproteins from *M. leprae* are capable to activate *in vitro* both monocytes and dendritic cells. In addition, lesions from leprosy patients with localized tuberculoid form displayed more strongly expression of TLR2 and TLR1 as compared with the lepromatous form of the disease. These data suggest the involvement of TLRs in the host defense against the mycobacteria.

Nerve damage is an important clinical hallmark of leprosy disease responsible for the patient morbidity. In this sense, the activation and expression of TLR2 have also been investigated in human Schwann cells (112). The lipopeptide that mimics the *M. leprae* 19-kDa lipoprotein, and can act as TLR2 agonist, induced an increase in the number of apoptotic cells during activation of Schwann cells (112). It was possible to identify the expression of TLR2 in Schwann cells present in lesions from tuberculoid patients, in addition to cells that had undergone apoptosis *in vivo* (112), providing a link between innate immune response and nerve injury in leprosy.

The presence of foamy cells highly infected is characteristic in lepromatous, but not in tuberculoid lesions. The foamy phenotype results from the capacity of *M. leprae* to induce and recruit host-derived lipids to bacterial-containing cells, forming lipid droplets (12). Interestingly, TLR6 is essential for lipid droplets biogenesis in *M. leprae*-infected Schwann cells, but not TLR2 (136). On the other hand, the formation of lipids droplets in *M. leprae*-bearing macrophages appeared to be only partially dependent on both TLR2 and TLR6 (12). These data suggest the involvement of alternative TLRs or additional receptors associated with the innate immune response for *M. leprae* recognition in leprosy.

Polycarpou et al. (137) demonstrated that *M. leprae* activates TLR4, by containing uncharacterized ligands, since the classic ligand agonist of TLR4 is LPS (138). TLR4 neutralizing antibody pretreatment decreased the production of TNF, IL-6, and CXCL-10 in human macrophages stimulated with *M. leprae* (137).

Furthermore, *M. leprae* upregulates TLR4 protein expression on macrophages from healthy subjects, but not in macrophages from BCG-vaccinated donors (137). Macrophages from non-vaccinated healthy donors treated with BCG present reduced TLR4 expression suggesting a role of TLR in the protective effect of BCG. Associated with this, the treatment of reversal reaction with corticosteroids decrease gene and protein expression of both TLR2 and TLR4 in skin lesion cells (139), indicating the involvement of receptors also in triggering the inflammatory process. A study linking the innate immunity pathways with the development of ENL suggested that recognition of DNA by TLR9 constitutes a major inflammatory pathway activated during ENL (140). The proinflammatory cytokines storm observed during ENL seems to be related to the massive release of mycobacterial TLR9 ligands during multidrug therapy (140). Moreover, the inflammatory response could be amplified by the binding of endogenous DNA to TLR9 (140), since expressive tissue destruction also occurs during ENL (141). Dias et al. (140) demonstrated a higher TLR9 expression in cells from ENL patients when compared with nonreactional lepromatous controls. In addition, significantly increased circulating levels of human and mycobacterial DNA–histone complexes were detected in ENL patients when compared with nonreactional controls (140). Furthermore, TLR9 agonists were able to induce the secretion of higher levels of TNF, IL-6, and IL-1β in ENL when compared with nonreactional patients and healthy individuals (140). The same effect was observed in the cells stimulated with lysed *M. leprae* (140). The use of a synthetic antagonist of nucleic acid-sensing TLRs suggested that this may be an alternative for the development of more effective drugs to treat ENL.

The genetic association demonstrated several single-nucleotide polymorphisms (SNPs) in TLR genes that may be associated with susceptibility or resistance to leprosy and leprosy reactions. However, most studies in this area focused mainly on the mutation of TLRs 1 and 2 and their correlation with the disease. The SNP within TLR1 (I602S) is associated with reduced responses to mycobacterial agonists (142). The TLR1 602S variant, but not the TLR1 602I variant, in heterologous systems showed the expected absence of the receptor on the plasma membrane (142). The 602S allele is associated with a reduced incidence of leprosy (142).

Previous studies showed that TLR1 variants N248S is a susceptibility factor for leprosy (143, 144). Additionally, PBMCs from individuals carrying 248S produce a lower TNF/IL-10 proportion levels after stimulation *in vitro* with *M. leprae*, but not with controls as LPS (TLR4 agonist) or PAM3CSK4 (TLR2 agonist) (144). Analysis of samples from patients that developed reactional episodes demonstrated that a TLR1 N248S-linked feature is associated with the development of disabilities and the progression from infection to disease (143).

Another transmembrane domain polymorphism in TLR1 (T1805G) was associated with susceptibility to leprosy, regulating the innate immune response (145). The group analyzed 933 Nepalese leprosy patients, 238 of whom with reversal reaction, and investigated the association of TLR1 variation with different clinical forms of leprosy or reversal reaction, demonstrating that the1805G allele is associated with protection from reversal reaction (145).

A TLR2 mutation in the lepromatous, but not in tuberculoid patients, was also identified (146). TLR2 from PBMCs from lepromatous patients presented a C to T substitution at nucleotide 2029 from the start codon. This modification was not identified in tuberculoid individuals (146). In fact, periphery monocytes from leprosy patients with modification in TLR2 (Arg677Trp) were significantly less responsive to cell lysate of *M. leprae* than subjects carrying wild-type TLR2 (147). Additionally, the secretion of IL-12 was lower in patients with TLR2 mutation (147). A study performed in Ethiopian patients investigated different polymorphisms in TLR2 (597C→T, 1350T→C, and a microsatellite marker) (148). The mutation-associated risk of developing leprosy was assessed. The microsatellite and the 597C→T polymorphisms were both associated with susceptibility to reversal reaction as predicted by reversal reaction.

The roles of TLR1 and 2 in leprosy and leprosy reactions were described and it may contribute for perspectives in leprosy management.

NLRs

The nucleotide-oligomerization domain (NOD) proteins are intracellular and cytoplasmic receptors. Previous data have demonstrated that the blockade of phagocytosis inhibits IL-1β and TNF production in response to *M. leprae*, suggesting that intracellular signaling is also required for macrophage activation after *M. leprae* infection. In addition, NF-κB activation and expression of TNF and IL-1β were observed in NOD1- and NOD2-transfected cells stimulated with *M. leprae* (149).

NLRPs are intracellular receptors that recognize PAMPs and induce the secretion of both caspase-1 and IL-1β in the context of inflammasome. SNPs in NLRP1 and NLRP3 genes were analyzed in Brazilian leprosy patients. The NLRP1 combined haplotype rs2137722/G-rs12150220/T-rs2670660/G was significantly more frequent in patients than in controls as well as in paucibacillary than in multibacillary patients (150). The NLRP1 combined haplotype rs2137722/G-rs12150220/A-rs2670660/G was associated with paucibacillary leprosy suggesting that NLRP1 might be involved in the susceptibility to leprosy (150).

Nod-like receptors may recruit and activate inflammatory caspases into inflammasomes or may trigger inflammation *via* different pathways including the NF-κB mitogen-activated protein kinase and regulatory factor pathways (151).

Polymorphisms in NOD2 are associated with leprosy susceptibility. Activation of monocytes *via* NOD2 induces preferentially the differentiation into dendritic cells, which was mediated by IL-32. Notably, IL-32 is able to induce monocytes from healthy donors or from tuberculoid patients to rapidly differentiate into DCs, which is more efficient than GM-CSF-derived DCs in presenting antigen to major histocompatibility complex class I-restricted CD8(+) T cells (152). In contrast, monocytes from patients with the lepromatous form of leprosy did not produce IL-32 in response to NOD2L and did not induce DC differentiation by a mechanism that is mediated by IL-10 (152). In tuberculoid patients there was a higher expression of NOD2 and IL-32 as well as the frequency of CD1b (+) DCs at the site of leprosy infection when compared with lepromatous patients (152, 153).

Complement Cascade

Lipoarabinomannan is a molecule from *M. leprae* that is associated with nerve damage. Curiously, previous studies demonstrated that LAM activates complement and previous study demonstrated the important role of complement in nerve damage in leprosy (117). Analysis of skin biopsies demonstrates that the percentage of CD3d, MAC, and LAM deposition is significantly higher in lepromatous when compared to tuberculoid patients (154). MAC deposition colocalizes with LAM and is found on axons in skin lesions of lepromatous patients. In tuberculoid lesions, the presence of T cells positive for CD3d was observed in surrounding granulomas without MAC deposition (154). Analysis of skin lesions from reactional patients demonstrated an increase in MAC immunoreactivity when compared to non-reactional leprosy patients (154). Immunofluorescence analysis showed an increase of C1q deposition in both reversal reaction and ENL lesions when compared to non reactional matched patients (124).

Lahiri et al. demonstrated that when disrupted, *M. leprae* could activate complement (155) and polymorphisms in genes of complement cascade suggest an association of complement genes with leprosy susceptibility (156).

Apoptosis

Analysis of skin lesion cells demonstrated that apoptosis is more frequent in tuberculoid and reversal reaction than in lepromatous cells (157–159). Lepromatous cells present increased expression of the antiapoptotic protein Bcl-2, suggesting that the decrease in cell death could contribute for sustains the infection (158).

The hypothesis of the involvement of apoptosis in the control of bacillary load was reinforced by *in vitro* studies that demonstrated that clofazimine, a compound used for the treatment of leprosy since the 1960s has the capacity to induce apoptosis in macrophages, suggesting that the antibacterial and anti-inflammatory properties of this drug are mediated by apoptosis (160). Analysis of apoptosis in skin cells from treated patients revealed that in both tuberculoid and lepromatous lesions, there is an increase in the frequency of apoptotic cells at 3 and 6 months after the start of the treatment (161). Analysis of lesions in either reversal reaction or ENL demonstrated a significant increase in apoptosis only in ENL lesions and those that were at 6 months of treatment (161).

Although several studies suggesting the antibacterial role of apoptosis in infected cells, there are evidences that in tuberculoid patients apoptosis is a mechanism that contributes to maintain the infection, instead of the pro inflammatory infiltrate and the presence of pro inflammatory cytokines. In tuberculoid lesions predominate a M1 phenotype, although few M2 cells were present in the skin lesions of these patients (16, 61). We have previously demonstrated that *in vitro* GM-CSF-differentiated monocytes (M1) stimulated with both *M. leprae* and apoptotic cells change their phenotype and express M2 cells-specific markers, such as CD163 and SRA-I. Moreover, the phagocytosis of apoptotic cells by *M. leprae*-infected macrophages increases the secretion of anti-inflammatory mediators as IL-10, TGF-β, and arginase, corroborating the hypothesis that in paucibacillary patients, although the presence of an effective cellular immune response, efferocytosis contributes for maintain few susceptible macrophages in skin lesions which contributes for sustain the infection (81).

The induction of apoptosis in Schwann cells stimulated with *M. leprae* was previously demonstrated (112, 113) and some studies associated apoptosis in Schwann cells as an important event for nerve damage. *M. leprae* induces demyelization in Schwann cells by a pathway that involves the activation of the MAPK (ERK 1/2). A previous study has demonstrated that the ganglioside 9-O-acetyl GD3 is associated with *M. leprae* entry in Schwann cells and that the blockade of this ganglioside may result in a reduced activation of the MAPK (ERK 1/2) pathway (162).

Autophagy

The canonical macroautophagy (hereafter termed autophagy) pathway is an evolutionarily conserved mechanism through which organelles and proteins are degraded and recycled by the lysosomal system to promote cellular and organismal homeostasis. The major hallmark of autophagy is the formation of double-membrane vesicles called autophagosomes, which engulf and driving intracellular targets for degradation. Autophagy impairment is widely implicated in the pathology of several diseases, including microbe infection, cancer, and metabolic, cardiovascular, and neurodegenerative disorders (163).

During infectious processes, autophagy helps the immune system by degrading intracellular microbes through a selective form of autophagy called xenophagy. The significance of autophagy in numerous infectious processes was established, including those caused by bacterial, parasitic, and viral pathogens, as well as the microbial strategies used to avoid or subvert autophagy and promote their own survival (164, 165). In contrast, the role of autophagy in leprosy pathogenesis remained unknown until recently. The first evidence that *M. leprae* can be targets for autophagy was revealed by transmission electron microscopy studies. It was observed that during the initial growth phase of *M. leprae* in macrophages, the mycobacteria are present in single membrane vacuoles with few nearby lysosomes, and the bacilli are intact. At the peak of the growth phase, the number of lysosomes increases and *M. leprae* is located in a large number of double membrane vacuoles.

During the stationary phase, macrophages have a vacuolar appearance and contain a significant number of lysosomes, *M. leprae* organisms are inside double membrane vacuoles, and most of these bacteria are degenerate (166). Chandi and Job (167) described the presence of double membrane phagosomes in macrophages after 40 min of *M. leprae* exposure, and after that, the lysosomes fuse with these *M. leprae*-containing vacuoles. These data provide evidences that *M. leprae* may have been the first bacterial pathogen to interact with the autophagic pathway and reinforces the role of autophagy in leprosy.

A genomewide association study of leprosy revealed that a polymorphism in the upstream autophagy activator gene NOD2 is a susceptibility factor to develop *M. leprae* multibacillary infection (168, 169). Interestingly, the polymorphisms in other autophagy-associated genes such as PARK2, VDR, and TLR2, are also correlated with the multibacillary leprosy susceptibility

(51, 146, 170–174). In other hand, these triggers of autophagy are preferentially expressed in the skin lesions of the auto limited tuberculoid clinical form (72, 135, 152). Subsequently, it has been suggested that the polymorphism in the autophagy gene IRGM, which is linked to susceptibility to Crohn's disease and tuberculosis (175–178), is associated with an increased risk of developing leprosy because it affects the production of inflammatory cytokines such as IFN-γ (179). In addition, increased IRGM expression was observed in monocytes and macrophages infected with *M. leprae*, as well as, monocytes from the self-limited tuberculoid form presented a higher expression of IRGM, as compared to cells of clinically progressive lepromatous patients (180). IRGM, an effector of IFN-γ-mediated autophagy, controls the autophagic pathway through their interaction with ULK1 and BECN1, governing the assembly of the initiation complex, and then together with ATG16L1 and NOD2, forms a molecular complex that promotes antimycobacterial defense (181, 182).

More recently, our group described an association between *M. leprae* death and targeting of mycobacteria to the autophagic pathway in human macrophages. It has been shown that the genetic silencing of the OASL antiviral protein, which is produced through the detection of *M. leprae* DNA mediated by STING sensor, increases the levels of autophagy and decreases the viability of the mycobacteria, being reversed by the autophagy blockade (183). Ma and cols (184) suggested that although autophagy could promote the elimination of intracellular pathogens, the induction of the autophagic pathway by *M. leprae* would be a mycobacteria pro-persistence factor. It has been reported that although activation of autophagy occurs in response to *M. leprae* infection in macrophages, it also promotes an IL-10-producing T cell-mediated anti-inflammatory response, which in a negative feedback cycle inhibits autophagy and allows *M. leprae* survival in macrophages (184). However, this work was based only on the alone use of CYTO-ID/CAT, an acidotropic dye from the monodansylcadaverine group recently developed to monitor autophagy in living cells (185), which is not recommended by autophagy experts (186).

Finally, we demonstrated the key role of autophagy in leprosy polarization (187). We showed that autophagy is differentially regulated between leprosy polar forms, uncovering an essential role for Beclin 1 protein in this process, which was upregulated in tuberculoid patients. In contrast, a higher expression of BCL2 protein was determined in lepromatous patients. While Beclin 1 is a key initiator of the functional formation of autophagosomes in mammals and may be induced by IFN-γ to activate autophagy, the BCL2 antiapoptotic protein inhibits autophagy by binding and sequestering Beclin 1 from the class III PIK3 complex (188). In tuberculoid skin lesion cells IFN-γ-induced autophagy

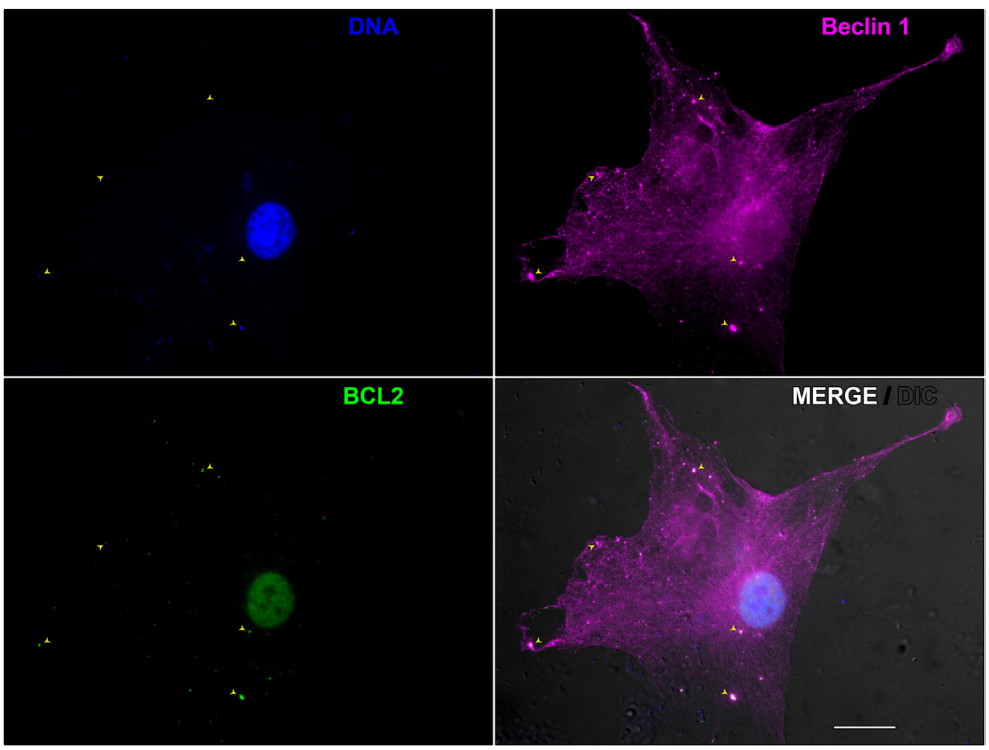

FIGURE 2 | Beclin 1-mediated autophagy during *Mycobacterium leprae* infection in skin lesion-derived lepromatous macrophage. Macrophages were isolated from the skin lesion of a lepromatous leprosy patient and cultured for 18 h in full nutrient medium. Cells were fixed and immunofluorescence for Beclin 1 (magenta) and BCL2 (green) was performed. Cellular and bacterial DNA were stained with DAPI (blue). Cell and mycobacteria morphology are shown by Nomarski differential interference contrast (gray). This image shows a lepromatous tissue macrophage interacting with *M. leprae*. BCL2 colocalizes with Beclin 1-entrapped mycobacteria (arrowheads) allowing *M. leprae* survival through autophagy inhibition. The modulation of autophagy has the potential to be useful in the leprosy treatment, as well as to prevent leprosy reactional episodes. Scale bar: 20 μm.

contributes for *M. leprae* control, whereas in lepromatous cells the BCL2-mediated block of the Beclin 1 autophagic pathway promotes the mycobacterial persistence (**Figure 2**). As previously described (184), we also observed an inhibition of autophagy triggered by live *M. leprae* infection in lepromatous macrophages, however, it can be reverted by IFN-γ treatment. In addition, the levels of autophagy were restored in lepromatous patients who developed the reversal reaction episodes, an inflammatory state associated with increased IFN-γ expression (187). Thus, autophagy is an important innate mechanism associated with leprosy immunopathogenesis.

PERSPECTIVES

The influential role of innate immunity in leprosy biology and their potential as therapeutic targets are now widely recognized. To gain a better understanding of these pathways and to discover new ones, new technologies such as single cell RNA sequencing studies are needed.

Future works should aim to determine further the roles of the neutrophils in host–mycobacteria interaction, with a focus in their role during disease progression. This review supports the role of neutrophils as effector cells and not only as migratory cells following chemoattractants in the context of leprosy. Another promising field that should be investigated by leprologists is the innate lymphoid cell (ILC) biology. ILCs have already been implicated in many studies including metabolism, tissue remodeling and protection against infection.

Although tissue resident macrophages have been extensively studied, phenotypic and functional characteristics of skin resident macrophages and its interaction with the skin sensory nervous system are not fully understood. Furthermore, the dynamic interaction of the resident and the migratory immune cells in the skin may improve our understanding of the immunological events that occur *in situ*. Of note, a recent report demonstrated that nitric oxide secreted by *M. leprae*-carrying macrophages directly damage nerve fibers, by inducing axonal and mitochondrial swelling followed by demyelination phenotype (119). This was a first report showing detrimental roles of infected macrophages that patrols the nerve and induces nerve pathology.

Local Innate immune mechanisms are crucial to determine the outcome of the different clinical forms and the reactional episodes in leprosy patients. The evaluation of the single cell gene expression using RNA sequencing (scRNAseq) emerged as a powerful tool in genomics. scRNAseq provides the expression profile of individual cells. Studies of scRNAseq in leprosy field is a valuable strategy and may shed light on the understanding of the functionality of each cell populations as well as the innate mechanisms induced by *M. leprae* that may contribute for the development of new strategies of control of the disease.

AUTHOR CONTRIBUTIONS

Concept of the review: RP and ES. Design and write the review: RP, VS, BJAS, BJS, AD, DE, and MP. Figures and legends: RP, VS, BJAS, MB, and ES. Final approval of the version to be published: RP, VS, and ES.

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Autoimmunity to Tropomyosin-Specific Peptides Induced by *Mycobacterium leprae* in Leprosy Patients: Identification of Mimicking Proteins

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Background: It has been shown earlier that there is a rise in the levels of autoantibodies and T cell response to cytoskeletal proteins in leprosy. Our group recently demonstrated a rise in both T and B cell responses to keratin and myelin basic protein in all types of leprosy patients and their associations in type 1 reaction (T1R) group of leprosy.

Objectives: In this study, we investigated the association of levels of autoantibodies and lymphoproliferation against myosin in leprosy patients across the spectrum and tried to find out the mimicking proteins or epitopes between host protein and protein/s of *Mycobacterium leprae*.

Methodology: One hundred and sixty-nine leprosy patients and 55 healthy controls (HC) were enrolled in the present study. Levels of anti-myosin antibodies and T-cell responses against myosin were measured by ELISA and lymphoproliferation assay, respectively. Using 2-D gel electrophoresis, western blot and MALDI-TOF/TOF antibody-reactive spots were identified. Three-dimensional structure of mimicking proteins was modeled by online server. B cell epitopes of the proteins were predicted by BCPREDS server 1.0 followed by identification of mimicking epitopes. Mice of inbred BALB/c strain were hyperimmunized with *M. leprae* soluble antigen (MLSA) and splenocytes and lymph node cells of these animals were adoptively transferred to naïve mice.

Results: Highest level of anti-myosin antibodies was noted in sera of T1R leprosy patients. We observed significantly higher levels of lymphoproliferative response ($p < 0.05$) with myosin in all types of leprosy patients compared to HC. Further, hyperimmunization of inbred BALB/c strain of female mice and rabbit with MLSA revealed that both hyperimmunized rabbit and mice evoked heightened levels of antibodies against myosin and this autoimmune response could be adoptively transferred from hyperimmunized to naïve mice. Tropomyosin was found to be mimicking with ATP-dependent Clp protease ATP-binding subunit of *M. leprae*. We found four mimicking epitopes between these sequences.

Conclusion: These data suggest that these mimicking proteins tropomyosin and ATP-dependent Clp protease ATP-binding subunit of *M. leprae* or more precisely mimicking epitopes (four B cell epitopes) might be responsible for extensive tissue damage during type1 reaction in leprosy.

Keywords: leprosy, myosin, epitopes, mimicking proteins, autoimmunity, tropomyosin

INTRODUCTION

Infectious agents of the environment are known to play a role in induction of an imbalance in the homeostatic mechanism of the host leading to an autoimmune disease (1). Hansen's disease (leprosy) is a chronic granulomatous disease caused by *Mycobacterium leprae* (*M. leprae*). *M. leprae* is an obligatory intracellular bacterium. The three cardinal signs used for diagnosis of leprosy are the presence of anesthetic skin lesion(s), enlarged peripheral nerve(s) and presence of acid-fast bacilli in the skin smear (2).

Upon entry into the host, *M. leprae* is selectively phagocytosed by non-professional phagocytic cells (MHC class II negative Schwann cells) in the peripheral nerve and grow taking advantage of immunologically privileged site (3, 4). In an endemic population, about 95 (5) to 99% (6) of infected individuals do not develop any overt disease. However, it has been found out to be very infectious in household contacts of lepromatous leprosy (LL) due to repeated exposure to *M. leprae* infection (7). The host immune response is responsible for disease manifestation and progression of leprosy.

Infection may initiate a continuous antigenic stimulus and may breakdown the tolerance of the host through several non-specific mechanisms leading to autoimmunity (8). Infection with *M. leprae* evokes considerable changes in the humoral immune system, which involves aberrant responses, often associated with autoimmune syndrome. Presence of some antigenic structures of *M. leprae* that can be immunogenic and are cross-reactive to self-proteins might be responsible for the growth of *M. leprae* in lepromatous type of leprosy (9) wherein T cell-mediated immunity to *M. leprae* is virtually absent. On the contrary, in tuberculoid leprosy and during type 1 reaction (T1R), these similarities may lead to a heightened T- cell response and extensive granuloma formation while *M. leprae* is not observed in the host tissues (9). Our group also reported the sharing of mimicking B cell epitopes between *M. leprae* and the cytokeratin-10 (10) and myelin basic protein (11) of host.

In leprosy patients, impairments of nerve and muscle functions are very common. More than 20% of leprosy patients have been shown to have motor deficits and paralysis of muscles (12). Further, *M. leprae* has been shown to be present between the striated muscle fibers of both tuberculoid and lepromatous patients (13–15). *M. leprae* was also shown to be present in smooth muscle fibers of skin, lips, and nipples in LL (16). Degenerative changes in muscle identified as "Leprous myositis" have also been reported (17, 18). Based on the above literature, we hypothesized that muscle weakness in leprosy patients might be due to presence of anti-myosin antibodies, and therefore, auto-reaction might play a role in muscle damage leading to loss of muscle functions in leprosy patients. Hence, we searched for the presence of mimicking protein/s between host myosin and *M. leprae*.

MATERIALS AND METHODS

Antigens

Non-irradiated *M. leprae* bacilli derived from armadillo was obtained from Colorado State University, Fort Collins, CO, USA (WHO Contract Number NIH-No1-AI-25469, Leprosy Research Support). *M. leprae* soluble antigen (MLSA) was obtained by sonication of cells of *M. leprae* according to published protocol (19). The protein content of MLSA was assessed by Bradford method (20). Myosin protein from porcine muscle (Cat. No. M0273) was acquired from Sigma-Aldrich Pvt. Ltd., USA.

Study Subjects

Human Subjects

A total of 169 leprosy patients were enrolled from the Outpatient department of National JALMA Institute for leprosy and Other Mycobacterial Diseases (NJIL&OMD) (ICMR), Agra for the study. Patients were categorized based on Ridley and Jopling scale (21) and were grouped as borderline tuberculoid (BT) ($n = 30$), borderline borderline (BB) ($n = 23$), borderline lepromatous (BL) ($n = 39$), LL ($n = 32$), BT patients with T1R ($n = 25$) and BL/LL with type 2 reaction or erythema nodosum leprosum (ENL) ($n = 20$). Healthy students and staff of the institution with no evidence for leprosy and any other disease were taken as healthy controls (HC) ($n = 55$) in the study.

This study was approved by Institute Human Ethics Committee, and all the subjects were enrolled after giving a written consent to participate in the study.

Animals

Outbred female New Zealand white rabbits and female mice of inbred BALB/c strain were obtained from the Central Drug Research Institute (CSIR), Lucknow. All the animals were kept in specific pathogen-free conditions in the Department of Animal Experimentations, NJIL&OMD, Agra, India. Present study was approved by Institute Animal Ethical Committee, and we followed the guidelines laid down by Animal Research Ethics Board at our institute.

Animal Experimentations

Hyperimmunization of Rabbit

Rabbits ($n = 3$ in each group) were hyperimmunized with protein concentration of 250 μg of MLSA emulsified with Freund's incomplete adjuvant (IFA) and 250 μg of porcine myosin to produce polyclonal antibodies against these proteins. Control group of rabbits ($n = 3$) was administered with normal saline emulsified with IFA. All the animals were boosted weekly with the same dose of antigens up to eighth week.

Hyperimmunization of Mice

Mice ($n = 15$) were hyperimmunized with 25 μg of MLSA and control group mice ($n = 10$) were inoculated with normal saline as described earlier (10).

Adoptive Transfer

Cells from hyperimmunized mice were adoptively transferred to naïve female mice as reported earlier by Singh et al. (10). Briefly, adoptive transfer was done in control group ($n = 5$) by intravenous (i.v.) inoculation into the tail vein of suspensions of splenocytes and lymph nodes cells obtained from control mice. Similarly, experimental group ($n = 5$) were inoculated with immune cells acquired from MLSA-hyperimmunized group. Third group ($n = 5$) was inoculated intravenously with T cells separated by nylon wool (22) taken from MLSA-hyperimmunized group.

Assessment of Anti-Myosin Antibodies by ELISA

Human Sera

ELISA was done for porcine myosin (Cat. No. M0273, Sigma-Aldrich Pvt. Ltd., USA) -reactive antibodies according to previously described protocol (11) with some changes. Porcine myosin (5 μg/ml) was coated into 96-well ELISA plate (flat bottom, Nunc Maxisorp, Denmark). ELISA was done according to previously published protocol (11) The absorbance was taken at 492 nM using Spectramax-M2 Reader (Molecular Devices, USA). The cutoff OD was calculated by adding average OD obtained in HC summed up with the value of twice SD.

Experimental Animals

ELISA protocol used for sera from experimental animals was same as described above under human sera except some minor changes in reagents.

Rabbit

Peroxidase conjugated anti-rabbit IgG (Sigma- Aldrich, USA) was used as secondary antibody.

Mice

Dilution of plasma was 50-fold, and secondary antibody was anti-mouse IgG peroxidase (Sigma- Aldrich, USA).

Effect of Myosin on Lymphoproliferation Assay

Lymphoproliferation assay was done as per the protocol described previously with some changes (10). Briefly, peripheral blood mononuclear cells were cultured in RPMI 1640 with 5% FBS in triplicate in presence of 10 μg/ml porcine myosin in Nunc-tissue culture plates (Denmark) and incubated in CO_2 incubator for 5 days (Forma Scientific Inc., USA) at 37°C with 5% CO_2 in air. Positive control culture was done with phytohemagglutinin. Cells were pulsed with 1 μCi/well of [^{3}H] thymidine after 5 days and incubated further for 18 h. Skatron cell harvester was used for harvesting the cells. Liquid scintillation counting (LKB Wallac, Finland) was used to determine the radioactivity incorporated into DNA. Stimulation index (S.I.) was calculated by using following formula:

$$\text{S.I.} = \text{Counts per minute } (\text{CPM}) \text{ of stimulated cells} / \text{CPM of unstimulated cells,}$$

S.I. > 2 was taken as significant stimulation.

Identification of Cross-Reactive Proteins Between Porcine Myosin and MLSA

Characterization of Cross-Reactive Proteins

Two-dimensional PAGE, isoelectric focusing, was carried out using the protocol described by Gorg et al., 2000 (23). Protein samples (100 μg of porcine myosin/MLSA) were loaded on IPG strips (Bio-Rad Laboratories, USA) of pH 3–10 for myosin, pH 4–7 for MLSA and length 7 cm. Proteins were separated in second dimension using 10% SDS-PAGE and transferred to nitrocellulose membrane (NCM) (24). Blotted NCM was blocked with 3% BSA (Sigma, USA) for 1 h, then incubated with pooled leprosy patients' sera (1:50) while NCM of separated proteins of MLSA was incubated with Myosin-hyperimmunized rabbit sera (1:50). These NCMs were incubated overnight at 4°C followed by three times washing with PBS containing 0.05% Tween-20 and incubated with peroxidase conjugated anti-rabbit IgG (1: 10,000) (Sigma-Aldrich, USA) for 1 h. Later, visualization of antigen antibody reactivity was done by color development with diaminobenzedine (Sigma, USA) solution. Capturing of image was done by Chemidoc (Bio-Rad Laboratories, USA).

MALDI-TOF Analysis

In-gel digestion with trypsin (25) was done according to previously published protocol (10). Mass spectra of digested peptides were analyzed using Mascot Wizard program (Matrix Science, Ltd., London, United Kingdom[1]). Peptide mass fingerprint of cross-reactive protein of porcine myosin with pooled leprosy patients' sera was submitted to Mascot search engine and search parameters used for the identification were peptide mass tolerance ±30 ppm, peptide charge state 1+, and maximum missed cleavages 1. However, search parameters used for the identification of the cross-reactive protein of MLSA by MS/MS ion search was peptide mass tolerance ±100 ppm, fragment mass tolerance ±0.5 Da, maximum missed cleavages 1.

B Cell Epitope Prediction

BCPREDS server 1.0 was used (aap prediction method) to identify B cell epitopes of the mimicking proteins.[2] Predicted B cell epitope length was of 20 amino acids and classifier specificity used was 75% (26).

Three-Dimensional Structure of Identified Protein

Structure of mimicking proteins of *M. leprae* and porcine myosin was predicted by submitting the sequence to Phyre2 server[3] (27). VMD viewer[4] was used for analysis of modeled structure (28).

[1]http://www.matrixscience.com (Accessed: November 11, 2009 and Accessed: September 4, 2017).
[2]http://ailab.ist.psu.edu/bcpred/predict.html (Accessed: September 6, 2017).
[3]http://www.sbg.bio.ic.ac.uk/phyre2/html/.
[4]www.ks.uiuc.edu/Research/vmd/.

Statistical Analysis

Data were analyzed using GraphPad prism software version 5.0 (GraphPad Prism, La Jolla, CA, USA). Cutoff value for ELISA data were expressed as mean ± 2SD and p value < 0.05 was considered as statistically significant. Under the respective figure or table legend specific test used for analysis has been mentioned. PD Quest Software (Bio-Rad, USA) was used to analyze 2-D blot data.

RESULTS

Levels of IgG Antibodies Against Myosin in Leprosy Patients' Sera

Highest mean OD value was obtained in the sera of T1R (0.416 ± 0.18) that was followed by LL (0.339 ± 0.13), ENL (0.322 ± 0.12), BL (0.302 ± 0.10), BB (0.275 ± 0.08), and TT/BT (0.264 ± 0.08). The mean OD value in sera of T1R patients' group was significantly higher than TT/BT ($p < 0.0001$), BB ($p < 0.0001$), BL ($p < 0.001$), LL ($p < 0.05$), and ENL ($p < 0.05$) group of patients (**Figure 1**). The cutoff OD value for myosin was found to be 0.282. Seropositivity of antibodies against myosin in the sera of all types of leprosy patients is shown in **Table 1**. Highest percent of seropositivity was observed in T1R (75%) followed by LL (56.25%), BL (50%), ENL (46.66%), BB (43.47%), and TT/BT (35%). The seropositivity of patients with T1R was found to be significantly higher than TT/BT ($p = 0.02$) using Fisher's exact test.

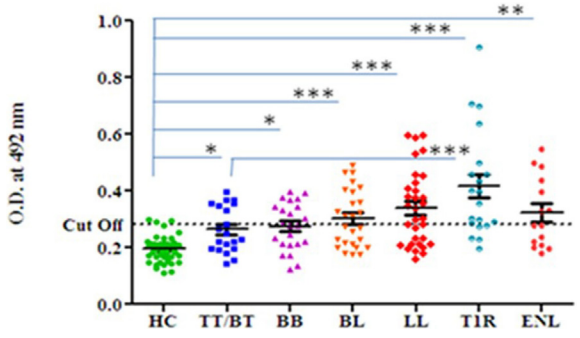

FIGURE 1 | Level of antibodies against myosin in leprosy patients across the spectrum and healthy controls (HC). Dotted horizontal line represents the cutoff OD value. Smooth line with vertical lines represents the mean OD value with SEM of each group. Each dot represents the OD value at 492 nm obtained from each individual. One-way ANOVA (and non-parametric) test and post-test used was Newman–Keuls multiple comparison test to find out the difference between OD value obtained in the sera of HC and leprosy patients (***p value < 0.0001, ** p value < 0.001, * p value < 0.05).

Lymphoproliferative Response of Leprosy Patients in the Presence of Host Myosin

The highest mean value of S.I. was obtained in T1R (4.06 ± 2.7) group of leprosy patients which was followed by BL/LL (2.71 ± 1.6), TT/BT (2.46 ± 1.3), and ENL (2.02 ± 1.4) patients.

The mean values of S.I. in the presence of myosin were found to be significantly higher in TT/BT ($p = 0.005$), BL/LL ($p = 0.004$), T1R ($p = 0.004$), ENL ($p = 0.05$) groups of leprosy patients in comparison to HC by using unpaired two-tailed t test (**Figure 2**).

Levels of IgG Antibodies Against Myosin in MLSA-Hyperimmunized Rabbit

Significantly higher levels of anti-myosin antibodies were observed in MLSA-hyperimmunized rabbit in comparison to control rabbit (average OD ± SD of MLSA hyperimmunized vs control 1.258 ± 0.16 vs 0.158 ± 0.03, $p < 0.05$). Highest levels of antibody against myosin (**Figure 3**) were observed at 35th day of immunization with MLSA.

IgG Antibody Levels Against Myosin in MLSA-Hyperimmunized Mice

It was observed that MLSA-hyperimmunized mice induce significantly elevated levels of anti-myosin antibodies in comparison to control mice ($p < 0.0001$). Mean level of antibodies against myosin was found to be significantly higher than pre-immunized (pre-immunized vs MLSA hyperimmunized 0.011 ± 0.009 vs 0.073 ± 0.035, $p < 0.0001$) and control group (control vs MLSA hyperimmunized 0.012 ± 0.012 vs 0.073 ± 0.035, $p < 0.0001$) at sixth week of inoculation with MLSA in female BALB/c mice by using one-way ANOVA (and non-parametric) and Bonferroni's multiple comparison post-test (**Figure 4**).

Adoptive Transfer With Immune Cells in Inbred Strains of Naïve Female BALB/c Mice

It was observed that significantly higher level of anti-myosin antibodies was observed in sera of adoptively transferred mice with nylon wool separated T cells (T cell vs pre-immunized 0.113 ± 0.0017 vs 0.090 ± 0.013, $p < 0.001$), splenocytes and lymph nodes cells (whole cell vs pre-immunized 0.1152 ± 0.027472 vs

TABLE 1 | Sero-positivity of anti-myosin antibodies in sera of leprosy patients and healthy controls (HC).

Subjects	HC	Leprosy patients					
	HC	TT/borderline tuberculoid	Borderline borderline	Borderline lepromatous	Lepromatous leprosy	Type 1 reaction	Erythema nodosum leprosum
Total number of individuals	45	20	23	26	32	20	15
Number of positive	3	7	10	13	18	15	7
Number of negative	42	13	13	13	14	5	8
Percentage positivity (%)	6.66	35*	43.47	50	56.25	75*	46.66

*p value < 0.05

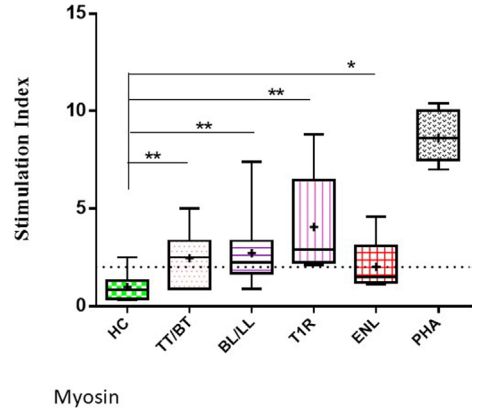

FIGURE 2 | Level of lymphoproliferation in the presence of myosin in leprosy patient across the spectrum. Graphical representation is done by Box and Whiskers. Each bar represents the minimum to maximum values with median as the horizontal line and SD as error bars. +sign in each bar represent the mean value. Dotted line represents the S.I. = 2 (**$p < 0.001$, *$p < 0.05$).

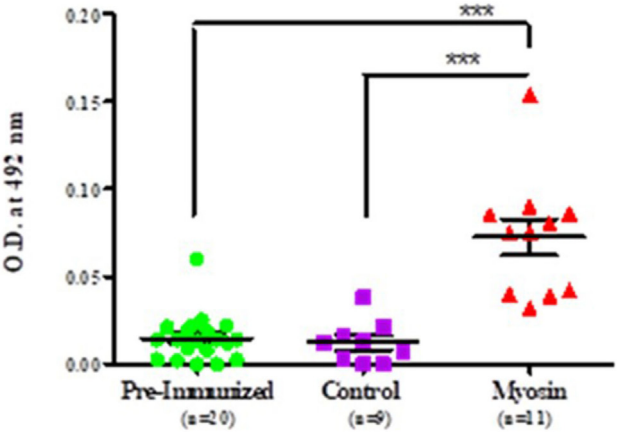

FIGURE 4 | Comparison in levels of autoantibodies against myosin in sera of *Mycobacterium leprae* soluble antigen-hyperimmunized female BALB/c mice, pre-immunized mice, and control mice. Each dot represents individual OD obtained from mouse plasma. Solid horizontal line with error bars represent mean OD with SEM (***$p < 0.0001$).

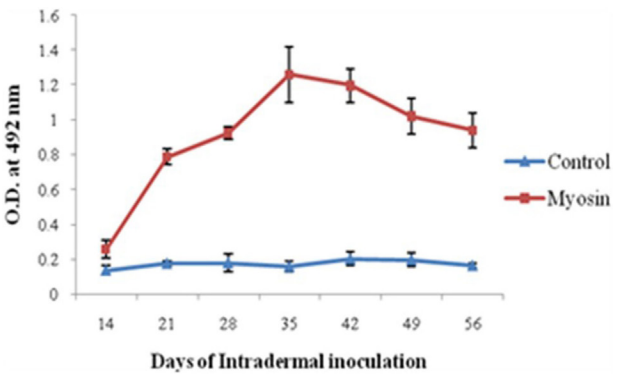

FIGURE 3 | Levels of antibodies against myosin in *Mycobacterium leprae* soluble antigen-hyperimmunized rabbit sera at different time intervals. Each dot with error bar represents the mean OD value with SD at different time intervals.

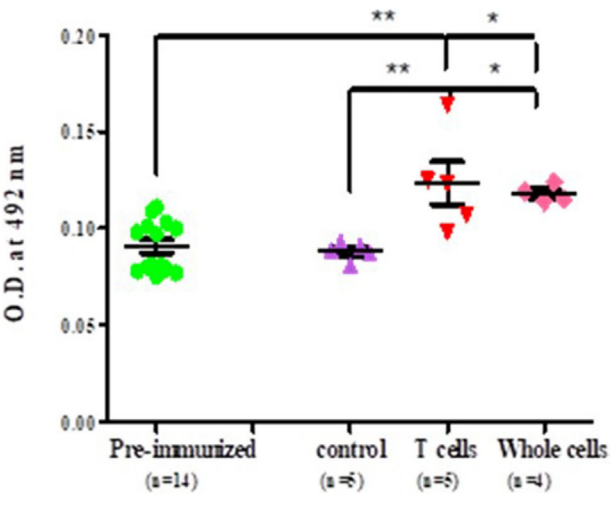

FIGURE 5 | Level of antibodies against myosin proteins in adoptively transferred naïve female BALB/c mice. Each dot represents the individual OD. Horizontal line with error bars represent mean OD value with SEM. Control group adoptively transferred with whole cells and experimental group adoptively transferred with T cells and whole cells.

0.0752 ± 0.004382, $p < 0.05$) in comparison to those of control and pre-immunized mice sera (**Figure 5**).

Cross-Reactive Proteins Between Host Myosin and Mycobacterial Components

It was observed that anti-myosin rabbit sera reacted with two isoforms of MLSA at ≈97 kDa, pI 4.5 and pI 7.0 (**Figures 6A,B**). Interestingly, we noted that pooled leprosy patients' sera reacted with myosin at ≈35 kDa, pI 4.6 (**Figures 6C,D**).

Identification of Cross-Reactive Proteins

The serum antibodies of leprosy patients reacted with tropomyosin alpha striated muscle isoforms (TM) (*Homo sapiens*) by MALDI analysis, whereas myosin- hyperimmunized rabbit sera that reacted with *Mycobacterium leprae* soluble antigen (MLSA) was identified as ATP-dependent C1p protease ATP-binding subunit (CLPC) of *M. leprae* (**Table 2**).

Identification of Mimicking B-Cell Epitopes Between Tropomyosin and Probable ATP-Dependent Clp Protease ATP-Binding Subunit of *M. leprae*

It was observed that four B-cell epitopes are mimicking epitopes of tropomyosin of host and probable ATP-dependent clp protease ATP-binding subunit of *M. leprae*. It was noted that $CLPC_{191–205}$ with $TM_{41–48}$ and $TM_{8–12}$, $CLPC_{237–248}$ with $TM_{49–60}$, $CLPC_{453–465}$ with $TM_{106–113}$ and $TM_{23–28}$ and $CLPC_{751–760}$ and $TM_{161–170}$ are putative mimicking epitopes (**Figure 7**).

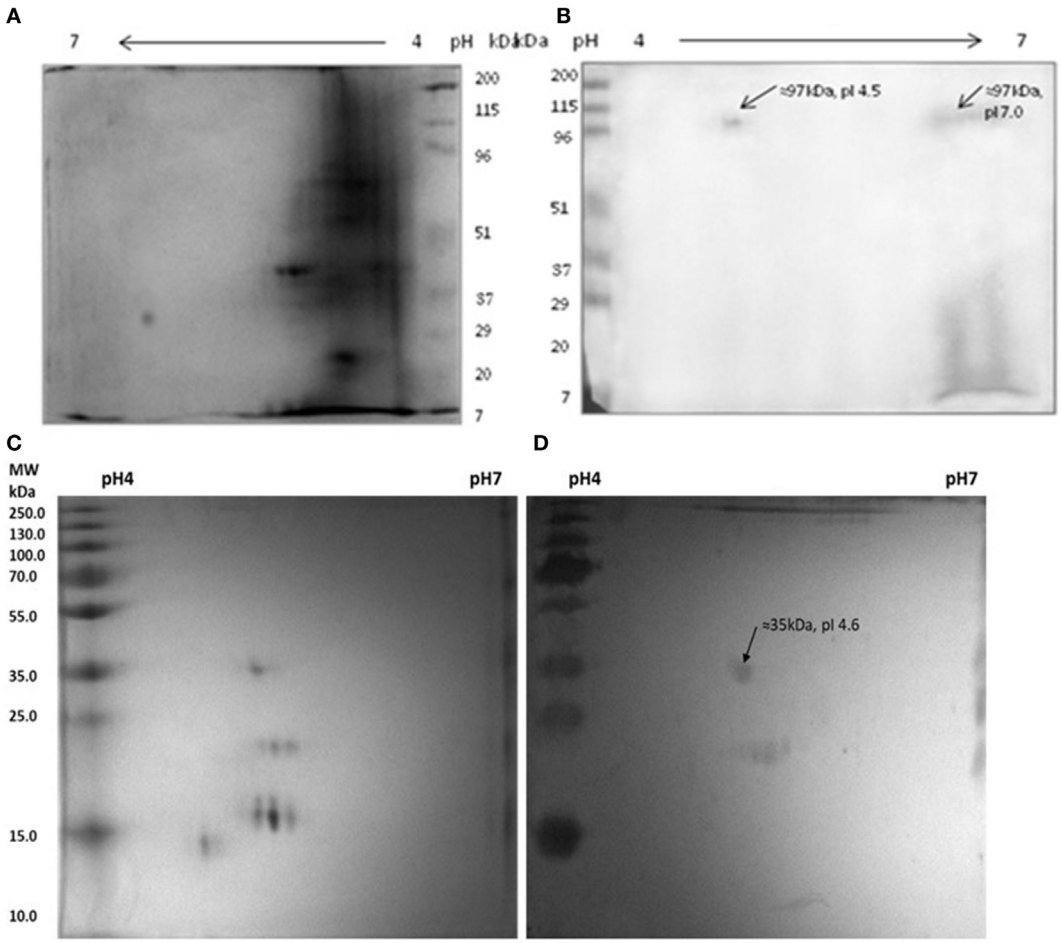

FIGURE 6 | Reactivity of anti-myosin rabbit sera with *Mycobacterium leprae* soluble antigen (MLSA) **(A,B)** pooled leprosy patients' sera with myosin **(C,D)**. **(A)** Protein profile of MLSA on 2-D gel stained with coomassie-blue, **(B)** Western blotting pattern of reactivity of anti-myosin rabbit sera with MLSA. **(C)** Protein profile of myosin on 2-D gel stained with coomassie-blue, **(D)** western blotting pattern of reactivity of pooled leprosy patients' sera with Myosin.

TABLE 2 | Cross-reactive proteins identified by MALDI-TOF.

Cross-reactive Protein	Protein identified	Accession number	Mascot Score	Nominal mass	pI	Sequence coverage (%)
Myosin cross-reacted with pooledleprosy patients' sera	Tropomyosin alpha striated muscle isoform (*Homo sapiens*)	AAT68295.1	93	32,690	4.67	42
MLSA cross-reacted with anti-myosin rabbit sera	ATP-dependent Clp protease ATP-binding subunit of *Mycobacterium leprae*	P24428	3	93,944	5.57	1

Three-Dimensional Structure of Tropomyosin and Probable ATP-Dependent Clp Protease ATP-Binding Subunit of *M. leprae*

Mimicking B-cell epitopes of both the proteins are highlighted on 3-dimensional structure of the proteins. It has been found that four putative mimicking B cell epitopes of CLPC of *M. leprae* and tropomyosin are present on the surface of the proteins (**Figures 8** and **9**).

DISCUSSION

In this study, we demonstrated the cross-reactivity of tropomyosin with sera of leprosy patients/*M. leprae* components using porcine myosin. The cross-reactivity is found in experimental animals also which are hyperimmunized with MLSA/porcine myosin. These results taken together suggest that common epitopes are shared between *M. leprae* and tropomyosin. Molecular mimicry is defined as epitopes shared between microbial antigens and host self-components (29) which may

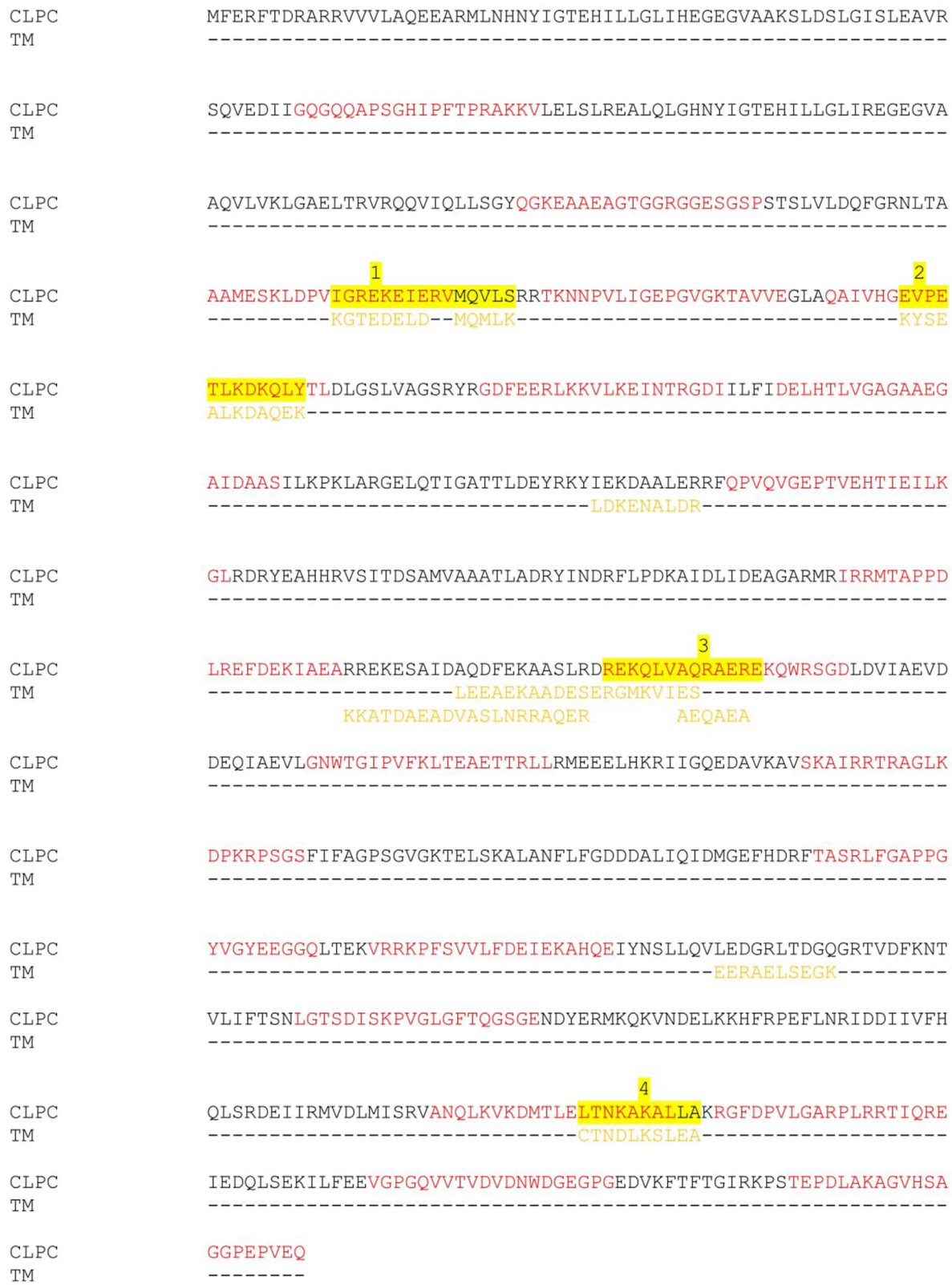

FIGURE 7 | Multiple sequence alignment of Probable ATP- dependent clp protease ATP-binding subunit (CLPC) of *Mycobacterium leprae* and B cell epitopes of tropomyosin (TM) of host. Red color—showing predicted B cell epitopes of CLPC of *M. leprae*. Purple color—showing predicted B cell epitopes of tropomyosin of host. Yellow color—highlighted sequences showing mimicking B cell epitopes of both the proteins.

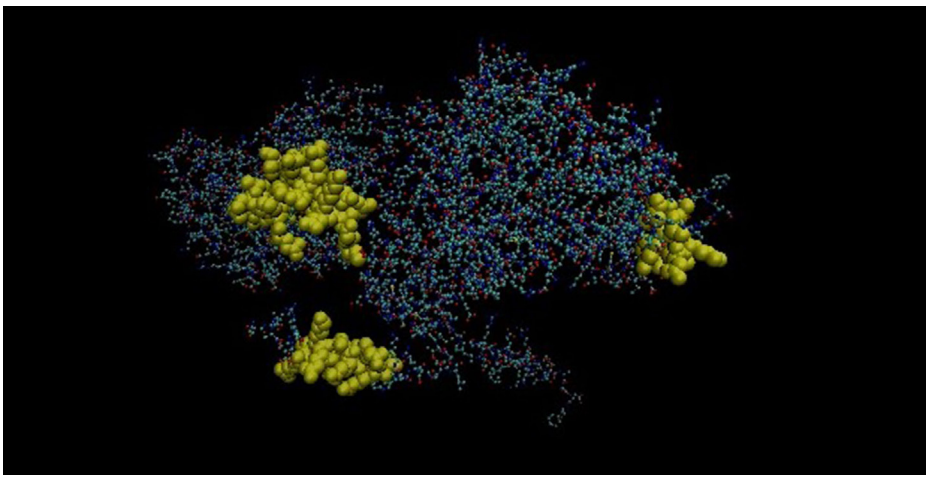

FIGURE 8 | Three-dimensional structure of Probable ATP-dependent clp protease ATP-binding subunit of *Mycobacterium leprae*.

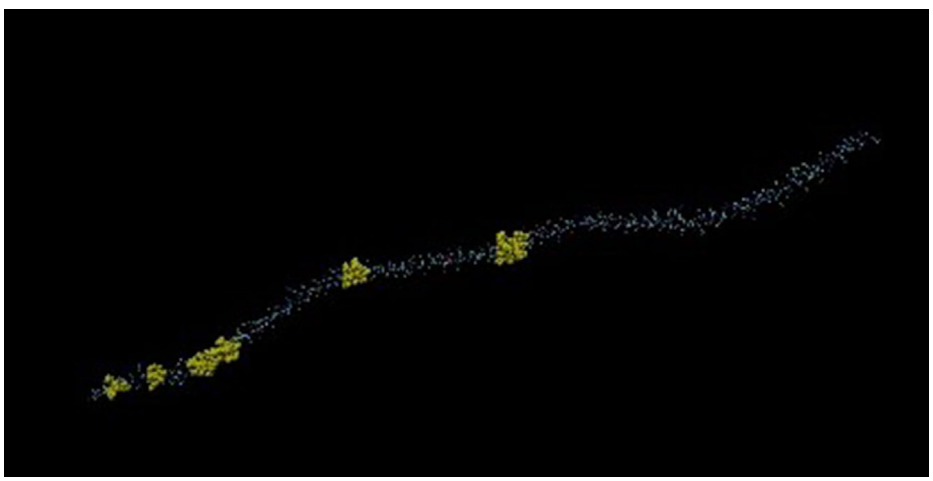

FIGURE 9 | Three-dimensional structure of tropomyosin. Yellow area showing the mimicking epitopes.

lead to autoimmunity, tissue injury and disease. We showed that significantly high levels of anti-myosin antibodies are present in all the groups of leprosy patients in comparison to HC. Highest level of anti-myosin antibodies was found in T1R (0.416 ± 0.18) which was followed by LL (0.339 ± 0.13), ENL (0.322 ± 0.12), BL (0.302 ± 0.10), BB (0.275 ± 0.08), and TT/BT (0.264 ± 0.08) (**Figure 1**). We observed significantly high lymphoproliferation with myosin in leprosy patients across the spectrum except ENL in comparison to HC (**Figure 2**). In the present study, porcine myosin was used to see the level of anti-myosin antibodies and lymphoproliferation in leprosy patients and experimental animals since, all the cytoskeletal proteins are conserved across the vertebrates and we observed high level of antibodies against this myosin in leprosy patients. We propose that molecular mimicry between putative epitopes of tropomyosin and *M. leprae* may potentially lead to loss of muscle functions in leprosy patients.

Leprosy is a chronic disease which affects both nerves and muscles. Leprosy is non-toxic disease and it was shown that most of the tissue and nerve damage occurs by host immune response to *M. leprae* antigens (30). Rambukkana et al. showed elegantly the immunological cross-reactivity between mycobacterial hsp 65 and human epidermal cytokeratin ½ (31). We recently reported existence of molecular mimicry between host cytokeratin-10 and HSP 65 (groEL2) of *M. leprae* (10) and between host myelin A1 and *M. leprae* 50S ribosomal L2 and lysyl tRNA synthetase proteins (11).

A central finding of this study is that MLSA induces antibodies against myosin in female BALB/c mice and this autoimmune reaction could be adoptively transferred to naïve mice. Hence, it supported our hypothesis that alteration in homeostatic mechanism may lead to autoimmune reaction and this autoimmunity is transferrable by autoreactive immune cells in naïve mice. Myosin reactive antibodies produced by immunization with MLSA could

be adoptively transferred to naïve mice even by T cell transfer could be explained by the proliferation of autoreactive B cells present in the secondary lymph nodes which are known to induce autoimmunity (32). It is also noted that MLSA induces antibodies against myosin in rabbit. It was earlier observed that mouse cytomegalovirus infection induces myocarditis in susceptible BALB/c mice by producing autoantibodies to cardiac myosin and it was concluded that there were common epitopes between both the proteins (33).

Presence of autoantibodies are common in leprosy patients (34). A key question is whether these autoantibodies are produced because of mimicking epitopes between host protein/s and *M. leprae* protein/s. Significantly elevated level of antibodies against myosin is observed in leprosy patients across the spectrum in comparison to HC indicates that anti-myosin antibodies are produced because of the presence of some cross-reactive regions between both the proteins. Significantly high lymphoproliferation with myosin antigen is also noted in leprosy patients in comparison to HC. It is possible that high CMI level with host antigen might also be because of similarity of myosin protein with *M. leprae* protein/s. This study indicates that the cross-reactivity is at the 35 kDa of porcine myosin with leprosy patients' sera and at 97 kDa of MLSA with anti-myosin rabbit sera. We propose that this cross-reactivity between myosin and MLSA may be because of presence of mimicking B cell epitopes in both the proteins. Further, these proteins are identified as tropomyosin of host and probable ATP-dependent clp protease ATP-binding subunit of *M. leprae*. We used porcine myosin for 2-D gel electrophoresis and western blotting but the reactive spot of porcine myosin with pooled leprosy patients' sera identified by MALDI-TOF analysis was tropomyosin. We expected to find myosin as the reactive spot but it turned out to be tropomyosin, and this reactivity might be because of the presence of tropomyosin in the porcine myosin that reacted with pooled leprosy patients' sera. Earlier reports from our group showing the presence of seven mimicking B cell epitopes of cytokeratin-10 and HSP 65 (10) and four mimicking B cell epitopes of myelin A1 and 50S ribosomal L2 and lysyl tRNA synthetase (11) were cross-reactive indicated their role in skin and nerve damage. Further, in the present study it is noted that four putative B cell epitopes are mimicking between tropomyosin and probable ATP-dependent clp protease ATP-binding subunit of *M. leprae*. These putative mimicking B cell epitopes might be responsible for "leprous myositis" leading to muscle damage in leprosy patients which has been reported earlier (17, 18). We have already reported in experimental mice that hyperimmunization with *M. leprae* antigen leads to lowering of Treg cells along with production of high levels of antibodies against *M. leprae* in addition to the production of high levels of autoantibodies against host proteins (10). Thus, these findings support our hypothesis that *M. leprae* infection can induce imbalance in homeostatic mechanism in immune system of the host and is responsible for the auto-reaction in leprosy patients.

For the first time, we identified the cross-reactive proteins between tropomyosin of host and probable ATP-dependent clp protease ATP-binding subunit of *M. leprae*. Further, it is noted that four B cell epitopes are putative mimicking B cell epitopes of both the proteins. We observed elevated level of antibodies against myosin and high level of CMI with myosin in leprosy patients in comparison to HC. The cross-reactive protein is at 97 kDa of *M. leprae* and at 35 kDa of myosin.

We also observed that this auto-reaction can be induced in experimental animals (rabbit and mice) after hyperimmunization with MLSA. This auto-reaction is transferrable to naïve mice with the help of immune cells. Hence, we conclude from our study that *M. leprae* infection can induce imbalance in the homeostatic mechanism of the host and can induce auto-reaction in leprosy patients. This induction in auto-reaction in leprosy patients is due to the presence of molecular mimicry between tropomyosin and probable ATP-dependent clp protease ATP-binding subunit of *M. leprae* which might be responsible for "leprous myositis" and muscular weakness.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Indian Council of Medical Research guidelines, National JALMA Institute for Leprosy & OMD Human Ethics Committee" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the "National JALMA Institute for Leprosy & OMD Human Ethics Committee". This study was carried out in accordance with the recommendations of "guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)," "National JALMA Institute for Leprosy & OMD Animal Ethics Committee". The protocol was approved by the "National JALMA Institute for Leprosy & OMD Animal Ethics Committee."

AUTHORS CONTRIBUTION

US conceived and designed the study; IS, KM, PS, VP, and AY performed the experiments and analyzed and interpreted the data; IS drafted the manuscript; KM, KK, DB, UG, and US critically reviewed the manuscript.

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Conflict of Interest Statement: 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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BCG and Adverse Events in the Context of Leprosy

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Background: Notwithstanding its beneficial immunoprophylactic outcomes regarding leprosy and childhood TB, BCG vaccination may cause adverse events, particularly of the skin. However, this local hyper-immune reactivity cannot be predicted before vaccination, nor is its association with protection against leprosy known. In this study we investigated the occurrence of adverse events after BCG (re)vaccination in contacts of leprosy patients and analyzed whether the concomitant systemic anti-mycobacterial immunity was associated with these skin manifestations.

Methods: Within a randomized controlled BCG vaccination trial in Bangladesh, 14,828 contacts of newly diagnosed leprosy patients received BCG vaccination between 2012 and 2017 and were examined for adverse events 8 to 12 weeks post-vaccination. From a selection of vaccinated contacts, venous blood was obtained at follow-up examination and stimulated with *Mycobacterium leprae* (*M. leprae*) antigens in overnight whole-blood assays (WBA). *M. leprae* phenolic glycolipid-I-specific antibodies and 32 cytokines were determined in WBAs of 13 individuals with and 13 individuals without adverse events after vaccination.

Results: Out of the 14,828 contacts who received BCG vaccination, 50 (0.34%) presented with adverse events, mainly (80%) consisting of skin ulcers. Based on the presence of BCG scars, 30 of these contacts (60%) had received BCG in this study as a booster vaccination. Similar to the pathological T-cell immunity observed for tuberculoid leprosy patients, contacts with adverse events at the site of BCG vaccination showed elevated IFN-γ levels in response to *M. leprae*-specific proteins in WBA. However, decreased levels of sCD40L in serum and GRO (CXCL1) in response to *M. leprae* simultaneously indicated less T-cell regulation in these individuals, potentially causing uncontrolled T-cell immunity damaging the skin.

Conclusion: Skin complications after BCG vaccination present surrogate markers for protective immunity against leprosy, but also indicate a higher risk of developing tuberculoid leprosy.

Clinical Trial Registration: Netherlands Trial Register: NTR3087.

Keywords: adverse events, BCG (re)vaccination, biomarker profiles, household contacts, protective immunity, leprosy, *Mycobacterium leprae*

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INTRODUCTION

Despite effective treatment of leprosy patients with multidrug therapy (MDT), the global number of new cases has not declined during the past decennium (1). A plausible explanation for this *status quo* could be that contacts of leprosy patients are prolonged and repetitively exposed to *Mycobacterium leprae* (*M. leprae*) before treatment of index cases is initiated, leading to continued bacterial transmission. Therefore, new tools and methodologies, such as immuno- and chemoprophylaxis regimens, are needed to interrupt transmission.

BCG vaccination offers variable protection against tuberculosis (2) and other mycobacterial diseases such as leprosy (3) and Buruli ulcer (4). Moreover, recently it has become clear that BCG can modulate the innate immune system also leading to protection through a mechanism referred to as trained immunity (5–7). The protective effect against TB thus induced in children by neonatal BCG vaccination influences cytokine responses to heterologous pathogens, an effect that is reported to be characterized by decreased anti-inflammatory cytokine responses, but increased IL-6 (5, 8).

In a previous study, immunoprophylaxis by BCG vaccination of contacts of newly diagnosed leprosy patients in Bangladesh conferred 56% protection, but was not affected by previous childhood BCG vaccination (9).

Although chemoprophylaxis does not protect a given individual from subsequent exposure to bacilli, the use of a single-dose rifampicin (SDR) in contacts in that study showed prevention of 56% in the first 2 years after chemoprophylaxis and treatment of the index case (10). Strikingly, if contacts had received BCG vaccination as part of a childhood vaccination program (as determined by the presence of a BCG-scar), the protective effect of SDR even reached 80%.

To investigate whether the effects of SDR and BCG can be complimentary, a cluster randomized controlled BCG vaccination trial is currently conducted in Bangladesh, analyzing the potential synergetic effect of these chemo- and immunoprophylactics by comparing the effect of BCG vaccination alone versus BCG followed by SDR after 8 to 12 weeks to prevent leprosy in contacts of new leprosy cases (designated the MALTALEP trial) (11).

In Bangladesh, BCG is routinely given to infants as part of the neonatal vaccination scheme as a prophylactic vaccine against tuberculosis. The coverage of BCG vaccination is estimated to be 98%[1]. Based on the visibility of BCG vaccination scars, 8,430 out of 14,779 contacts (57%) within this trial had received BCG vaccination at birth. However, since not all individuals receiving BCG develop a visible scar (12), this number is probably higher.

BCG vaccination has been reported to cause adverse effects within BCG childhood vaccination programs in endemic areas (13–16) as well as in BCG naïve individuals in leprosy and TB non-endemic areas (17–20). In the current study, we investigated the number and nature of adverse events occurring after BCG vaccination in the MALTALEP trial.

In addition, to investigate whether these adverse events can provide further insight into the protective effect of BCG, we analyzed cytokine production in *M. leprae*-antigen-stimulated whole-blood assays (WBA) of 13 contacts developing adverse events and 13 contacts matched for age and gender, lacking such complications.

MATERIALS AND METHODS

Study Population

Newly diagnosed leprosy patients and their household contacts (HCs) were recruited on a voluntary basis between 2012 and 2017 (**Table 1**). Leprosy was diagnosed based on clinical and bacteriological analysis and classified according to Ridley and Jopling (21). Leprosy patients were treated according to WHO standards. Contacts of consecutively diagnosed new leprosy patients were included in the districts of Nilphamari, Rangpur, Thakurgaon, and Panchagarh, in the northwest of Bangladesh (11). Each contact group consisted of around 15 contacts and were randomly assigned to receive BCG or BCG plus rifampicin. Immunization with BCG was given to all included contacts, when the index case received the second dose of MDT. At intake, before BCG vaccination, all contacts were examined for a BCG scar on the left upper arm. After 8 to 12 weeks, vaccinated contacts were reviewed for adverse events during follow-up examination. Contacts were categorized as household members (sharing either roof, kitchen or both) or direct neighbors. Contacts were excluded from the study according to criteria described previously.

Leprosy Prevalence

During this study, the prevalence in the four districts (Nilphamari, Rangpur, Panchagarh and Thakurgaon) in the northwest of Bangladesh was 0.82 per 10,000 with a new case detection rate of 0.98 per 10,000 (monthly report of the Rural Health Program of these four districts).

TABLE 1 | Characteristics of contacts with or without complication after BCG vaccination.

	Contacts with complication after BCG (% of total)	Contacts without complication after BCG	Total contacts who received BCG	*p*-value
Contacts	50	14,778	14,828	n.a.
Male	23 (0.34%)	6,677	6,700	0.91
Female	27 (0.33%)	8,101	8,128	
Child (5–16 years)	21 (0.43%)	4,829	4,850	0.16
Adult	29 (0.29%)	9,949	9,978	
No BCG scar visible	20 (0.32%)	6,336	6,356	0.68
BCG scar present	30 (0.35%)	8,430	8,460	
Vaccination status unknown	0	12	12	n.a.
Index with MB	19[a] (4.08%)	447	466	0.08
Index with PB	26[b] (2.42%)	1,047	1,073	

[a]*One household with a multibacillary (MB) index had two contacts with a BCG complication.*

[b]*One household with a paucibacillary (PB) index case had two contacts with a BCG complication, another household even had four contacts with a BCG complication.*

[1]http://www.who.int/immunization/monitoring_surveillance/data/bgd.pdf (Accessed: March 19, 2018).

Ethics

The MALTALEP trial is performed according to standard Good Clinical Practice (GCP) guidelines.[2] Participants were informed about the study objectives, the samples, and their right to refuse to take part in or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrollment from all participants. For illiterate people a thumb print was taken, and for minors under 16 years of age, the guardian's additional consent was obtained. All patients received treatment according to national guidelines. Participants were informed about the potential adverse events of the trial, that free consultation and treatment would be offered in case of adverse events and requested to report any suspected adverse events to the responsible field worker. Ethical approval of the study-protocol was obtained through the National Research Ethics Committee (Bangladesh Medical Research Council; Protocol no. BMRC/NREC/2010-2013/1534).

BCG Vaccination

Vaccination was performed between September 2012 and February 2017. BCG was administered intradermally. The BCG vaccine used in this trial (Japan BCG Laboratory, Tokyo, Japan) is also used in the routine neonatal vaccination program of Bangladesh. Vaccines were stored at the State Immunization Program facilities in the four different districts of the study area and kept at 0–4°C throughout the fieldwork.

Adverse Events

All contacts receiving vaccination were provided with a vaccination card with details on how to reach the researcher in case of questions or adverse events. Contacts with self-reported adverse events were examined by field staff. Additionally, all contacts were examined 8 to 12 weeks after administration of the BCG. Data on adverse events were collected on the MALTALEP Contact Registration forms and on a separate BCG complication form (11). In the case of an adverse event following BCG complication, contacts were referred to the state tuberculosis medical officers for treatment. Ulcers were considered abnormal if they were larger than 10 mm diameter in size, or if they presented in combination with fever and malaise. Contacts were also checked for the presence of lymphadenopathy, abnormal scarring and keloids and if the course of the complication was different than normal. To document the size of the ulcers, pictures were taken of each BCG complication case and stored in a database.

Samples for Immunological Analysis

Blood was drawn from 15 contacts who developed an adverse event after receiving BCG vaccination. Two contacts were excluded from the analysis, because they later developed leprosy. Cytokine levels in WBA of 13 contacts with adverse events were analyzed and compared with those in contacts without (a scar or ulcer of <10 mm). WBA were performed for both groups and anti-phenolic glycolipid-I (PGL-I) serology cytokines and chemokines concentrations in supernatants were assessed.

[2] www.ich.org (Accessed: March 19, 2018).

Whole Blood Assays (WBA)

Venous blood was drawn from contacts at the time BCG complications occurred, which was on average 7.9 weeks after receiving BCG. As a control group, contacts without complications were tested. Controls were matched for age and gender as well as time point at which blood was drawn (on average 7.7 weeks; **Table 2**). Heparinized blood (4 mL) was directly added to microtubes pre-coated with *M. leprae* whole cell sonicate (WCS), *M. leprae*-unique recombinant proteins ML2478 and ML0840 (designated Mlep) (22), or without antigen stimulus (designated NIL) (11, 23). After 24-h incubation at 37°C materials were frozen at −20°C, shipped on dry ice to the LUMC, and stored at −80°C until analysis.

Cytokine-Chemokine Analysis

sCD40L, EGF, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-17A, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AB/BB, PDGF-AA, RANTES, TGF-α, TNF-α, TNF-β, VEGF, and Eotaxin (CCL11) in WBA supernatants were measured with the Milliplex magnetic bead kit (Merck, USA) on 96 well multiscreen filter plates (Millipore, USA) using the Bio-Plex-100-suspension-array-system (BioRad, Veenendaal) and analyzed using the Bio-Plex Manager software 6.1 (Bio-Rad Laboratories, Veenendaal, The Netherlands) (22). After pre-wetting the filter with assay solution, supernatant samples (25 µL) were added to the plates, together with 25-µL assay buffer and 25-µL beads, and the plates were incubated overnight at 4°C. After two washing steps with 200-µL wash buffer using a vacuum pump (Millipore, USA), 25-µL detection Ab mixture was added per well, and plates were incubated at room temperature in the dark for 1 h on a plate shaker at 300 rpm. Streptavidin-PE solution (25 µL per well) was added and incubated for 30 min at room temperature in the dark. After two washes, 150-µL Sheath Fluid was added to each well, and the plates were placed in the Bio-Plex System. From each well, a minimum of 50 analyte-specific beads were analyzed for fluorescence. A curve fit was applied to each standard curve according to the manufacturer's manual. Sample concentrations were interpolated from these standard curves. Analyte concentrations outside the upper or lower limits of quantification were

TABLE 2 | Characteristics of contacts with BCG-related complications and matched controls.

	Complications	No complications
Number of contacts	13	13
Average age (years)	33.8	36.2
Number of females	8	8
Number of males	5	5
Average no. of weeks between BCG and WBA	7.9 (1.0–13.5)	7.7 (4.0–10.0)
Presence of BCG scar before study	8	6
Average size of BCG scar/ulcer (in mm)	14.8 (4.5–27)	3.4 (2.5–4.5)
Received SDR before blood drawing	1[b]	3[a]
Received no SDR	12	10

[a] *All controls received SDR 2 weeks before blood was drawn.*

[b] *The contacts with a complication after BCG vaccination received SDR 4 weeks before experiencing the adverse event at 13 weeks post vaccination.*

assigned the values of the limits of quantification of the cytokine or chemokine.

PGL-I and *M. leprae* WCS

Synthesized disaccharide epitope [3,6-di-O-methyl-β-D-glucopyranosyl(1→4)2,3-di-O-methylrhamnopyranoside], similar to *M. leprae*-specific PGL-I glycolipid, coupled to human serum albumin (synthetic PGL-I; designated ND–O–HSA) and *M. leprae* WCS, generated with support from the NIH/NIAID Leprosy Contract N01-AI-25469, were obtained through the Biodefense and Emerging Infections Research Resources Repository[3] (24).

PGL-I ELISA

IgM and IgG antibodies against synthetic PGL-I were detected as previously described adapted for the use of specific IgM and IgG antibody detection (22, 25). A synthetic analog of the *M. leprae*-specific PGL-I (ND–O–HSA) was coated onto high-affinity polysorp Immulon 4HBX 96-well Nunc ELISA plates (Thermo Scientific, Rochester, NY, USA) using 200 ng/well in 50-µL 0.1-M sodium carbonate/bicarbonate pH 9.6 (i.e., coating buffer) at 4°C overnight. Unbound Ag was removed by washing with PBS containing 0.05% Tween 20 (washing buffer) six times and wells were blocked with PBS containing 1% BSA (Roche Diagnostics, Germany) and 0.05% Tween 80 for 1 h at room temperature. 50 µL of 1:400 diluted serum/plasma (PBS/0.01% BSA as dilution buffer) was added to the wells and incubated for 2 h at room temperature. After incubation, wells were washed six times with washing buffer, followed by the addition of 50 µL of 1:8,000 antihuman IgM-HRP (Sigma A6907) or 1:4,000 antihuman IgG-HRP (DAKO P0214) and incubated for 2 h at room temperature. Following washing, the wells with the wash buffer, 50 µL 3.3′,5.5′-tetramethylbenzidine (TMB) was added and the color reaction was stopped using H2SO4 after 10–15 min. The absorbance was determined at wavelength of 450 nm. Samples with an optical density (OD_{450}), after correction for background, >0.20 were considered positive. The cutoff for positivity was determined by a threefold multiplication of the average value for non-endemic control individuals.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA, USA),[4] SPSS Statistics 24,[5] and R Version 3.3.0 (R, Vienna, Austria).[6] A chi-square test was performed for contacts who developed BCG complications to identify potential differences compared with the control contacts' characteristics (**Table 1**). A significance level of $p \leq 0.05$ was used.

For identification of an immune biomarker signature associated with skin complications after BCG vaccination, a global test was used (26), which provided hierarchical clustering of the cytokines/chemokines based on absolute correlation difference and average linkage. Moreover, the Mann–Whitney *U* test was performed to identify differences in group mean levels of host markers. The statistical significance level used was $p \leq 0.05$. For significantly different markers in both the global test and Mann–Whitney *U* test, the diagnostic potential was assessed by receiver operating characteristic curve (ROC) analysis to determine the area under the curve (AUC). The cutoff values for optimal sensitivity and specificity were determined by calculating the Youden's Index (27). To construct a biomarker profile, a linear discriminant analysis (LDA) was performed in SPSS. Analytes were ranked based on the pooled within-group correlations between discriminating variables and standardized canonical discriminant functions. The six most contributing analytes to the discriminant function were selected to construct a biomarker profile. The profile was constructed stepwise, determining the optimal sensitivity and specificity for each step. The optimal cutoff was determined per analyte after which each individual was designated positive or negative for all analytes separately.

RESULTS

Occurrence of Adverse Events After BCG Vaccination

Out of the 14,828 contacts who received BCG vaccination within the trial, 50 (0.34%) presented with vaccination-related adverse events (**Table 1**). The most common adverse events were skin ulcers (Table S1 in Supplementary Material; **Figure 1A**). A total of 40 contacts (80%) developed large skin ulcers varying between 10 and 35 mm; four of these also had axillary lymphadenopathy and one had enlarged lymph nodes. One ulcer was 8 mm, but was included as adverse event because the contact also reported malaise and mild fever. Keloids (**Figure 1B**) were present in eight contacts, of whom three were small (<1 cm) and three were >1 cm. One contact developed a persistent keloid, which was first signaled 1 year after receiving BCG vaccination. When excluding the contact with persistent keloid, the average time between BCG vaccination and initiation of complication in the 50 contacts was 5.5 weeks.

Variations in BCG-Vaccination-Related Adverse Events

In four contacts, adverse events manifested differently: one woman developed an abscess, which was incised and drained at home 3 months after vaccination, then developed intermittent fever and was treated unsuccessfully with various antibiotics of unknown kind provided by different doctors. After 1 year, the contact was admitted for investigation, because of an erythematous nodule (2 cm × 2 cm) surrounded by scarring. She was re-incised by a plastic surgeon upon suspicion of a deep-seated abscess. The histological report of the biopsy showed a keloid scar (**Figure 1B**).

A second contact had a persistent pustule of 5 mm 5 months after receiving BCG, felt weak, and had coughed for the past 2 months. She only had a 2-day history of fever and was tested sputum-negative for acid-fast bacilli (AFB). The pustule was not opened, but kept clean and dry and healed after a course of flucloxacillin.

[3] http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx (Accessed: March 19, 2018).
[4] http://www.graphpad.com (Accessed: March 19, 2018).
[5] http://www.spss.com.hk (Accessed: March 19, 2018).
[6] http://www.R-project.org (Accessed: March 19, 2018).

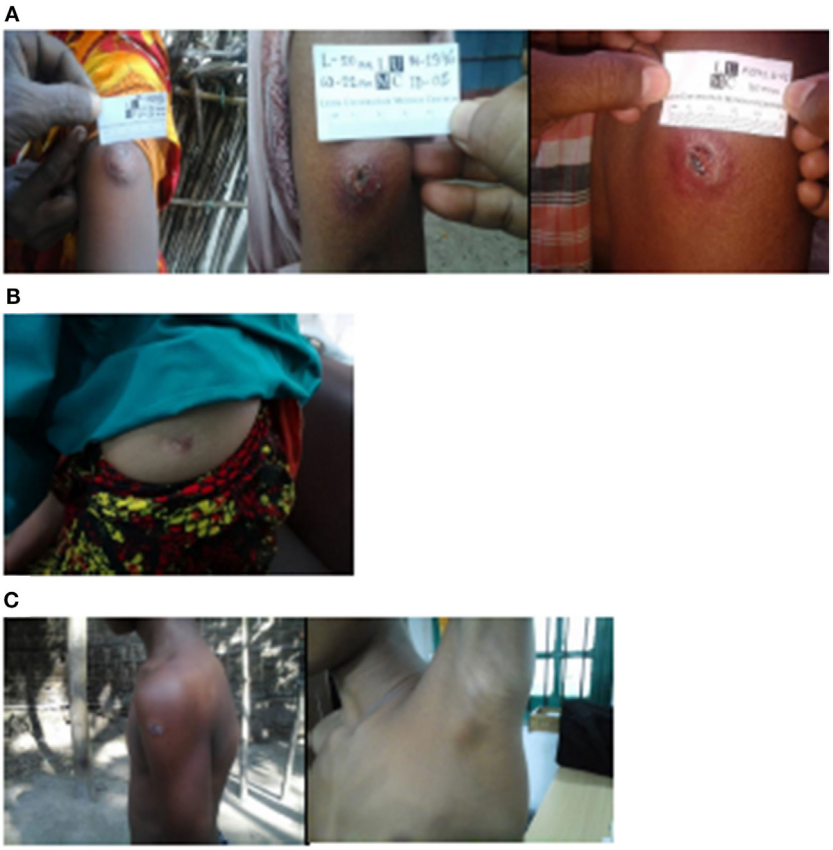

FIGURE 1 | Representative examples of skin complications after BCG vaccination. **(A)** Three contacts with big ulcers (>10 mm). **(B)** A contact with keloid (picture taken before operation). **(C)** A contact with an ulcer and lymphadenitis who developed leprosy at follow-up.

A third contact had developed a large scar (12 mm × 10 mm) and many small ulcers on both arms and legs after receiving BCG. She received unknown medication from an outside doctor and the lesions healed.

Finally, the fourth contact presented with an ulcer at the BCG injection site of 10 mm × 15 mm and mild left axillary lymphadenopathy. Already before BCG vaccination, the contact had a history of occasional fever and pain palpable on the ribs, which was treated with pain killers. He had no known contact to TB patients, and was sputum- and X-ray negative for TB.

In addition to adverse events, two contacts also developed leprosy following BCG vaccination (**Figure 1C**). One had a small keloid, and the other had an ulcer of 15 mm × 20 mm with lymphadenitis (**Figure 1B**).

The average age at the time of the adverse event was 30 years, with a range of 6 to 80 years. Similar numbers of males and females were identified with adverse events (**Table 1**). More than half (60%) received a revaccination, based on the presence of a BCG scar. A higher number of children aged between 5 and 16 years old (as aged under 5s were excluded) developed BCG adverse events compared with adults (0.43% versus 0.29%); however, this number was not statistically significant ($p = 0.16$; **Table 1**). A slightly higher but statistically not significant number of contacts who received BCG for the second time developed adverse events compared with those who lacked a BCG scar (0.35% versus 0.32%; $p = 0.68$). Despite that an almost double amount of contacts developed adverse events when the index patient had multibacillary (MB) leprosy, compared with paucibacillary (PB) leprosy, this increase was not statistically significant either ($p = 0.08$).

Among the 13 contacts with an adverse event after BCG from whom blood was analyzed, nine had large ulcers >10 mm, one patient had an ulcer of 8 mm, but with general malaise, one had a keloid, one had a big scar, and one had an enlarged lymph node.

Anti-PGL-I IgM Levels

To estimate whether the extent of seropositivity in contacts of leprosy patients could already indicate whether complications could occur after BCG vaccination, the levels of anti-*M. leprae* PGL-I IgM antibodies, as estimated by the optical density at 450 nm (OD_{450}), were measured in sera of 26 individuals: 13 with and 13 without BCG complications (**Figure 2**). Three contacts from both groups were seropositive for IgM against PGL-I ($OD_{450} > 0.2$), but no significant differences were observed between both groups.

Immune Profiles Coinciding With Adverse Events After BCG Vaccination

To assess what type of immune profile (i.e., combinations of cytokines in *M. leprae*-stimulated WBA) is associated with

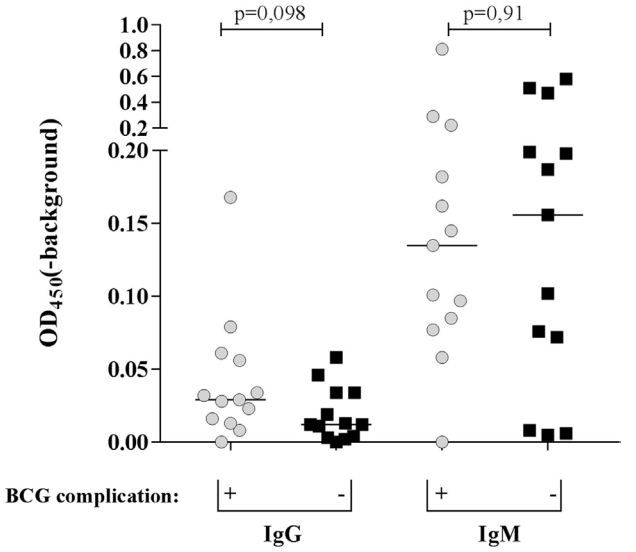

FIGURE 2 | *Mycobacterium leprae* phenolic glycolipid-I (PGL-I)-specific antibodies in contacts of leprosy patients with or without BCG-induced skin complications. IgG and IgM antibodies directed against synthetic PGL-I (ND–O–HSA) were determined by ELISA. Samples with OD_{450} (corrected for background OD) >0.2 were considered seropositive. No statistically different levels of IgG and IgM antibodies were observed between the contacts with (+ ; gray dots) or without (–; black squares) complications.

BCG-related complications, a global test (26) was performed on all 32 cytokines stratified by stimulus used in the WBA (**Figure 3**). This analysis showed that three analytes were significantly different between the two contact groups: decreased levels of $sCD40L_{NIL}$ (soluble cluster of differentiation ligand 40, without stimulation) and GRO_{WCS} (growth-regulated oncogene, in response to *M. leprae* WCS) were significantly associated with occurrence of BCG complications ($p = 0.03$ and 0.013, respectively; **Figures 3** and **4**). In contrast, increased levels of IFN-γ in response to *M. leprae*-specific proteins (IFN-γ_{Mlep}; $p = 0.012$) were observed in individuals developing BCG complications (**Figures 3** and **4**). Individually these three markers enable a good distinction between contacts with BCG-related complications and those without, showing an AUC of 0.75 for sCD40L and 0.78 for both GRO_{WCS} and IFN-γ_{Mlep} (**Figure 3**). Using a LDA three additional markers $CCL4_{NIL}$, IL-6_{Mlep}, and $GCSF_{NIL}$ that were decreased in individuals with adverse events, were identified, that improved the signature for adverse events. Next, the six analytes were ranked based on their contribution to the discriminant function and sequentially added to the biomarker profile (**Table 3**) and scored for each individual as positive or negative based on the optimal cutoff. This showed that optimal sensitivity (100%) was observed for the combination of $sCD40L_{NIL}$, IFN-γ_{Mlep}, and GRO_{WCS} showing 76% specificity and an AUC of 0.94 ($p < 0.0001$). On the other hand, optimal specificity (100%) was achieved by a five marker profile ($sCD40L_{NIL}$, IFN-γ_{Mlep}, GRO_{WCS}, $CCL4_{NIL}$, and IL-6_{Mlep}), with a sensitivity of 84% and an AUC of 0.96. The cutoff of >3.5 indicates that none of the contacts without complications scores positive for more than 4 out of 5 markers, thereby showing addition of markers improves the specificity. The five marker profile was optimal, as addition of a sixth marker slightly decreased the AUC from 0.96 to 0.93 (**Table 3**).

DISCUSSION

Within a cluster randomized controlled BCG vaccination trial in contacts of leprosy patients in Bangladesh, adverse events were observed in 0.34% of the recipients. These complications consisted primarily (80%) of skin ulcerations and were associated with increased Th1 immunity, inflammation and reduced T-cell regulation in WBA.

Although serious adverse events after BCG vaccination are rare, as many as 95% of BCG recipients have an uncomplicated, local reaction at the site of inoculation, characterized by the appearance of a pustule in combination with pain, swelling, and erythema within 2 to 3 weeks after vaccination. In approximately 70% of the cases, ulceration with drainage occurs at the vaccine site after about 6 weeks, resulting in a lesion of about 5 mm in diameter. Lesions usually heal within 3 months with permanent residual scarring at the vaccination site. Rare local abscesses and ulcers usually occur between 1 and 5 months post-vaccination, but adverse events have also been reported after longer periods of time (28). Lymphadenopathy occurs in the drainage area of the vaccinated site, so is most common in the axilla and sometimes in the cervical lymph nodes (28). Even more uncommon are serious adverse events such as osteitis, osteomyelitis and disseminated infection (19). Disseminated disease following BCG vaccination occurs usually with immunosuppression, such as HIV-infection (16) or genetic immune deficiency (29), which develops in less than one in a million (20).

The incidence of adverse events of 0.34% in this study is comparable with the 0.02 to 5% described in previous studies (13–15, 18, 28). A trial evaluating the incidence of adverse events to primary and booster BCG vaccination in schoolchildren in Salvador, Bahia (Brazil) (14) observed a rate of 0.35 per 1,000 vaccinations, without lethal cases or disseminated infections. Although not statistically significant, adverse events after booster vaccinations were approximately twice the rate compared with primary vaccination with BCG. The median time to onset of complications was 26 days, 12 days shorter than observed in Bangladesh. Similarly, 0.38 out of 1,000 vaccinated individuals developed complications in a study in the Brazilian Amazon (15). In contrast, the risk in the group receiving a revaccination was only >1.05 in the group receiving a first dose, similar to what we found in our Bangladesh study (0.35 versus 0.32%; $p = 0.68$).

The presence of a BCG scar is considered a highly sensitive indicator of the vaccination status as 92% of individuals aged 1–4 months at vaccination, develops a visible scar at 7–12 months of age, which declines to 84% at 4 years (12). When BCG is given to an infant before they are 1 month old, 90% has a scar at 7–12 months of age and 76% has a scar at 4 years. In this study, we used the absence of a BCG scar to designate the lack of previous (childhood) vaccination. However, since 16–24% of BCG vaccinated individuals do not develop a scar, it could be that a larger number of individuals actually received a BCG booster in the MALTALEP trial than is estimated solely based on the presence of a scar.

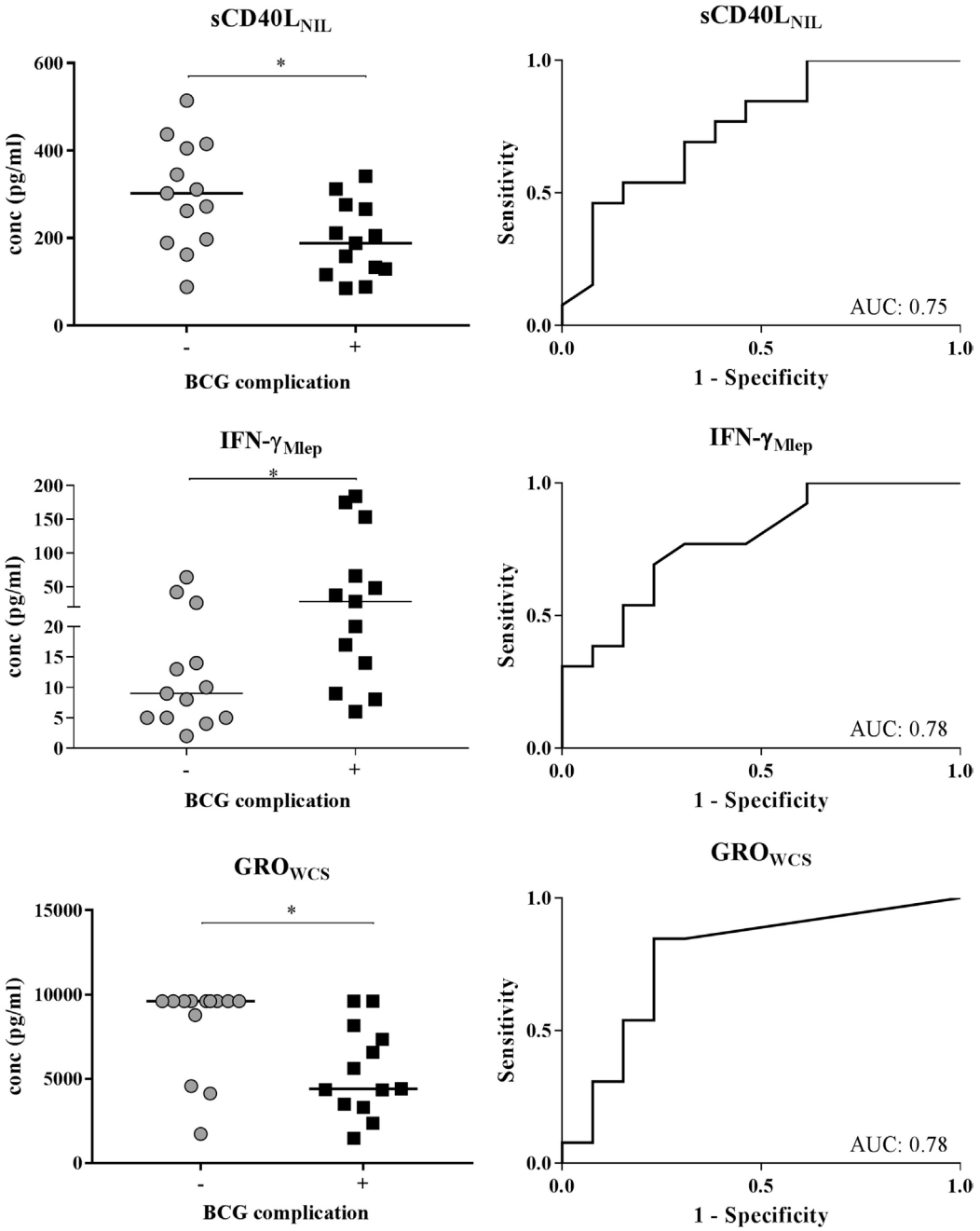

FIGURE 3 | Cytokine concentrations in 24-h whole-blood assays (WBA) with or without stimulation with *Mycobacterium leprae* (*M. leprae*) unique proteins (Mlep) or *M. leprae* whole cell sonicate (WCS) in contacts with and without BCG complications (left panels). The global test (26) indicated that sCD40Lmed, GROwcs, and IFN-γ_{Mlep} were significantly different between BCG-vaccinated contacts of leprosy patients with BCG-related complications and those without. This was confirmed by a Mann–Whitney *U* test. *$p < 0.05$–0.01. Receiver operating characteristic curves (ROCs) were computed and the area under the curve (AUC) is indicated for each analyte (right panels). The limits of detections for sCD40Lmed were 1.5–10,000, for GROwcs were 12.5–9,600. and IFN-γ_{Mlep} were 2–10,000.

The development of leprosy after BCG vaccination can be considered an ultimate adverse event. In a previous study (30), we observed an unexpectedly high proportion of new leprosy patients (mainly PB and leprosy type-1 reactions) among apparently healthy HCs of leprosy patients within the first 3 months after BCG vaccination (0.4% of vaccinated contacts). Of these, 43% had a BCG scar before vaccination in the trial. However, it remains unclear whether BCG vaccination merely catalyzes the formation of clinical symptoms in individuals who are bound to develop leprosy, or whether patients would not have developed the disease without this vaccination.

Several recent studies show that BCG alters the innate immune system by trained immunity (5–7). The protective effect against TB induced by neonatal BCG vaccination coincides with

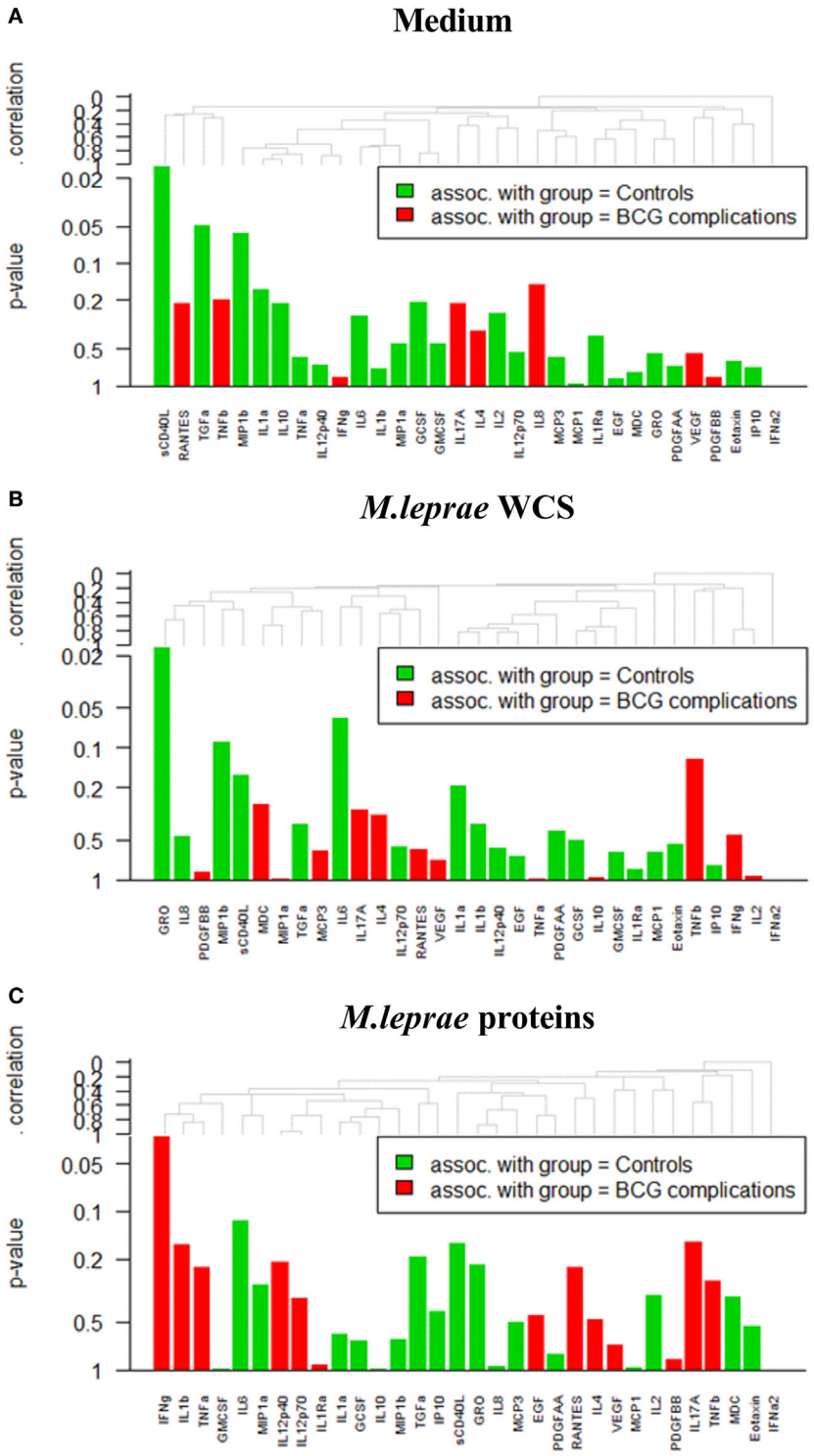

FIGURE 4 | Results of whole-blood assays (WBAs) in contacts with and without BCG complications in **(A)** medium (designated NIL). **(B)** *Mycobacterium leprae* whole cell sonicate (designated WCS). **(C)** ML2478/ML0840 recombinant proteins (designated Mlep) **(C)**.

protection against heterologous pathogens. This effect is characterized by decreased anti-inflammatory cytokine responses, but increased IL-6 in unstimulated samples (8). In another study, a BCG vaccination-induced increase in IL-6, EGF, and PDGF-AB/BB and decrease in IP-10, IL-2, IL-13, IL-17, GM-CSF, and GRO was observed in response to various non-specific innate immunity

TABLE 3 | Ability of analytes to distinguish contacts with adverse events in whole blood assays.

	Single markers				Signature			
Step	**Analyte**	**Correlation**	**Stimulus**	***p*-value**	**AUC**	**Sens.**	**Spec.**	**Cutoff**
1	sCD40L	0.086	NIL	0.0262	0.75	85%	54%	<289
2	IFN-γ	0.076	Mlep	0.0124	0.83	62%	92%	>1.5
3	GRO	0.070	WCS	0.0126	0.94	100%	76%	>1.5
4	CCL4	0.066	NIL	0.1254	0.94	92%	85%	>2.5
5	IL-6	–0.055	Mlep	0.2234	0.96	84%	100%	>3.5
6	GCSF	0.043	NIL	0.2428	0.93	85%	92%	>3.5

Step-by-step addition of analytes ranked by absolute size of correlation within discriminant function. For each step, the analyte that was added to the signature specific for occurrence of BCG vaccination-related adverse events, the absolute size of correlation generated from the linear discriminant analysis, the stimulus, p-value (Mann–Whitney U test), area under the curve (AUC), and the sensitivity (sens.) and specificity (spec.) based on the optimal cutoff are shown. The following three different stimuli used were: M. leprae whole cell sonicate (WCS), ML2478/ML0840 recombinant proteins (Mlep), or without antigen stimulus (NIL).

stimuli (PAM3Cys, *C. albicans*, and *S. aureus*). Along with this cytokine biomarker signature, increased CD69 expression on NK cells was observed as well (Dockrell, 2017 #483).

T-helper 1 (Th1) host-cellular immunity is generally considered to be key in controlling mycobacterial infections (31). However, clinical presentation of tuberculoid leprosy as well as type-1 (reversal) reactions also coincides with strong *M. leprae*-specific Th1 immunity and high levels of pro-inflammatory cytokines (32).

Despite the apparent homology between the mycobacteria, BCG but not *M. leprae* can stimulate monocytes to initiate a protective type-1 cascade (33). Moreover, *in vitro* exposure of monocytes from healthy donors to *M. leprae* (or *M. leprae* PGL-I) reduced levels of Th1-type cytokines and expression of macrophage type-1 (Mϕ1) cell surface markers (33). In contrast, *ex vivo* stimulation of peripheral blood mononuclear cells (PBMCs) with BCG or purified protein derivative of tuberculin (PPD) from 10-week-old infants in South Africa, who had received neonatal BCG vaccination, showed upregulation of mϕ1-associated genes whereas mϕ2 associated genes were down-regulated (34), indicating BCG-induced protective immunity. Also, in response to *M. leprae*, monocytes from these infants released higher levels of inflammatory cytokines TNF-α and IL-1β compared with monocytes from unvaccinated infants (33). Similarly, cytokine profiles of infants from the United Kingdom receiving BCG vaccination (35) showed that a higher number of IFN-γ^+ TNF-α^+ IL-2^+ multifunctional $CD4^+$ T cells were associated with growth inhibition of mycobacteria. Although T-cell activation (HLA-DR^+CD4^+ T cells) was a risk factor for TB disease, increased numbers of BCG-specific T cells secreting IFN-γ were detected in BCG vaccinated infants without TB (36). These studies indicate that pro-inflammatory Th1 immunity, although not the only factor, is associated with BCG-induced protection against tuberculosis. Similarly, the Mitsuda reaction measures whether an adequate immune response to an intradermal injection of the heat-killed leprosy bacilli (lepromin) is initiated, as it has a good prognostic value for susceptibility (when negative) or resistance (when positive) to the lepromatous form of leprosy (37). In line with that it was also observed that individuals that showed large local reactogenicity after intradermal BCG administration or lepromin injection are reported to have less risk for leprosy onset (38).

In a BCG vaccination study in 12 tuberculin skin test (TST) and Quantiferon negative, BCG- naïve adults in The Netherlands, local skin reactions varied strongly between individuals (17). It was observed that BCG vaccination induced significant Th1-type immunity ($CD4^+$ IFN-γ^+, IL-2^+ TNF-α^+ and $CD8^+$ IFN-γ^+ T cells) in those that presented with high local inflammation responses, with a peak 8-week post-vaccination. Of note is that BCG vaccination significantly increased regulatory $CD8^+$ T cells such as $CD25^+$ $Foxp3^+$ $CD39^+$ $CD8^+$ T cells as well as $CD25^+$ $Foxp3^+$ $CD39^+$ LAG-3^+ $CCL4^+$ $CD8^+$ T cells in low inflammation responders.

Similarly, individuals who developed (skin) complications in Bangladesh also produced higher levels of IFN-γ in response to *M. leprae* antigens around 8 weeks (average 7.9) post-vaccination, although at least 8 out of 13 contacts with BCG complications were not BCG-naïve and the *a priori* chance of exposure to mycobacteria was considerably larger. In contrast to the Dutch cohort, CRP levels were high in both groups and did not differ significantly (Figure S2 in Supplementary Material).

Of note in the current study are the lower levels of $sCD40L_{NIL}$ and GRO_{WCS} that were significantly associated with BCG complications, concomitantly with elevated IFN-γ levels in response to *M. leprae* unique proteins ($IFN\text{-}\gamma_{Mlep}$). GRO (CXCL1) is expressed by macrophages, neutrophils and epithelial cells and has neutrophil chemoattractant activity. Although the role of GRO in leprosy pathology has not been investigated, increase in GRO levels can reduce severity of multiple sclerosis (39). This neuroprotective role for CXCL1 could well be consistent with the onset of complications upon its reduction after *M. leprae* WCS stimulation as observed in our study. Moreover, in UK-born, BCG-vaccinated infants the levels of GRO in response to non-specific innate immunity stimuli were suppressed as well, in line with our finding in Bangladesh (5).

Recently, it was shown that higher levels of sCD40L present in serum of patients with Behçet's disease caused a strong stimulus on the production of reactive oxygen species (40). Thus, the reduction in sCD40L observed in contacts with complications could indicate a weaker ability to combat BCG bacilli locally leading to tissue destruction at the vaccination site.

Besides induction of activated T cells, BCG vaccination can also induce regulatory T cells (Tregs), in particular $CD8^+$ T cells which dampen the inflammatory response to mycobacteria (41, 42) and lead to inadequate killing of mycobacteria (43). Likewise,

Tregs have been isolated from lepromatous leprosy patients, who in contrast to tuberculoid patients display reduced Th1 immunity and capacity to kill *M. leprae* bacteria (44). The breakdown of T-cell regulation, in favor of inflammation, underlies the etiology of tissue damage in tuberculoid leprosy and leprosy reactions (45).

Regulatory T cells can suppress Th1 cells through the secretion of CC chemokine ligand 4 (CCL4) (42). In this study, a reduction in CCL4 (although not significant) could indicate decreased T-cell regulation in individuals with complications, causing a shift in the equilibrium toward excessive Th1-type immunity with corresponding inflammation at the BCG vaccination site. However, further research will be required to identify in detail the cellular subtypes involved. Furthermore, the leprosy contacts with high inflammatory responses after BCG vaccination could therefore also be more likely to develop tuberculoid leprosy. In line with this hypothesis are the two cases out of the 50 contacts in this study with BCG complications, who developed border line tuberculoid leprosy (BT).

CONCLUSION

The rate of documented adverse events after BCG vaccination in the studied Bangladesh cohort of leprosy patients' contacts was low (0.34%), and comparable to studies in other countries.

Contacts with BCG complications showed increased *M. leprae*-specific Th1-type immunity but a tendency of reduced T-cell regulation in WBA with corresponding inflammation at the BCG vaccination site indicating improved protection against *M. leprae*. In addition, these individuals may also be at a higher risk of developing tuberculoid leprosy after *M. leprae* infection.

ETHICS STATEMENT

The MALTALEP trial is performed according to standard Good Clinical Practice (GCP) guidelines. Participants were informed about the study objectives, the samples, and their right to refuse to take part in or withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. For illiterate people, a thumb print was taken, and for minors under 16 years of age, the guardian's additional consent was taken. All patients received treatment according to national guidelines. Participants were informed about the potential adverse events of the trial, that free consultation and treatment would be offered in case of adverse events and requested to report any suspected adverse events to the responsible field worker. Ethical approval of the study-protocol was obtained through the National Research Ethics Committee (Bangladesh Medical Research Council; Protocol no. BMRC/NREC/2010-2013/1534).

AUTHOR CONTRIBUTIONS

This research project was designed by the authors JR and AG. Patients were enrolled and a clinical diagnosis was performed and registered by the field staff under supervision of KA and RR. The laboratory testing was done by AH, SE, and LW. The data were analyzed by RR, AH, JR, and AG. The paper was written by RR and AG. All authors agreed with manuscript results and conclusions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00629/full#supplementary-material.

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Emerging Concepts of Adaptive Immunity in Leprosy

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Leprosy is a chronic intracellular infection caused by the acid-fast bacillus, *Mycobacterium leprae*. The disease chiefly affects the skin, peripheral nerves, mucosa of the upper respiratory tract, and the eyes. The damage to peripheral nerves results in sensory and motor impairment with characteristic deformities and disability. Presently, the disease remains concentrated in resource-poor countries in tropical and warm temperate regions with the largest number of cases reported from India. Even though innate immunity influences the clinical manifestation of the disease, it is the components of adaptive immune system which seem to tightly correlate with the characteristic spectrum of leprosy. *M. leprae*-specific T cell anergy with bacillary dissemination is the defining feature of lepromatous leprosy (LL) patients in contrast to tuberculoid leprosy (TT) patients, which is characterized by strong Th1-type cell response with localized lesions. Generation of Th1/Th2-like effector cells, however, cannot wholly explain the polarized state of immunity in leprosy. A comprehensive understanding of the role of various regulatory T cells, such as Treg and natural killer T cells, in deciding the polarized state of T cell immunity is crucial. Interaction of these T cell subsets with effector T cells like Th1 (IFN-γ dominant), Th2 (interluekin-4 dominant), and Th17 (IL-17+) cells through various regulatory cytokines and molecules (programmed death-1/programmed death ligand-1) may constitute key events in dictating the state of immune polarization, thus controlling the clinical manifestation. Studying these important components of the adaptive immune system in leprosy patients is essential for better understanding of immune function, correlate(s) the immunity and mechanism(s) of its containment.

Keywords: polarized immunity, natural killer T cells, regulatory T cells, Th 17, programmed death-1-programmed death ligand-1

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INTRODUCTION

Leprosy is regarded as a stigmatized disease even today. Even though prevalence has fallen substantially in the past few decades, its transmission continues and the disease remains a major public health problem, especially in many third world countries. The chronic infectious disease is caused by the acid-fast, rod-shaped *Bacillus*, *Mycobacterium leprae*. It results in extensive damage to the skin, eyes, mucosa of the upper respiratory tract, and peripheral nerves, in some cases leading to sensory and motor impairment with characteristic deformities and disability (1). Worldwide, two to three million people are estimated to be permanently disabled because of leprosy (2). India has the largest number of cases, with Brazil second, and Burma third (2). Although the reported number of registered cases worldwide has declined in the past two decades, the number of new cases registered each year has remained almost same (3). For the immunologists, however,

leprosy still garners a lot of attention mainly because *M. leprae* infection which evokes distinct polarized T cell responses in humans, which correlates with the clinical manifestations. The two polar forms of leprosy, known as tuberculoid type (TT) and lepromatous leprosy (LL), have clinical, microbiological, and immunological linkage [(4, 5), **Figure 1**]. TT is characterized by fewer skin lesions, low numbers of bacteria in lesions, and histologically well-formed granulomas containing abundant CD4+ T cells. On the other hand, LL is characterized by numerous infiltrative skin lesions, large numbers of bacteria in lesions, and poorly formed granulomas with fewer lymphocytes (6). However, most leprosy patients display a pathogenesis somewhere in between and are classified as either borderline tuberculoid (BT) or borderline lepromatous (BL) (4). Leprosy reactions known as type 1 reactions (T1R) (**Figure 1**) are common in these immunologically unstable borderline groups and involve an upregulation of the host response to *M. leprae* antigens (5). In patients with the disseminated LL, a reaction known as erythema nodosum leprosum (ENL) or type 2 reactions (T2R) is frequent, being observed in almost half of these patients receiving antimicrobial therapy (1).

POLARIZED IMMUNITY IN LEPROSY: POSSIBLE CAUSES

Several factors may be involved in regulating the polarization of newly activated naïve T cells into mature Th1 or Th2-like effector cells (7): *viz*., the local cytokine milieu; the presence of immunologically active hormones; the dose and route of antigen administration; the type of antigen-presenting cell stimulating T cells; and the "strength of signal" of the T-cell receptor for the MHC-antigen complex. The most important among these is the cytokine milieu surrounding the newly activated T cell. In the context of leprosy, reciprocal changes in cytokine expression in TT vs. LL along with complex cytokine regulatory networks have been evidenced at the site of infection (8). However, the important question is: which of these factors serve as the initial determinant of the polar immune responses to *M. leprae* ? Given the extremely high relatedness of leprosy bacilli genomes worldwide (9), bacterial diversity is unlikely. This leaves differential host responses as the most likely mechanism.

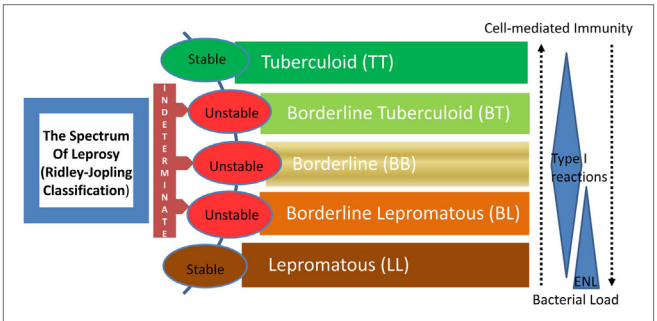

FIGURE 1 | The spectrum of leprosy: Ridley–Jopling classification and the relationship with host immunity. ENL, erythema nodosum leprosum or type 2 reaction.

Polarized T cell response (Th1/Th2 biased) to *M. leprae* is believed to be a critical element in the pathogenesis of leprosy and its varied clinical manifestations (8). The generation of Th1 effector cells chiefly producing the cytokine interferon-gamma (IFN-γ) vs. Th2 effector cells producing interluekin-4 (IL-4) have been held primarily responsible for the polarized state of immunity. During reversal reaction, lesional sites have demonstrated presence of CD4+ *M. leprae*-responsive T cells with a polarized type 1-like phenotype (10). However, the immune response manifested at the pathologic site(s) of leprosy is an extremely complex process, particularly in the light of recently evidenced remarkable heterogeneity of T cell subsets (11). Proportional enrichment of selective T cell subsets, particularly at the pathologic sites, determines the bulk T cells response (12). The major focus of the review is on the functionality of various relatively infrequent, yet significant lymphocyte subsets, which subsequently regulate the host's cellular immune response and consequently disease pathogenesis. Numerous smaller subsets of lymphocytes have been identified in the past few decades which play critical roles in shaping the host immunity *via* their T cell response. These include natural killer T cells (NKT), regulatory T (Treg) cells, γδ T cells, and the very recently identified regulatory B cells. These cells have been demonstrated to exert regulatory influences on the generation of various effector T cells, such as Th1, Th17, and Th9-like cells (13–15).

THE NKT CELLS

First described in 1987 (16, 17), NKT cells are a unique subset of mature T cells co-expressing a semi-invariant Vα24Jα18 T cell antigen receptor (TCR)α chain and surface markers characteristic of NK cells. The semi-invariant TCR on iNKT cells recognizes glycolipids bound to monomorphic CD1d molecules. The most prominent and characteristic function of NKT cells is the early rapid production of immune-regulatory cytokines, such as IL-4, IFN-γ, and TNF-α upon their activation (18).

Earlier studies on the tissue origin and developmental pathway of iNKT cells from Taniguchi's group (19) have suggested that iNKT cells develop extra-thymically, particularly in the liver. However, others have demonstrated that the majority of iNKT cells, like conventional T cells, are generated in the thymus (20, 21). The finding that not only peptides, but also glycolipids can serve as a source of antigen recognized by these NKT cells opened up new vistas in the study of antigen processing and presentation (22). The ability of nonpolymorphic CD1 molecules to present structurally diverse glycolipids to T cells has generated interest on these fascinating lipid–protein interactions. Since, NKT cells exercise a determining influence on a variety of immune responses in mice, ranging from autoimmunity to tumors and infections (23–25), significant interest has been generated to study their roles in human diseases as well.

Invariant iNKT cells (26, 27), which have a limited diversity of their TCR chains recognize glycolipid antigens from certain bacteria that are presented by CD1d, a nonpolymorphic antigen-presenting molecule (25). CD1-restricted T cells appear to play a major role in immune responses to mycobacteria. However, results of studies in mouse models are inconsistent. For example,

although CD1d-deficient mice did not differ significantly in susceptibility to *Mycobacterium tuberculosis* (28), NKT cells predominate in the granulomatous reaction to *M. tuberculosis* cell wall preparations, and such granulomas do not form in NKT cell-deficient Jα2812/2 mice (29). Furthermore, NKT cells of normal mice respond to mycobacterial infection by decreasing IL-4 and increasing IFN-γ production (30), changes that aid the host response to mycobacteria, since IFN-γ plays a critical role in pathogen clearance.

Leprosy-specific studies on NKT cells (31) have shown mycobacterium-reactive double-negative T-cell lines derived from skin lesion of a leprosy patient responded to subcellular fractions of mycobacteria in the presence of CD1-expressing antigen-presenting cells (APCs). However, lipoarabinomannan-depleted soluble cell wall fraction did not induce detectable T-cell proliferation. Recognition of purified lipoarabinomannan from *M. leprae* was restricted by CD1b, and T cells lysed lipoarabinomannan-pulsed monocytes in a CD1b-restricted manner. Lipoarabinomannan also induced these T cells to secrete large amounts of IFN-γ. Upon examination of leprosy patients, they found few CD1+ cells in LL leprosy lesions. In contrast, there was a strong upregulation of CD1+ cells in the granulomatous lesions of patients with TT leprosy or reversal reaction (32). These cells were also CD83+, a marker for dendritic cells, indicating a strong correlation between CD1 expression and cell-mediated immunity in leprosy. Interestingly, administration of granulocyte-macrophage colony-stimulating factor, a cytokine which can promote dendritic-cell activation, to LL leprosy patients induced infiltration of CD1+ cells into the lesions (33, 34). NKT cells were also found in T-cell-reactive leprosy, but when compared with the granulomas in cutaneous sarcoidosis these cells were undetectable (35). They studied the TCR Vα repertoire and found that all patients with T-cell-reactive leprosy showed a very restricted T-cell-reactive Vα repertoire with a strong bias toward the use of the Vα6 and Vα14 segments. Unpublished data from our laboratory have given clear indications that NKT cell-derived cytokines control the ensuing effector T cell responses on activation with lipid antigens and further help in dictating the overall T cell response and manifestation of the disease. All of these studies strongly suggest that NKT cells play a determining role in regulating the varied type of immune responses as evidenced in leprosy affected individuals.

THE Treg CELLS

Regulatory T cells, on the other hand, are essential for maintaining peripheral tolerance, preventing autoimmune diseases, and limiting chronic inflammatory diseases (36). However, in case of chronic infections, they also limit such beneficial effect by suppressing the host immunity. During an infection, immune regulation is the result of the host's response to the infection in a bid to maintain or restore a homeostatic environment and/or it can be actively induced by the pathogen to promote pathogen survival, like in the case of *M. leprae* (37). The presence of T cells with suppressive or anergic activity was discovered a long time back when they were known as suppressor T cells (38, 39). These same cells were shown to produce IL-10 and generated *in vivo* during infection (40). Recently, it has emerged that there are several specialized subsets of Treg cells, which contribute to the elaborate regulatory network within the infected host.

Based on their origin, generation, and mechanism of action, two main subsets of Treg cells have been identified: one is the naturally occurring CD4+CD25+ Treg cells (natural Treg cells), which mainly develop in the thymus and regulate self-reactive T cells in the periphery (41). Others are the inducible Treg cells, which develop in the periphery from conventional CD4+ T cells after exposure to signals, such as regulatory cytokines, immunosuppressive drugs, or APCs conditioned by microbial products (42). Both types of Treg cells, by virtue of their capacity to control the intensity of effector responses have been shown to have a major role in infection (12, 43). Treg cells mediate their suppressive capacity on inflammatory effector T cells, such as Th1, Th17, and Th9 cells both by contact dependent as well as contact-independent manner (36). From a functional perspective, Treg cells can be grouped into four basic "modes of action:" the various potential suppression mechanisms used by these include suppression by inhibitory cytokines, suppression by cytolysis, suppression by metabolic disruption, and suppression by modulation of dendritic cell maturation or function (44). Inhibitory cytokines, IL-10 and TGF-β, have been the focus of considerable attention as mediators of Treg cell induced suppression (45–47).

Differential trafficking of these Treg cells to the diseased sites are thought to be under the influence of tissue chemokine response elicited at the site of lepromatous lesions. This in turn is believed to determine the local immunity of BT/TT and BL/LL forms of leprosy. The tissue chemokine response at the lepromin DTH site and lesions of various forms of leprosy determines the recruitment of effector T cells at the lesional levels in leprosy patients (48). Therefore, subset composition of T cells infiltrating the pathologic/lesional site(s) of leprosy patients appears to be the key element in deciding the local immunity in leprosy, which may dictate the clinical manifestation of the disease. Some of these subsets have been demonstrated to be hierarchy in nature and known to exert significant influence on the effector T cells, and thus regulate the immune response at the pathologic site(s) of various chronic infectious diseases, including leprosy. These include the FoxP3 positive Treg cells as one of the most potent hierarchic cell type suppressing the effector T cell function with eventual regulation of immune response elicited by the host during intracellular infections, such as tuberculosis and leishmaniasis (49). Over representation of Treg cells either in peripheral compartment or more particularly at the pathologic site(s) has been shown to be of critical importance in determining the local immunity, thus dictating the outcome of the disease among patients suffering from various forms of tuberculosis (12). In leprosy as well, works have suggested that Tregs are present in increased numbers in LL patients, and they may have a pathogenic role in leprosy patients harboring uncontrolled bacillary multiplication (50). CD25+ Treg cells have also been shown to play a role in *M. leprae*-induced Th1 unresponsiveness in LL (51). FoxP3+ inducible Tregs producing the immunosuppressive cytokine TGF-β may also downregulate the T cell responses leading to antigen-specific anergy associated with LL (52).

Recent studies have revealed (53) that T2R or ENL patients have significantly lower number of circulating and *in situ* Tregs than T1R patients and controls with concomitant increase in pro-inflammatory cytokines such as TNF-α and IFN-γ produced by Th1 lymphocytes.

THE TH17 CELLS

Very recently, a third subset of T helper cells, Th17 cells, has been identified based on their cytokine production profile. These cells produce IL-17A (also referred to as IL-17), IL-17F, and IL-22, cytokines involved in neutrophilia, tissue remodeling and repair, and production of antimicrobial proteins. Th17 cells differentiate in response to the STAT3-activating cytokines IL-6, IL-21, and IL-23 along with TGF-β and IL-1β (54). They are abundant at mucosal interfaces, where they contain infection with pathogenic bacteria and fungi (55). Skin-homing T helper cells that produce IL-22, but not IL-17, have also been described in humans, and they may represent a new T cell subset with distinct effector functions (56). It is believed that the differentiation of CD4+ T cells that produce IL-17 and IL-22 is influenced by the composition of the intestinal microbiota and by the presence of innate immune cells mainly the neutrophils that amplify the Th17 cell response.

For long, Th1 cells were considered to be the major effectors in multiple autoimmune diseases, while Th2 cells were involved in atopy and asthma. In recent times, however, Th17 cells have been implicated as culprits in a plethora of autoimmune and other inflammatory diseases in mice and humans. Many diseases that were previously associated with Th1 cells, e.g., experimental autoimmune encephalomyelitis (EAE, a model for multiple sclerosis), collagen-induced arthritis, and some forms of colitis, were shown to be caused by IL-23-dependent Th17 cells or other IL-17-producing lymphoid cell types (57–59). Conversely, defects in the Th17 cell differentiation axis may predispose the host to bacterial and fungal infections at mucosal surfaces (60). Th17 cells mediate their pro-inflammatory function by (i) recruiting neutrophils, (ii) activating macrophages, and (iii) enhancing Th1 effector cells (54). Much of the inflammatory damage previously ascribed to type 1 response is now thought to depend on IL-17 and IL-23 (the cytokine responsible for supporting Th17 response *in vivo*) (58).

CD4+ Th17 cells have been recently identified in borderline cases of leprosy (61), which highlighted their importance in infectious diseases as well. A persistent and very relevant concept is that an imbalance between Th17 and Treg cell function may be critical in the immunopathogenesis of many disease states (62). This concept is highlighted in a leprosy-specific study, where IL-10+ produced by Treg cells in BL/LL patients correlates significantly with polarized immunity highlighted by lesser IL-17 by CD4+ T cells in the same group. Blocking of IL-10/TGF-β resulted in the reversal of effector immune response (IL-17) in BL/LL with higher frequency of Th17 cells (63). This indicates that by negating the influence of suppressive cytokines we can successfully gain back immune responsiveness. The presence of Th17 cytokines (IL-6, IL-17, and IL-23) *in vitro* results in reduction of FoxP3 expression on Tregs simultaneously, possibly leading to increase in IL-17-producing CD4+ cells in BL/LL (63). This further suggests that the generation of antigen-specific Treg cells is very much dependent on the environment of cytokines they are exposed to. Hence, these cells may be targeted for reversal of effector response in BL/LL patients proving to be an important mode of immune modulation in the immunocompromised hosts to revive the immune response.

An imbalance in Treg and Th17 populations has also been observed in patients with leprosy reactions (53, 64). Studies done in biopsies from T2R patients showed a decrease in Tregs and associated cytokines, TGF-β and increase in cells producing IL-6, IL-21, and IL-17. On the other hand, T1R patients are showing the opposite trend with increased Tregs and reduced IL-17+ cells. This increase in inflammatory cytokines along with downregulation of Tregs may be responsible for the lesional inflammation characterizing T2R reactions.

THE PROGRAMMED DEATH-1(PD)-1-PROGRAMMED DEATH LIGAND-1 (PD-L1) PATHWAY

T cell responses during parasitic infections are tightly controlled by co-stimulatory or co-inhibitory molecules. It is well known that interactions between PD-1 and its ligand, PD-L1 can inhibit the effector functions, such as proliferation, cytokine production, and survival of the T cells, thus balancing the tolerance, autoimmunity, infection, and immunopathology (65, 66). On infection with *M. tuberculosis,* protective T cells are generated in the infected host. However, T-cell-mediated immunity does not easily eradicate these bacteria because they have evolved effective strategies to overcome the host defense mechanisms (67). Studies have identified various virulence-associated genes and intracellular survival mechanisms of mycobacteria (68). The PD-1 signaling pathway is activated during persistent infection with various microorganisms and contributes to the impairment of protective immunity (69–71). A recent study showed that *in vitro* blockade of PD-1 signaling with the specific antibody enhanced IFN-γ production by T cells of TB patients on stimulation with *M. tuberculosis* antigen (72). In pulmonary TB patients, inhibiting this signaling pathway rescues *M. tuberculosis*-specific IFN-γ producing T cells from apoptosis (73). Similarly, persisting infection with pathogens like *Helicobacter pylori* and *Porphyromonas gingivalis*, showed elevated expression of PD-L1 on gastric epithelial cells and monocytes, suggesting a potential involvement of PD-L1 in promoting chronic infections (74).

Leprosy-specific studies show reduced expression of the positive signaling co-stimulatory molecules, CD28 and CD86 on T-cells, consistent with the LL anergy, in contrast to TT patients which displayed increased expression of the negative signaling molecules CD152 and PD-1 (75). This may represent a probable means of modulating an exacerbated immune response and avoiding immunopathology. However, another recent study in leprosy reveals elevated surface expression of PD-1 on T cells, NKT, and Treg cells and its ligand PD-L1 on APCs, such as monocytes and B cells, in BL/LL as compared to BT/TT leprosy patients (63). The authors have demonstrated that the PD-1/PD-L1 pathway preferentially suppress IFN-γ against TNF-α in

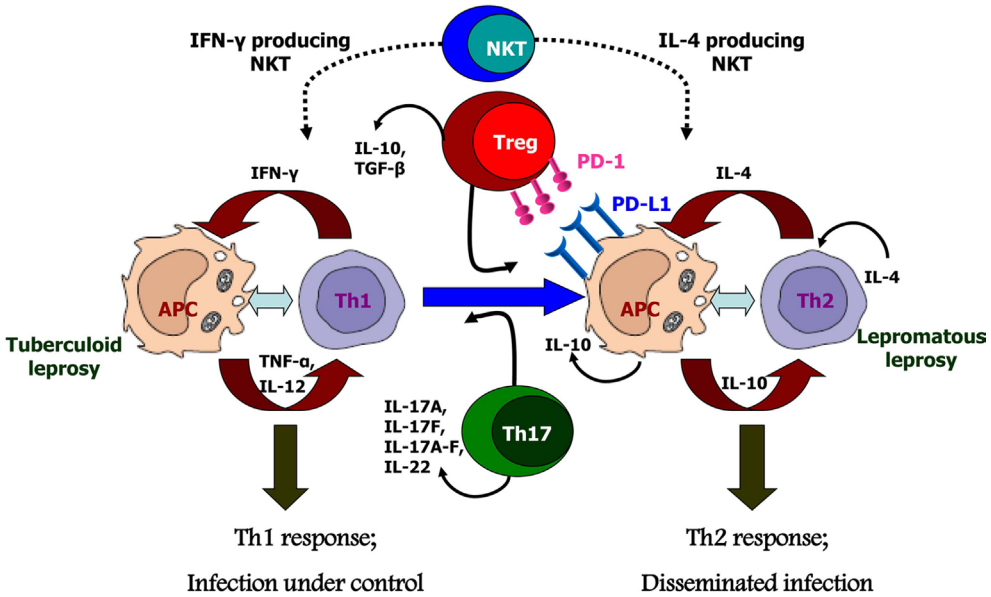

FIGURE 2 | Possible causes for polarized host immunity in tuberculoid type (TT) vs. lepromatous leprosy questioning the well-established Th1–Th2 paradigm. Natural killer T cells which are initial responders producing either Th1 cytokines like IFN-γ or Th2 cytokines like interluekin-4 depending on the basal cytokine response of the host. Tregs cells which are predominantly suppressive in nature and produce cytokines IL-10 and TGF-β, along with increased expression of programmed death-1 and its ligand, programmed death ligand-1 on antigen-presenting cells; these cells are found in significant numbers in lepromatous leprosy patients. Th17 or T helper 17 cells which produce the cytokines IL-17 and IL-22 demonstrate inflammatory phenotype and are, therefore, found in increased numbers in TT leprosy patients.

BL/LL which is touted as the designate cytokine for generating protective immune response in the immunosuppressed host. This may also be one of the contact-dependent mechanisms utilized by Treg cells for immune suppression of effector T cells. These findings raise the possibility that the antigen-specific T-cell response is impaired by several inhibitory mechanism(s), thereby allowing mycobacterial persistence.

CONCLUSION

However, it needs to be emphasized that no single mechanism of suppression can account for the kind of *M. leprae*-specific T cell anergy evidenced in LL. The diversity of effector mechanisms characteristic of NKT affords versatility capable of restraining diverse types of inflammatory responses in different tissues. Likewise, both Tregs and Th17 cells can exert beneficial as well as pathogenic effects depending on the physiology of the infected host. Other cell subsets, such as Th9 (76) or γδ T cells, have also been identified in leprosy patients, but their exact roles have not been defined till date. The intricate mechanisms governing differentiation and functions of these pro- and anti-inflammatory cells are yet to be discerned and pose major challenges ahead. Therefore, in conclusion, we can state that as seen in other chronic granulomatous diseases, NKT and Treg cells along with Th17 and the PD-1-PD-L1 pathway play crucial roles in the outcome of the host–parasite interactions in leprosy (**Figure 2**). Providing a balanced level of function for these cell subsets is the key to achieving an appropriate level of parasite control without inducing immunopathology. This would be a major goal in the management of this still-challenging infectious disease.

AUTHOR CONTRIBUTIONS

Author has planned and structured the review article.

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REVIEW
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Type I Interferons, Autophagy and Host Metabolism in Leprosy

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For those with leprosy, the extent of host infection by *Mycobacterium leprae* and the progression of the disease depend on the ability of mycobacteria to shape a safe environment for its replication during early interaction with host cells. Thus, variations in key genes such as those in pattern recognition receptors (*NOD2* and *TLR1*), autophagic flux (*PARK2*, *LRRK2*, and *RIPK2*), effector immune cytokines (*TNF* and *IL12*), and environmental factors, such as nutrition, have been described as critical determinants for infection and disease progression. While parkin-mediated autophagy is observed as being essential for mycobacterial clearance, leprosy patients present a prominent activation of the type I IFN pathway and its downstream genes, including *OASL*, *CCL2*, and *IL10*. Activation of this host response is related to a permissive phenotype through the suppression of IFN-γ response and negative regulation of autophagy. Finally, modulation of host metabolism was observed during mycobacterial infection. Both changes in lipid and glucose homeostasis contribute to the persistence of mycobacteria in the host. *M. leprae*-infected cells have an increased glucose uptake, nicotinamide adenine dinucleotide phosphate generation by pentose phosphate pathways, and downregulation of mitochondrial activity. In this review, we discussed new pathways involved in the early mycobacteria–host interaction that regulate innate immune pathways or metabolism and could be new targets to host therapy strategies.

Keywords: leprosy, tuberculosis, innate immunity, autophagy, type I interferon, metabolism, host-directed therapy

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INTRODUCTION

Leprosy is caused by *Mycobacterium leprae* or *Mycobacterium lepromatosis*. Here, we will discuss mechanisms of infection and host–pathogen interaction mediated by *M. leprae*. The heaviest exposed population, including the household and family members and social contacts of patients, is considered to have the highest risk of developing leprosy, but the disease will not necessarily progress during their lifetime. Thus, mycobacterial infection is a necessary, but not sufficient cause of leprosy progression. During the natural course of the disease, it has been suggested that once *M. leprae* infects an individual through the airways, the bacteria can come into the lungs and be phagocytosed by resident macrophages. The mycobacteria can infect epithelial cells in the nasal mucosa and penetrate the organism, while host cells initiate an innate response to eliminate the pathogen (1). Intracellular mycobacteria are able to use different strategies to circumvent potential bactericidal peptides: (i) mimic a viral response; (ii) upregulate lipid metabolism; or (iii) downregulate pro-inflammatory cytokines, which is generally associated with a cascade of pro-mycobacteria events (2–4). These virulence strategies are related to other pathogens, such as *Mycobacterium tuberculosis*, suggesting that virulent mycobacteria can share common mechanisms of host colonization (5–7). Thus, by understanding the critical pathways related to the subversion

of antimicrobial responses, researchers can understand the conditions for successful mycobacterial infection and, perhaps, the disease progression. Actually, these novel pathways, which use different strategies during *M. leprae* infection have already been described, but a better understanding of these phenomena could help us interfere, reverse or halt the disease progression.

In this regard, the *M. leprae* genome is highly conserved, and the strain circulating worldwide has remained basically the same for the past 1,000 years (8). So, the decline of leprosy in Europe does not account for genetic changes in *M. leprae* that could impact bacterial virulence. Currently, it is clear that very few differences are observed between strains isolated from different clinical forms of the disease. One possible conclusion is that the various stages and clinical forms observed in cases of leprosy are similar due to the host genetics (9).

Large-scale studies have contributed to the identification of new candidate genes and pathways to help understand this complex puzzle. These strategies provide insights not only about leprosy but also about other immune-based and/or infectious diseases. In fact, the most successful genome-wide association studies (GWASs; or genomic scans) were performed in leprosy, as compared, for example, to tuberculosis studies, in which no genes were consistently pinpointed. Several genes have been associated with leprosy, such as *NOD2*, *PARK2/PARCG*, *LRRK2*, *RIPK2*, *TNF/LTA/HLA*, *LACC1*, *IL10*, *TLR*1, and microRNA (miR)-146a (10–14). Single-nucleotide polymorphisms in these genes were replicated consistently in different populations and have been assigned a functional role in leprosy susceptibility. Whole exome sequencing and rare variant analysis have implicated several novel candidates that still need to be validated. Most of these confirmed associations have a modest odds ratio value, but few other infectious diseases have a clear association with key genes that demonstrate consistent results, which can be replicated in populations with different ethnic backgrounds. Interestingly, the most important genes or pathways that emerge after *M. leprae* infections in studies using microarray gene expression are type I interferon (IFN), autophagy and mitochondrial, and lipid metabolism (15). Therefore, different large-scale approaches in the literature are revealing distinct, but complementary pathways that clearly outline the strategies used by *M. leprae* to destabilize antimicrobial responses and establish a safe environment for continuous bacterial replication. We have depicted main pathways associated with disease susceptibility in a way that how could we potentially regulate lipid and mitochondrial metabolism and immuno-inflammatory responses toward a reversion of the phenotype to accelerates treatment and develop new prevention strategies? Hence, in this article, we will discuss seminal findings that reveal critical mechanisms of innate immunity and host metabolism with a direct impact on the disease outcome where modulation could be path toward disease control.

TLR-2/1-MEDIATED ANTIMICROBIAL RESPONSE IN LEPROSY

In the early stages of mycobacterial infection, macrophages and other cells of the innate immune system are able to rapidly recognize pathogen-associated molecular patterns through exposure to an extensive repertoire of pattern recognition receptors (PRRs). These transmembrane receptors mediate the activation of several signaling pathways in response to intracellular pathogens and initiate important immune events, such as cell differentiation and antimicrobial programs (16). The most recognized toll-like receptors (TLRs) have been observed to mediate the immune recognition of mycobacteria (17). Among these, the TLR-2/1 heterodimer was responsible for recognizing mycobacterial lipoproteins, activating a pro-inflammatory response and releasing vitamin-D-dependent antimicrobial peptides (18). Genetic analysis has demonstrated that polymorphisms in the *TLR1* gene are associated with leprosy susceptibility, and these variations have a functional effect that includes structural modifications to the protein and alterations to TNF/IL-10 log ratio values in the supernatants of *M. leprae*-stimulated peripheral blood mononuclear cells (13, 16). These individual variations exemplify the ability of the host's immune system to initiate an efficient antimicrobial response against mycobacteria.

Other components also contribute to TLR-2/1 signaling. miR-21, which is highly expressed in the disseminated form of leprosy, it is a suppressive mechanism of host antimicrobial TLR-2/1-mediated genes that affect the production of critical cytokines, such as IL-1β and IL-10 (19). Recently, a novel component of a TLR-2/1-mediated antimicrobial programme has been described. The *S100A12* gene, which encodes the calgranulin C protein, is highly expressed in response to the activation of the TLR-1 receptor. This gene codifies an antimicrobial peptide that is able to kill *M. leprae* directly (20). Also, *S100A12* is more expressed in skin lesions of tuberculoid (TT) patients than in those of lepromatous (LL) patients (20). Since TLR-2/1 signaling pathways regulate this gene, differences in disease susceptibility could be linked to variations of *TLR1* expression and the activation of this signaling pathway among patients and healthy volunteers. Thus, pattern recognition is essential for controlling mycobacterial growth by regulating optimum levels of the TNF/IL-10 ratio during the period of infection, while miR-21 levels could counterbalance or impair an adequate antimicrobial response (19).

NOD2 SIGNALING PATHWAY

In the past few years, independent GWASs in leprosy and inflammatory diseases such as Crohn's disease (CD) have revealed a common genetic fingerprint and a considerable overlap of susceptibility mechanisms among these pathologies (10, 21, 22). As demonstrated in mycobacterial diseases, the risk variants of inflammatory bowel disease (IBD) comprise genes that are active in the early stages of the host response suggesting that the continuous interaction between host and pathogens shapes genetic factors that are predisposed to IBD (23). An important signaling pathway identified by a GWAS was the nucleotide-binding oligomerization domain containing 2 (NOD2)-mediated immune response, where variants of genes involved in this signaling pathway are also implicated in susceptibility to *M. leprae* infection and CD (10).

NOD2 is an intracellular component of NOD-like receptors that detects muramyl dipeptide (MDP), which is a cell wall

structure. *M. leprae* presents a distinct MDP compared with other mycobacteria (24). However, even with these structural modifications, *M. leprae* MDP maintains the capacity to trigger the NOD2 response. Upon recognizing MDP, NOD2 is able to initiate a leucine-rich repeat kinase 2 (LRRK2)-dependent pro-inflammatory response, as well as other cellular processes, such as autophagy (25). LRRK2 is a downstream component of NOD2 signaling, which enhances the inflammatory cytokine production that is required for antimicrobial activity in the presence of macrophages (25). For this reason, the *LRRK2* gene is similar to the many critical genes involved in the NOD2-mediated response that is associated with leprosy susceptibility, CD, and Parkinson's disease (PD) (10). Unbalanced LRRK2 activity is related to excessive inflammation, which leads to tissue damage. It has been reported that a specific mutation in the *LRRK2* gene is associated with acute inflammation in both leprosy and CD cases, supporting the assumption that these diseases share common pathological mechanisms (26). Furthermore, a recent study has found that functional variations in *LRRK2* genetically link CD to PD, affecting cellular processes such as kinase activity and autophagy (27).

In like manner, genetic variation at *NOD2* is reported to be associated with exacerbated inflammatory responses in leprosy reactions that could modulate downstream pathways, such as LRRK2 activation (28). Notably, NOD2 activation induces the differentiation of monocytes into dendritic cells (DCs) in an IL-32-dependent manner (29). This DC activation triggers autophagy, a process required for bacterial handling, antigen presentation and generation of CD4 T cell response (30). Individuals suffering from CD present a defective activation of these processes, which are still poorly investigated in leprosy. In addition to the genetic relevance of the NOD2 response to leprosy susceptibility, some advances in functional studies have demonstrated that this signaling pathway is upregulated in patients with paucibacillary leprosy when compared with those that manifest the disseminated (multibacillary) form of the disease (29). These findings show that the activation of the NOD2 response is an essential link between innate and adaptive immunity, and aberrant NOD2 signaling results in impairment of antimicrobial activity and defective antigen presentation in leprosy (**Figure 1**).

TYPE I IFN AND AUTOPHAGY: THE HETEROGENEITY OF DNA SENSING IN MYCOBACTERIAL INFECTIONS

In parallel, other mycobacterial components trigger innate immune responses. A classical view of phagocyte–mycobacteria

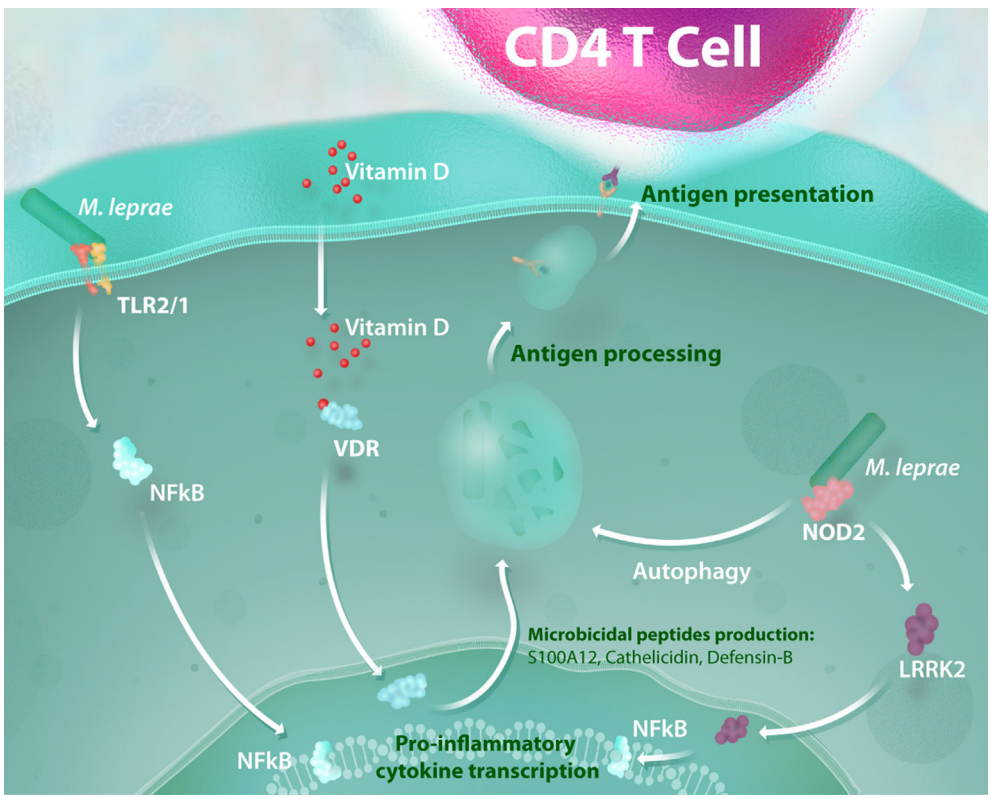

FIGURE 1 | Antimicrobial activity and NOD2-induced autophagy mediate the link between innate and adaptive immunity in mycobacterial infection. The recognition of mycobacterial lipoproteins by the TLR-2/1 heterodimer is a critical way to initiate a pro-inflammatory response and activation of a vitamin-D antimicrobial program against intracellular pathogens such as *Mycobacterium leprae*. Mycobacterial muramyl dipeptide sensing by NOD2 receptors enhances the inflammatory response in a leucine-rich repeat kinase 2 (LRRK2)-dependent manner and activates autophagic mechanisms. All of these processes lead to mycobacterial killing and are essential for bacterial handling, antigen presentation, and consequent generation of an effective CD4 T cell response.

interaction supports the view that virulent bacilli are able to persist within phagosomes, preventing their fusion with lysosomes to achieve a safe environment for replication (31, 32). This interpretation has been extended and updated in light of new data, which suggest that a breach in the phagosomal membrane and cytosol contamination during the course of an infection leads to a permissive response (6). Mechanisms of phagosome maturation are arrested and punctured during mycobacterial infection, which involves virulence factors that manipulate important host response against intracellular infection.

The ESX-1 secretion system is a determinant of mycobacterial virulence that is presented in pathogenic mycobacteria, such as *M. tuberculosis* and *M. leprae*, and it is responsible for the secretion of (CFP-10) and early secreted antigenic target 6 kDa (ESAT-6) proteins (5). The absence of this secretion system in virulent mycobacteria such as *Mycobacterium bovis* BCG supports the importance of those proteins for the success of mycobacterial infection (33). Just after infection, virulent mycobacteria express the ESX-1 system, exporting ESAT-6, which is able to create a fissure in the phagosomal membrane (34). Consequently, ESX-1-mediated pore formation allows an equalization of phagosomal and cytosol content. This process is essential for bacteria to acquire nutrients from the host cell and deliver virulence factors capable of downregulating host responses against the pathogen (5, 35). The leakage of mycobacterial DNA from phagosomes into the cytosol strongly activates the host cell cytosolic surveillance pathways, triggering both a type I IFN response (6) and autophagy (36, 37), which comprise pro- and antibacterial responses, respectively (6). Furthermore, ESX-1 activity and cytosolic recognition of mycobacterial DNA is also involved in the activation of caspase-1, promoting the formation of the inflammasome complex and regulation of IL-1β secretion (38, 39).

Type I IFN (IFN-α/β) activation was originally characterized as a pathway involved in controlling virus infection. However, in the past decade, a number of reports have described a type I IFN transcriptional signature in the pathogenesis and progression of tuberculosis (40) among other mycobacterial diseases. The production of IFN-β may inhibit IL-1β activation, which plays a critical role in the elimination of *M. tuberculosis* (41). IFN-β-mediated suppression of the host bactericidal mechanisms is also noticed in leprosy. An inverse correlation between IFN responses (type I and II) is observed in the clinical spectrum of leprosy. Paucibacillary patients preferentially express type II IFN (IFN-γ) and, consequently, its downstream antimicrobial genes, preventing the spread of mycobacteria; by contrast, the IFN-β program is prominent in multibacillary patients (42). The IFN-β response can induce IL-27-dependent IL-10 activation, which in leprosy, is a well-known immune suppressive mechanism that favors mycobacterial growth and dissemination (43).

Interferon-β induction is redundant, and it involves a large repertoire of nucleic acid sensors (44). *M. tuberculosis* models have been used to generate most of the existing data on type I IFN trigger mechanisms for infections, and this area has not been fully explored in leprosy studies. Once released into the cytosol, extracellular mycobacterial DNA ligates to a double-strand DNA sensor (6). In this context, different studies reported that cyclic GMP-AMP synthase (cGAS) is the primary sensor for mycobacterial DNA (39, 45, 46). After DNA recognition, cGAS is able to produce the second messenger cyclic GMP-AMP, a potent ligand of the stimulator of interferon genes (STING), TANK-binding kinase 1 (TBK1), interferon regulatory factor 3 (IRF3) signaling pathway exhibiting a transcriptional profile of the type I IFN response that antagonizes the host's antimicrobial programmes (6).

Conversely, cGAS-mediated DNA sensing and STING/TBK1 activation is also required for mycobacterial targeting of the ubiquitin-dependent autophagy pathway, an efficient mechanism that eliminates intracellular pathogens and links innate and adaptive immune responses by enhancing antigen presentation (37, 45, 47). However, only one-third of the intracellular mycobacteria in the host are delivered for autophagic degradation, suggesting that virulent mycobacteria have an active mechanism to evade autophagy (36). The paradoxical mechanisms of DNA sensing during mycobacterial infection are not clearly understood, but they involve a type of bifurcation that could be dependent on multiplicity of infection. Thus, it is likely when infected by a low number of mycobacteria, the host can preferentially load autophagy and control the infection. If a higher mycobacterial burden is presented during infection, a pro-mycobacteria response is directed.

Many factors may be involved in the heterogeneity of DNA sensing following infection. Determining the immunological status of a host at the early stages of host–pathogen is critical to define the course of infection. An initially permissive environment favors bacterial colonization and triggers virulent mechanisms. The increase of the mycobacterial burden and consequent virulence released into the host cell contribute to an imbalance of the DNA-mediated response, driving type I IFN production that, in turn, leads to an impairment of the host antimicrobial mechanisms (2, 6). Host genetic variation in the PRRs of genes that mediate mycobacterial interactions could also modulate the bacilli uptake (9), as well as the activation of an inflammatory response that directly affects downstream signaling pathways, such as cGAS/STING signaling. However, in large-scale screenings, no evidence has been found that major genes or consistent effects in this pathway are associated with leprosy. Mutations in *TMEM173*, which encode STING, are related to selective STING activity, and such activity is able to disrupt IRF3 phosphorylation without affecting other activities of TBK1 (48). These findings support the hypothesis that variation in genes that encodes key DNA sensing components may contribute to the heterogeneity of DNA-mediated responses. Previous research suggests that other cytosolic sensors, such as AIM2 inflammasome, may interact competitively with the mycobacterial DNA implicated in the balance of STING-mediated responses (49).

The targeting and delivery of *M. tuberculosis* for autophagic degradation occurs by a recruitment of the host's ubiquitin chains, a process that depends on Parkin (*PARK2*), an E3-ubiquitin ligase (37). Intracellular *M. tuberculosis* avoid ubiquitin or proteasomal host systems. More than one decade ago, the gene *PARK2*, which encodes Parkin, was associated with leprosy susceptibility (11); this suggests that Parkin also controls ubiquitination and autophagy levels during *M. leprae* infection. A more recent study

has showed that multibacillary patients demonstrated autophagy impairment, while paucibacillary ones exhibited strong autophagy upregulation (50). The research revealed how live *M. leprae* actively downregulates the autophagic machinery of human monocytes to generate a protected intracellular niche for bacterial replication. Following this research, our group described a transcription profile of the type I IFN response in both human Schwann cells and macrophages following *in vitro* infection with live *M. leprae*. *OASL* [2′-5′-oligoadenylate synthetase (OAS) like] was the most differentially expressed interferon-stimulated gene in our study (2). OASL is a member of the OAS family, a group of proteins with a recognized antiviral action, although its function in bacterial infections is poorly understood. OASL can play a dual role following activation: the ubiquitin-like domain of OASL can interact with RIG-I, a double-strand RNA sensor, leading to type I IFN activation enhancement (51). Conversely, viral double-stranded DNA can induce an OASL-mediated type I IFN inhibitory effect by blocking cGAS/STING signaling (52). Upon *M. leprae* infection, macrophages are able to produce high levels of OASL in a STING-dependent manner. This production is associated with the persistence of *M. leprae* inside the cell as OASL inhibits autophagic mechanisms that are essential for mycobacterial clearance (2) (**Figure 2**). However, the mechanisms for the OASL-mediated blockage of autophagy need to be explained. The OASL–cGAS interaction, as it occurs during double-stranded DNA virus infection, could also be investigated in mycobacterial infection to improve our understanding of how OASL modulates cGAS/STING-mediated autophagy. Moreover, investigating the interactions of OASL with other molecules in its ubiquitin-like domain may help us understand the role of OASL in the regulation of immune responses against intracellular infections. Thus, these data suggest that OASL participates in the fine-tuning of infection outcomes by regulating DNA sensing pathways.

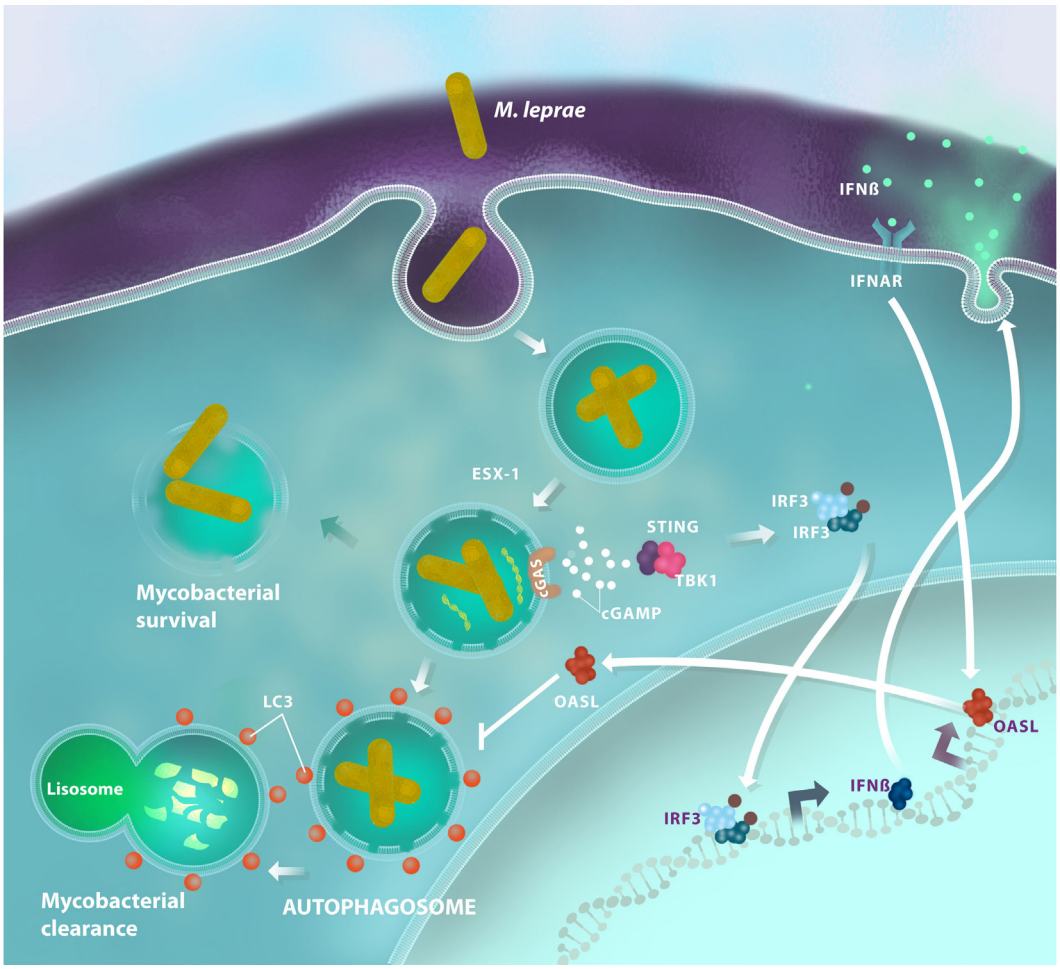

FIGURE 2 | Antimicrobial autophagy is inhibited in *Mycobacterium leprae* infections through the activation of the type I interferon (IFN) pathway. After being inside the host cell, *M. leprae* is able to disrupt the phagosomal membrane by a mechanism that is dependent on the mycobacterial ESX-1 secretion system. Then, bacterial DNA activates the cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING)/TANK-binding kinase 1 (TBK1) pathway and promotes interferon regulatory factor 3 (IRF3) translocation, which induces IFN-β production. In response to an autocrine and/or paracrine IFN-β stimulus, macrophages increase *OASL* expression. OASL production inhibits bacterial clearance, blocking LC3-dependent autophagy, and promotes mycobacterial survival by creating a permissive microenvironment for sustainable growth and disease progression.

METABOLIC IMMUNITY IN LEPROSY

Using microarray analysis, researchers have pointed out important changes in metabolic pathways in bacterial infections such as *M. leprae* (15, 53). Determining the ability of intracellular pathogens to modulate the host metabolic pathways has provided an understanding of the infection in pathogenic terms (54). *M. leprae* must adjust the cytosol to its requirements, and the breach of the phagosomal membrane releases bacterial components that will trigger a metabolic switch.

When infected by intracellular pathogens, immune cells are able to increase nitric oxide synthetase inducible (iNOS) and indoleamine 2,3-dioxygenase-1 (IDO-1) activity. These enzymes catalyze the degradation of L-arginine and L-tryptophan, respectively, resulting in local amino acid deprivation (55). While iNOS generates nitric oxide radicals, IDO-1 leads to the production of kynurenine metabolites (56). This metabolite activates the aryl hydrocarbon receptor, promoting the conversion of naive CD4 T cells into Foxp3+ regulatory T cells (57). DCs are able to increase *IDO1* expression and activity in response to IFN-γ (56), and *IDO1* is highly activated in leprosy patients (58). Genetic variations in the *IDO1* gene are related to differential activation of regulatory T cell function and correlated with autoimmune disease development (59). IDO-1-mediated L-tryptophan deprivation is an innate response against viral replication. However, it is ineffective against mycobacterial infection. Despite the drastic reductive evolution in the *M. leprae* genome, all enzymes involved in L-tryptophan anabolism have been maintained. *M. leprae* infection activates the IDO-1 signaling pathway (55, 60, 61) in an iron and IL-10-dependent manner. Thus, the L-tryptophan deprivation does not affect *M. leprae* survival (56). Transforming growth factor beta, which is highly expressed in leprosy patients (58), is able to maintain high IDO-1 expression in DCs through phosphorylation of its immune-based inhibitory tyrosine motifs, leading to a sustained immunoregulatory effect (62).

Glucose plays a central role in energy metabolism as a carbon source. In addition, glucose is a highly versatile precursor of amino acids, coenzymes, fatty acids, and cholesterol. After phosphorylation, this molecule can follow a catabolic pathway such as that of glycolysis, generating energy and carbon to be burned in the mitochondria. Alternately, it can follow an anabolic pathway, such as the pentose phosphate pathway (PPP), which generates carbons and reducing equivalents, in the form of nicotinamide adenine dinucleotide phosphate (NADPH) to synthesize lipids, nucleotides, and aromatic amino acids (63). In both leprosy and tuberculosis, it was found that the bacilli increases glucose uptake in the infected host cells in a glucose transporter 1-dependent manner (64, 65). Modulation of glucose metabolism was noticed in *M. leprae*-infected Schwann cells (64) (**Figure 3**) while this event has been demonstrated in human macrophages infected by *M. tuberculosis* (65). The hypothesis that these mechanisms also occur in *M. leprae*-infected macrophages needs to be investigated.

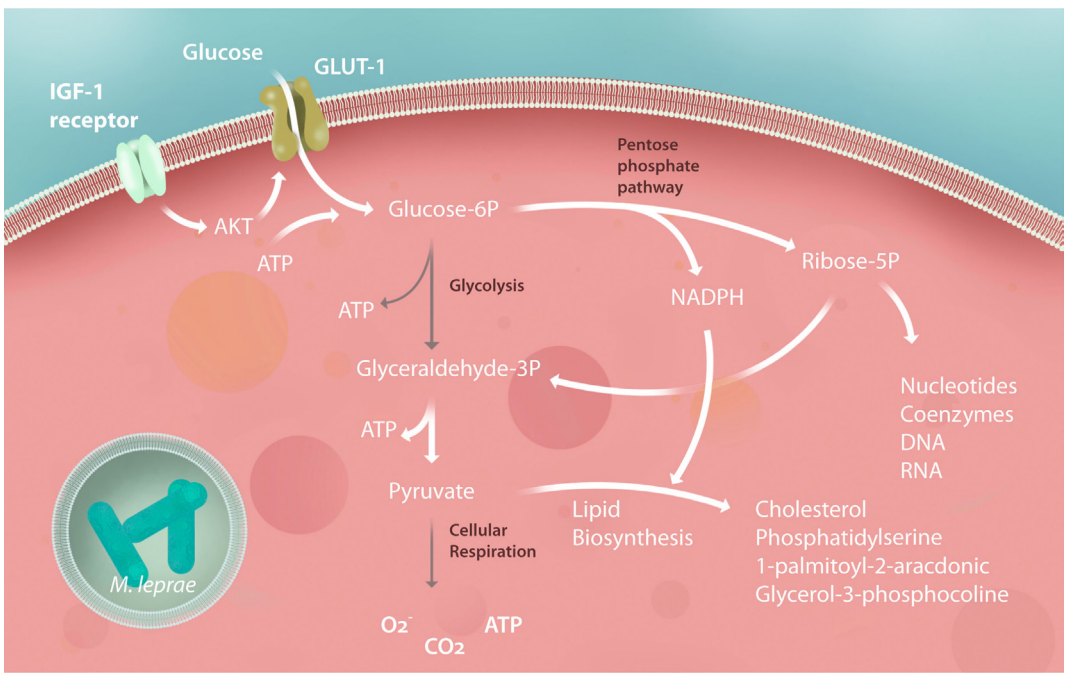

FIGURE 3 | Schwann cell central metabolism is subverted by *Mycobacterium leprae*. After infection, Schwann cells increase their insulin-like growth factor (IGF) expression, upregulating glucose transporter 1 (GLUT-1) and glucose uptake by Akt signaling. Glycolysis is downregulated, feeding the pentose phosphate pathway (PPP) with carbons used to synthesize building blocks to promote Schwann cell dedifferentiation and proliferation, generating during this process the reducing power [nicotinamide adenine dinucleotide phosphate (NADPH)] responsible for pumping up lipid biosynthesis. Pyruvate generated by the PPP is rapidly converted to citrate and subsequently converted to lipids, virtually stopping the tricarboxylic acid cycle, respiration and mitochondrial energy potential of the Schwann cells. All of these modulations are crucial for subverting the host immunity against the mycobacteria and, consequently, to the success of the *M. leprae* infection, representing potential for new host-target therapy strategies to halt leprosy progression. The gray arrows represent downregulated pathways.

Studies on the host carbohydrate metabolism during infection have demonstrated that many pathogens, including viruses such as immunodeficiency virus (HIV), hepatitis C virus (HCV), Mayaro, transmissible gastroenteritis virus, and human cytomegalovirus, can increase host cell glucose uptake to provide biosynthetic precursors for their replication (66–71). Furthermore, the synthesis of immune-active lipids, such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine, is able to generate a strong anti-inflammatory response when oxidized (72). In *M. leprae* infection, live bacteria are able to avoid free radical generation by using carbons from the electron transport chain (ETC) for lipid synthesis (64). To support the positive feedback of this pathway, *M. leprae* mediates an increase in the production of insulin-like growth factor (IGF)-1 in both macrophages and Schwann cells (73). IGF-1 is one of the main regulators of glycolysis metabolism. In macrophages, IGF-1 can impair the host antimicrobial activity and increase lipid metabolism (73). Otherwise, IGF-1 shares a high amino acid homology with insulin (74), and the structure of its receptors is closely related to post-receptor signaling (75). This signaling activation involves glucose uptake with subsequent lipid synthesis and storage in lipid bodies. Indeed, glucose uptake can be positively modulated by the IGF-1 receptor through the activation of the PI3K signaling pathway. Thus, virulent mycobacteria cause a metabolic switch that drives the cell toward the production of several micronutrients, macronutrients, and electron acceptors in response to infection.

After *M. leprae* infection, Schwann cells redirect glucose from the glycolysis pathway to the PPP through the activation of G6PD, increasing the carbon flux to lipid synthesis. The PPP generates ribose-5-phosphate and NADPH, as the main products that sustain cell proliferation, lipid biosynthesis, and the regeneration of oxidized glutathione, which is the main free radical scavenger of human cells (63). *M. leprae* is highly dependent on the host PPP because G6PD inhibition by pharmacological interference and RNA interference associated with G6PD knockdown decreases the viability of intracellular mycobacteria (64). During its adaptation, *M. leprae* has developed another mechanism to live inside human cells: shutting down the cell's mitochondria (64). The dissipation of the mitochondrial inner membrane electric potential after infection demonstrates the suppression of the ETC. This is probably due to the redirection of carbons to lipid synthesis for the formation of lipid bodies in infected cells, and it will increase long chain fatty acids in cytosol, responsible to mitochondrial permeability transition pore opening and consequent electric potential dissipation (3, 64, 76).

Gene expression analysis of skin lesions of lepromatous patients revealed upregulation of *SREBF1*, a host gene involved in lipid synthesis (77). Together with this observation, a mass spectroscopy analysis revealed that these patients' skin lesions were enriched with cholesterol (77) and other immune-active lipids, such as oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC), prostaglandins E2 and D2, lipoxin A_4, and omega-3 and omega-6 (72, 78). Live *M. leprae* can actively induce and support adipophilin, adipose differentiation related protein, and perilipin expression in macrophages, promoting lipid accumulation within the phagosome (77). In this context, host lipid synthesis and its deposition in infected tissues have been associated with pathogenesis and infection success in leprosy (79), with special involvement of cholesterol. In contrast to *M. tuberculosis*, *M. leprae* is not able to use cholesterol as a carbon source (80). However, during the reductive evolution of the genome, *M. leprae* maintained an enzyme of paramount importance to its survival, 3β-hydroxysteroid dehydrogenase, which is a catalyst in the first step of cholesterol degradation: the oxidation of cholesterol to cholest-4-en-3-one (cholestenone) (80). In clinical applications, avoiding cholesterol synthesis by treating infected macrophages with statins, inhibitors of HMG-CoA reductase, has a strong impact on intracellular *M. leprae* and *M. tuberculosis* viability (81). Based on microscopy data from our previous study, in which we demonstrated the ability of *M. leprae* to recruit and surround itself with lipid bodies (3), we hypothesized that *M. leprae* could use lipids to cover and hide its surface antigens from innate immune receptors in the cytosol.

Altogether, these host metabolic alterations are essential for immune response modulation and infection success. For that reason, new strategies based on host metabolite identification could, in the near future, contribute to preclinical diagnosis. The development of fast, highly sensitive, and non-invasive diagnostic tests is paramount for the control of this disease. As an example, it was demonstrated that it is possible to identify leprosy patients through detection of leukotriene E4 by gently pressing silica plates against their skin for a few seconds (82). Based on the fact that *M. leprae*-infected Schwann cells increase their glucose uptake by about 40% (64), we propose, as another example, that full body imaging of the glucose analog fludeoxyglucose using positron emission tomography could represent a potential non-invasive alternative for diagnosing pure neural leprosy.

LIPID METABOLISM DEREGULATION ASSOCIATED WITH INFLAMMATION IN LEPROSY

Several diseases are associated with deregulation of the host lipid metabolism, favoring an exacerbated inflammatory process that contributes to immunopathogenesis. In an experimental model of arteriosclerosis, for example, the lipid accumulation process and atherosclerotic plaque development are mediated by the production of monocyte chemoattractant protein-1 (MCP-1), which recruits monocytes to the inflammatory site. Largely differentiated from anti-inflammatory macrophages with an M2 profile, which has a foamy phenotype, these monocytes are rich in lipid droplets (83). MCP-1-mediated recruitment of peripheral monocytes was also observed in a zebra fish model of *Mycobacterium marinum* infection. In this model, MCP-1 produced by infected resident macrophages actively participated in the recruitment of monocytes to the infection site by a mechanism that was dependent on the STING signaling pathway (84). In the context of *M. leprae* infection, in the absence of *OASL*, a gene induced by type I IFN, there is a drastic decrease in the levels of MCP-1 and the intracellular viability of the bacilli in *M. leprae*-infected macrophages (2). Indeed, MCP-1 induction can be mediated by STING either by a type I IFN-dependent pathway or by an independent pathway

(85–87). These data, taken together, suggest a scenario in which the induction of the type I IFN pathway participates in MCP-1 induction. The enhancement of MCP-1 aids the recruitment of monocytes at the site of infection and promotes the differentiation of monocytes into macrophages with a M2 phenotype, exhibiting high levels of IL-10 and prostaglandin E2 (PGE2) (88). Lipid bodies are sites of production of eicosanoids, such as PGE2, leukotriene B4 (LTB4), and lipids, including cholesterol. This could explain the characteristic phenotype of foamy macrophages that present in the skin lesions of patients with lepromatous leprosy, as well as the abundance of immunological mediators, such as IL-10, IL-4, PGE2, and MCP-1, in these lesions (89).

In a *M. tuberculosis* murine model, IL-1β triggered PGE2 production as a protective response toward mycobacterial clearance and it is also negatively regulated type I IFNs. Curiously, highly susceptible mice (IL-1β knockouts, for example) can be rescued using PGE2 and zileuton, which is an inhibitor of 5-lipoxygenase that blocks LTB4 and, consequently, TNF (41, 90). Genetic polymorphisms of *LTB4* demonstrate an important association with the development of severe tuberculous meningitis, in which the inadequate balance of the inflammatory response that is mediated by TNF and LTB4 may aggravate the disease progression (90). The importance of the host's lipid metabolism regulation, which can affect the availability of nutrients to the pathogen as well as the production of inflammatory mediators, is increasingly evident. Host-based therapies are currently under development with the goal of metabolic drugs that could be interesting adjuvants in the mycobacterial diseases treatment, such as leprosy and tuberculosis.

Thus, ongoing mycobacterial survival is associated with enhancements to lipid metabolism. After infection takes place, mycobacteria cause a shift in the host cell gene expression that leads to lipid uptake through the receptor induction of cholesterol (15, 77) and the formation of lipid bodies (91). Strong modulation of lipid synthesis pathways in host cells by *M. leprae* or *M. tuberculosis* has been observed, and it has been suggested that lipid droplets work as a nutrient reservoir for *M. tuberculosis* (7). Although *M. leprae* are unable to remove carbons from cholesterol (80), both *M. leprae* and *M. tuberculosis* seem to take shelter within lipid bodies, which are formed abundantly by host cells (91). Therefore, as an example, a pharmacological approach to compensate for the induction of this crucial pathway for *M. leprae* survival would be the use of statins as an adjuvant in combination with multidrug therapy. Results from experimental models (81) suggest that modulation of autophagic mechanisms could also promote the antimicrobial response against *M. tuberculosis* and decrease inflammation-mediated immunopathology (31, 82, 83). Recently, mammalian target of rapamycin pharmacological agents, including rapamycin or AMPK targets such as metformin, have been tested in clinical trials as an adjuvant therapy in tuberculosis; these tests have been successful and can be applied in leprosy (92–94).

CONCLUSION

An infectious disease is a result of a specific and successive combination of events that can only culminate in complete progression if the bacteria are able to block several restrictive antimicrobial mechanisms. The last 10 years of research have been remarkable for revealing novel genes associated with leprosy, including complementary approaches such as genomic scans or GWASs and microarray analysis. Combining these data produce a clear overview of the mechanisms induced by bacteria to survive within hostile and sterile cellular cytosol. Gene-sensing mycobacterial components such as *NOD2* and *TLR1* and pathways that regulate autophagy (*PARK2*, *LRRK2*, and *RIPK2*) are intrinsically antimicrobial, but they can be opposed and inhibited by the emergence of type I IFN induction. In this scenario, double-stranded DNA receptors and STING/TBK1/IRF3 signaling drive a pro-mycobacterial response.

The fact remains that it is very difficult to define the chronology of these events or even the precise moment when the disease progression takes place in the infected individual. The rationale here is that defining these steps carefully and observing the fine-tuning of genotypic influences on phenotypes can help to halt the disease progression in infected people. Consequently, the current challenge is to combine results from *in vitro* and genotype-to-phenotype studies toward the development of host-directed therapies.

AUTHOR CONTRIBUTIONS

TP, LB-S, FL, and MM contributed equally to this manuscript. RM contributed with the "Metabolic immunity in leprosy" topic. All authors participated in the conception, design and writing of this review.

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Nerve Growth Factor and Pathogenesis of Leprosy: Review and Update

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Neurotrophins are a family of proteins that regulate different aspects of biological development and neural function and are of great importance in neuroplasticity. This group of proteins has multiple functions in neuronal cells, as well as in other cellular populations. Nerve growth factor (NGF) is a neurotrophin that is endogenously produced during development and maturation by multiple cell types, including neurons, Schwann cells, oligodendrocytes, lymphocytes, mast cells, macrophages, keratinocytes, and fibroblasts. These cells produce proNGF, which is transformed by proteolytic cleavage into the biologically active NGF in the endoplasmic reticulum. The present review describes the role of NGF in the pathogenesis of leprosy and its correlations with different clinical forms of the disease and with the phenomena of regeneration and neural injury observed during infection. We discuss the involvement of NGF in the induction of neural damage and the pathophysiology of pain associated with peripheral neuropathy in leprosy. We also discuss the roles of immune factors in the evolution of this pathological process. Finally, we highlight avenues of investigation for future research to broaden our understanding of the role of NGF in the pathogenesis of leprosy. Our analysis of the literature indicates that NGF plays an important role in the evolution and outcome of *Mycobacterium leprae* infection. The findings described here highlight an important area of investigation, as leprosy is one of the main causes of infection in the peripheral nervous system.

Keywords: nerve growth factor, leprosy, *Mycobacterium leprae*, pathogenesis, immunology

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INTRODUCTION

The most extensively studied neurotrophin is nerve growth factor (NGF). This growth factor consists of 118 amino acids and has a molecular weight of 130 kDa. NGF was discovered as a growth factor that participates in the responses of sympathetic and sensory neurons regulating differentiation, neuronal regeneration, and the perception of pain (**Figure 1**) (1–5). In sensory neurons, NGF specifically binds to the TrkA receptor with high affinity and to receptor p75 with low affinity. The binding of NGF to receptor p75 seems to optimize the activity of TrkA (**Figure 2**) (6, 7). In skin and immune cells, NGF is produced by the proteolytic cleavage of its precursor, proNGF. proNGF is translocated to the lumen of the endoplasmic reticulum, transported through the exocytic pathway, and converted to its active mature biological form, NGF (3). The roles of NGF in physiological and pathological processes have been studied in several systems. Specifically, the significance of this molecule in responses to infectious and inflammatory diseases has recently been investigated in experimental

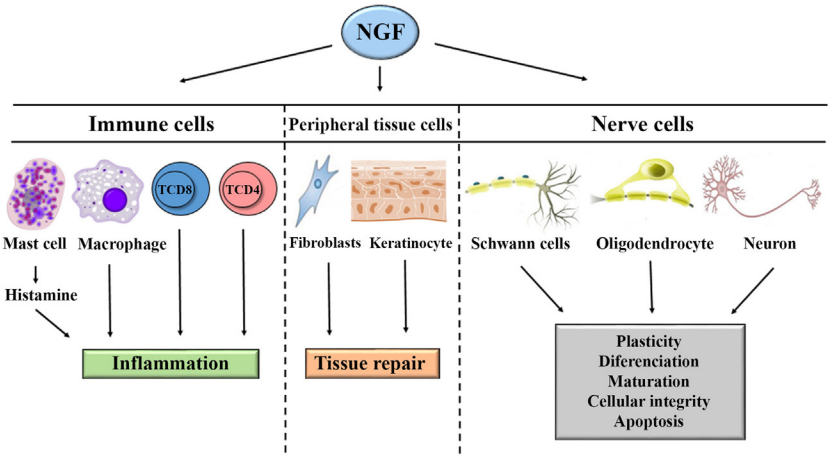

FIGURE 1 | General mechanism of action of nerve growth factor (NGF) in the systemic immune response, neuroinflammation, regeneration, and tissue repair. NGF acts on lymphocytes, mast cells, and macrophages to induce inflammation. NGF acts on fibroblasts and keratinocytes to induce tissue repair. NGF acts on oligodendrocytes, Schwann cells, and neurons to induce repair or apoptosis.

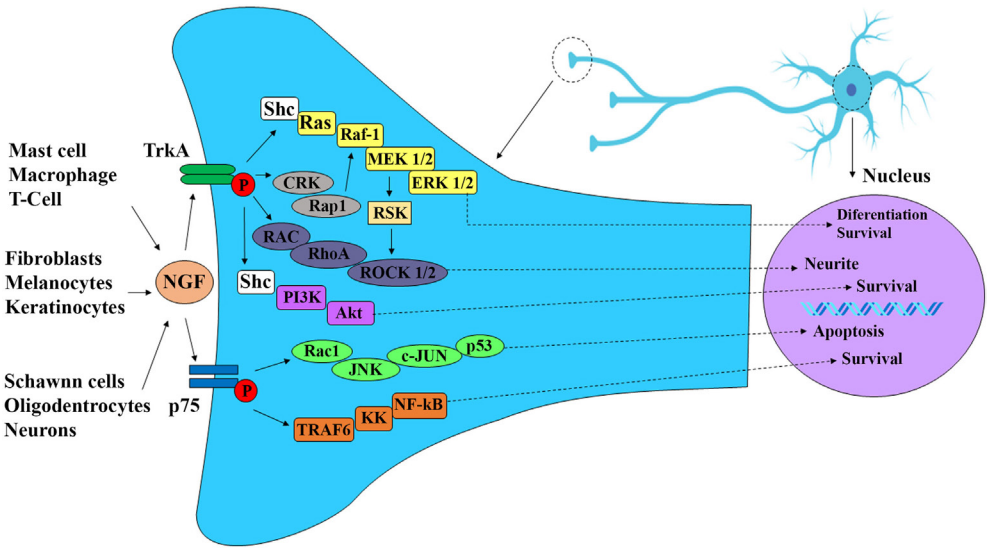

FIGURE 2 | Nerve growth factor (NGF) signaling cascade. NGF is produced during development and into maturity by immune and nerve cells as well as peripheral effector cells, such as keratinocytes, melanocytes, and fibroblasts. NGF exhibits high affinity to receptor TrkA and low affinity to receptor p75. The binding of NGF through receptor TrkA induces autophosphorylation of TrkA receptor and activation of P13K/Akt pathway leading to neuronal differentiation, survival, and neurite. NGF binding to receptor p75 activate c-Jun kinase (JNK) and NF-κB pathways resulting in neuronal apoptosis and survival respectively.

models of Chagas disease, respiratory syncytial virus infection, herpes simplex virus infection, myalgic encephalomyelitis, and other diseases (8–15).

DEFINITION AND BIOLOGICAL ASPECTS

Nerve growth factor was first described by Rita Levi-Montalcini (16), who showed its importance in the development, differentiation, maturation, and preservation of the integrity of sympathetic and sensory neurons (17). NGF is involved in modulating the sensitivity of peripheral nerve fibers to heat and pain in physiological and pathological events, such as genetic, metabolic (diabetes mellitus), and infectious neuropathies (18). Further supporting a relationship between NGF and leprosy, Scully and Otten and others by previous studies reported the involvement of NGF in sympathetic and sensory neuron apoptosis (**Figures 1** and **2**) (19–21).

Nerve growth factor is endogenously produced as proNGF during development and into maturity by immune and nerve cells, as well as peripheral effector cells, such as keratinocytes, melanocytes, smooth muscle cells, fibroblasts, and Schwann cells (5, 17, 18). It is also synthesized in other organs, such as the gonads, thyroid, parathyroid, and exocrine glands (e.g., salivary glands) (5, 21, 22). The expression and receptor binding affinity of NGF, as well as the duration and intensity of cellular events

triggered by proNGF activation, determine its specific activity in effector cells or neurons (21, 23, 24).

Following its synthesis, NGF enters nerve endings by endocytosis, a process that permits NGF transport to the nerve cell body through contact with NGF receptors (NGF-R) located on the plasma membrane (25, 26). Several studies have described the roles of NGF in the central nervous system (CNS). In adults, NGF is involved in plasticity. However, the promotion of neuronal survival by NGF has only been observed in cholinergic neurons. NGF regulates the size of the cell body, dendritic branching, and neuronal connectivity (27, 28). In the peripheral nervous system, NGF acts on sympathetic and sensory neurons derived from the neural crest (29).

Nerve growth factor has been suggested to be involved in apoptosis of sympathetic and sensory neurons (20, 21). The binding of NGF to glial cells (i.e., oligodendrocytes and Schwann cells) through receptor p75 induces proapoptotic effects. However, these effects depend on NGF binding intensity and Trk receptor interactions (1, 30). Receptor p75 is a low-affinity accessory receptor for the TrkA receptor family to which all neurotrophins can bind. Receptor p75 seems to modulate the activity of TrkA and its intracellular signaling cascade. These receptors are present not only in nerve cells and CNS tumors, but also in immune cells such as macrophages, lymphocytes, and mast cells. These cells synthesize, store, and secrete proNGF, which suggests a possible role for NGF in the regulation of immune responses during inflammatory, infectious, and autoimmune processes (31, 32).

Thus, in addition to its primary functions in the nervous system, NGF participates in inflammatory processes and immune responses. NGF concentrations are increased during tissue inflammatory processes. Increased NGF levels can produce hyperalgesia through direct activation of nociceptors, which leads to CNS activation and neurogenic inflammation. Moreover, this process leads to local events comprising activation-induced release of histamine and increased numbers of mast cells and other immune system cells (7, 19).

Labouyrie et al. described the relationship between NGF and cells in normal and pathologic human lymphoid tissues and showed that NGF is involved in inflammatory or lymphoproliferative disorders (33). Immunoreactivity to the NGF-R (TrkA) has been observed in tissues, such as the thymic epithelium, cryptic tonsillar epithelium, epithelioid cells, multinucleated macrophages, and follicular dendritic cells. These findings demonstrate a relationship between NGF and the immune system. Owing to its production and activity in immune cells, NGF is thought by some to be a cytokine that participates in immune system events controlling the immune response in inflammatory and infectious processes (32, 34).

Different inflammatory and autoimmune diseases lead to altered expression of NGF. Increased anti-NGF antibody levels have been detected in patients with rheumatoid arthritis, systemic lupus erythematosus, and thyroiditis and are thought to contribute to the immune dysfunction and nerve damage observed in these diseases (21, 35, 36). However, the relationship between NGF autoantibodies and NGF expression remains unclear. Further studies are needed to investigate the effects of NGF autoantibodies on the expression of NGF and its receptors in autoimmune disease.

Other studies have shown an association between NGF and immune response mechanisms (37–42). For instance, Lambiase et al. have investigated the expression of NGF and the TrkA receptor in CD4+ T cells (38). Santambrogio et al. have shown that B cells express and secrete NGF, which in turn regulates the expression and secretion of calcitonin gene-related peptide by these cells *via* the TrkA receptor. This process has previously been described for sensory neurons (39). The immune response components interferon-β and interleukin (IL)-1β increase NGF-R expression and activate its signaling pathways in neurons and participate in the control of apoptosis in these cells (40). T and B lymphocytes, dendritic cells, and macrophages express NGF, TrkA, and the p75 neurotrophin receptor (**Figure 2**). In the context of innate immunity, NGF is related to the activation of IL-1β, nod-like receptor protein 1, NLRP3, and caspase-1 and contributes to inflammasome activation. TNF-α can induce differentiation and neuronal maturation through its interaction with NGF, which is in turn involved in the neuronal survival process (41, 42). Nevertheless, further investigation is needed to better elucidate the role of NGF in controlling the immune response in other cell types (41–43).

NGF, IMMUNE RESPONSE, AND LEPROSY

Leprosy is a chronic disease caused by *Mycobacterium leprae*, an intracellular bacillus that leads to loss of sensibility, innervation, intra-epidermal damage, and lesions associated with the loss of myelin in Schwann cells (44). Clinically, the different forms of the disease are characterized in part by the immune response patterns of the host (19, 44). According to the classification by Ridley and Jopling, leprosy has five clinical forms based on clinical, histopathological, immunological, and bacilloscopic criteria: borderline-tuberculoid, borderline-lepromatous, borderline-borderline, tuberculoid, and lepromatous (45). The initial stage of infection is referred to as the indeterminate form. The World Health Organization (WHO) classifies patients with leprosy as multibacillary or paucibacillary for treatment purposes. These classifications are made based on the identification of the bacilli and the number of lesions. Bacilloscopy is not always possible. Therefore, according to the WHO, the number of lesions can be used to classify patients into three groups: paucibacillary single-lesion leprosy (one skin lesion), paucibacillary leprosy (two to five skin lesions), and multibacillary leprosy (more than five skin lesions) (46).

In the tuberculoid form of the disease, the lesions are granulomatous and the individual displays an intense cell-mediated immune response (Th1) that prevents the proliferation of the bacillus. In the lepromatous form, the cell-mediated immune response is characterized by an anti-inflammatory cytokine profile (Th2) that contribute to multiplication of the bacillus in macrophage phagosomes. In the borderline form, the patients exhibit immunological and histopathological characteristics that vary between those of the tuberculoid and lepromatous forms (47).

Nerve injury-associated tissue damage is arguably the most important clinical consequence of leprosy (47). In the process of leprosy-associated neuropathy, the presence of bacilli in nerve endings and Schwann cells induces a response mediated by macrophages and other cells that eventually leads to the appearance

of immune-mediated lesions. In this process, cytokines, such as TNF-α, IL-6, and IL-17, may contribute to the evolution of neural lesions and deformities that are characteristic of some forms of the disease. The immune response and inflammation are not only defined by the presence of chemical mediators, such as cytokines and chemokines. Rather, inflammatory edema is also very important in the neuropathy associated with leprosy, which can induce degeneration of neural fibers. There are strong positive correlations among the levels of NGF, NGF-R, and TGF-β in patients with leprosy. This indicates that the above factors have synergistic actions that reduce tissue damage resulting from nerve injury (48). Study by Antunes et al. (48) in patients with the neuritic form of leprosy (pure neural form or primary neural form), it was observed that the NGF-R immunoexpression was lower in nerve fibers and Schwann cells when compared to normal controls. In this study, hypoesthesia was correlated with decreased expression of NGF-R and protein gene product (PGP) 9.5, and electroencephalographic changes were observed in patients with altered immunolabeling for neurofilaments and PGP 9.5. These data point to a key role of NGF in the pathogenesis of neural lesions in leprosy.

Leprosy is the most common cause of non-traumatic neuropathy and is a classic example of an infectious neurodegenerative disease of the peripheral nervous system. It is estimated that more than one-fourth of patients with leprosy have some degree of disability and that about half of these patients have grade 2 disability corresponding to permanent neurological damage (44–59).

Studies have shown that different levels of NGF are associated with lepromatous and tuberculoid leprosy such that higher levels of NGF are associated with lepromatous forms and low levels of NGF are associated with tuberculoid forms of the disease (60). Specifically, Facer et al. have demonstrated the importance of NGF in leprosy (44). In patients with leprosy with and without lesions, TrkA receptors were shown to be present in subepidermal fibers and TrkA receptor messenger RNA was produced in the skin. The authors of the above study also evaluated the integrity of nerve endings and found that the presence of NGF in keratinocytes was correlated with deficient thermal sensation. Anand et al. detected low NGF levels in nerve and skin lesions of patients with leprosy and demonstrated that these low NGF levels contributed to the loss of NGF-dependent nociceptive fibers in damaged skin (19). Another study by Anand demonstrated that the loss of interaction between keratinocytes and nerves in affected skin drastically reduced the flow of NGF (61). Schwann cells produce NGF in response to axonal degeneration. However, while the levels of NGF are sharply reduced in the affected nerve trunks in patients with neuropathic lesions, there is a local increase in NGF levels in patients with chronic cutaneous hyperalgesia (61). The use of anti-NGF antibodies may be effective in treating hyperalgesia in patients with neuropathy and compromised nerve endings. In addition, physiological combinations of NGF, NT-3, and glial cell line-derived neurotrophic factor may assist in the reestablishment of homeostasis, and may thus be used in the treatment of neuropathic pain (61).

Other studies have suggested that NGF may restore pain sensitivity. Thus, NGF could play an important role in the prevention of ulcerations resulting from nociception loss, as observed in leprosy and other peripheral neuropathies (50, 61). Some reports indicate that anti-NGF treatment may promote analgesia in patients with hyperalgesia, which suggests a modulatory role for NGF in nociception (61).

Immunostaining of damaged tissues using anti-NGF and anti-NGF-R antibodies revealed higher expression of NGF in patients with lepromatous leprosy and lower expression of NGF in those with the indeterminate form of the disease (49). In lepromatous forms of leprosy, higher mean expression levels of NGF and its receptor are associated with larger and more diffuse lesion patterns and greater nerve involvement. The presence of NGF is generally more apparent in patients with the highest probability of nerve damage (i.e., those with lepromatous leprosy and young patients) (**Figure 3**) (52, 53).

Several important findings have elucidated the role of NGF as an immune response mediator (38). In addition to its well-documented involvement in the differentiation and growth of neurons, there is growing evidence that NGF exerts a broad spectrum of effects on immune cells. As a result, NGF is considered a pleiotropic molecule involved in different functions (i.e., neuropeptide modulation and tissue healing) that influence neural development, immune function, and injury responses (54).

Imbalance in the proNGF/NGF ratio, increased expression levels of TNF-α and p75 neurotrophin receptor, and impaired TrkA phosphorylation have been reported in microvascular preparations from Cre-proNGF transgenic mice when compared to normal control animals (43). TNF-α can induce differentiation and neuronal maturation through its interaction with NGF, which is in turn involved in the neuronal survival process (62). Endogenous TNF-α expression induced by NGF leads to a positive feedback loop comprising Akt activation through TNF receptor 2. This pathway promotes the survival of normal neural cells. Some studies suggest that TNF-α and IL-1β control the activity of NGF in human synovial fibroblasts modulating the physiopathology of osteoarthritis (41, 62).

The relationship between NGF and TGF-β in glial cells has been described in a study on mice and rats with spinal injury (37). Following injury, TGF-β1 levels increased, which in turn led to NGF messenger RNA and protein expression in the glial cells of these animals. In leprosy, the main target of *M. leprae* is the Schwann cell, which is the predominant cell type in the peripheral nervous system. Degradation of Schwann cells by *M. leprae* favors the development of peripheral neuropathy, which is the leading cause of morbidity in patients with leprosy. NGF may act as a protective factor for Schwann cells, and low levels of NGF may contribute to the development of neuropathy (38, 39).

Studies of systemic diseases (i.e., diabetes mellitus and osteoarthritis) have revealed an interaction between NGF and TGF-β (62, 63). Neurotrophins play a crucial role in the differentiation and survival of nerve cells. The characteristic positive correlations among NGF, NGF-R, and TGF-β in the clinical forms of leprosy highlight the interdependence of these factors (49).

Studies of lesions of patients with borderline leprosy have revealed strong correlations between TGF-β and NGF, and TGF-β and NGF-R ($r = 0.8722$ and $r = 0.7257$, respectively), with highly significant p-values for the two correlations ($p < 0.0001$ and

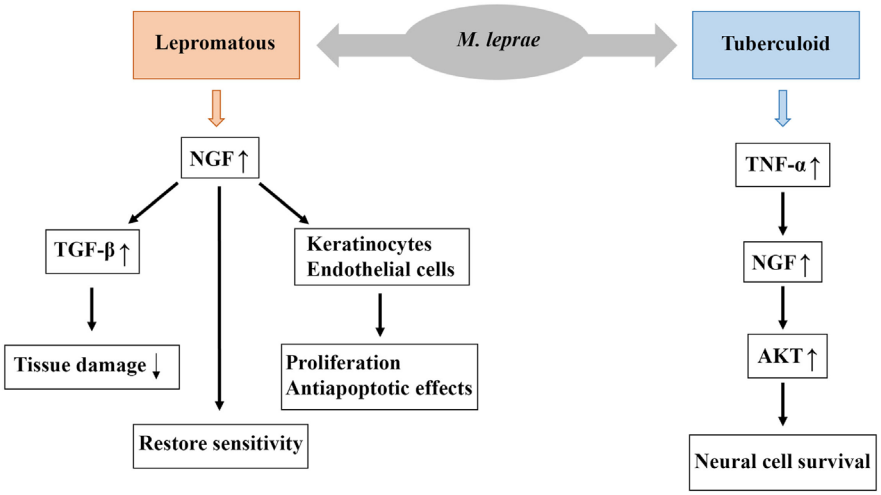

FIGURE 3 | Possible biological role of nerve growth factor (NGF) in the pathogenesis of leprosy. Higher levels of NGF are associated with lepromatous forms, and this increased NGF expression stimulates the expression of TGF-β, which reduce tissue damage resulting from nerve injury. Moreover, NGF restore sensitivity and exerts proliferative and antiapoptotic effects on keratinocytes and endothelial cells. Low levels of NGF are associated with tuberculoid forms and this low expression of NGF stimulates the expression of TNF-α which may contribute to the evolution of neural lesions. Low levels of NGF may also contribute to the development of neuropathy, such as sensitivity loss, nerve demyelination, and degeneration.

$p = 0.0015$, respectively) (48, 49). The borderline form of leprosy is immunologically dynamic and oscillates between the two polar forms. In patients with borderline-tuberculoid, borderline, and borderline-lepromatous leprosy, the progressive reduction in the cell-mediated response from the borderline-tuberculoid to the borderline-lepromatous form is accompanied by more extensive skin lesions and nerve damage, as well as increased bacillary burden and antibody levels (63, 64). In borderline forms of the disease, neurological manifestations resulting from immunological instability are characterized by nerve trunk impairment and frequent reactional episodes, which lead to early and asymmetrical nerve injuries and physical disability. This process is caused by increased numbers of bacilli in nerve branches close to Schwann cells (49). Cunha analyzed the relationships between the clinical forms of leprosy and episodes of neuritis. They found that patients with the borderline form of leprosy are 2.69 times more likely to progress to neuritis than those with the lepromatous form of the disease (65, 66).

During healing, NGF activates processes involved in the restoration of innervation (65, 67). In addition to inducing fibroblast migration, NGF exerts proliferative and antiapoptotic effects on keratinocytes and endothelial cells (68, 69). Moreover, NGF may be important for potentiating injury-specific responses through proinflammatory effects on neutrophils, eosinophils, mast cells, and T lymphocytes (67). The interactions of NGF in the tissue microenvironment are complex, and its relation to TNF-α, which can induce apoptosis in Schwann cells by binding to specific death receptors, may lead to antagonistic effects. This is because NGF can activate survival signals in the target cell. In fact, the same cytokine can have antagonistic effects depending on its interactions with specific receptors and the intracellular cascade activated after the activation of these receptors (**Figure 3**) (41–43, 62).

Cell-mediated immune responses may be beneficial against bacterial infections; however, inflammation can lead to irreversible tissue damage. Nerve injury occurs in approximately 10% of patients with paucibacillary leprosy and 40% of patients with multibacillary leprosy and is particularly acute in patients with reverse reactions (69, 70).

TGF-β participates in the tissue repair process as an anti-inflammatory agent during intense inflammation by inducing nerve and tissue regeneration (67, 71). Higher TGF-β expression in patients with the lepromatous form of the disease is associated with a higher frequency of apoptosis in lesions, especially within Schwann cells (72). The positive correlation between TGF-β expression and NGF expression may be associated with the protection and regeneration of nerve endings (60).

In lepromatous leprosy, neurological manifestations progress slowly over many years and involve small nerve branches and multiple nerve trunks within the skin (73, 74). Peripheral nerve injury, or any pathological condition that causes an interruption between the target organ and the nerve cell body, acts as a signal that induces non-neural cell populations (e.g., fibroblasts) to produce NGF. The induction of NGF synthesis in these cells is also modulated by cytokines, which invade the site of nerve injury where nerve regeneration is initiated (74).

The expression of p75 receptor in peripheral nerve cells is induced by loss of contact between the target organ and the axon (60). Some studies suggest that the p75 receptor is involved in axonal NGF uptake, reflecting the importance of NGF in efficient nerve regeneration. Patients with lepromatous leprosy have nerve trunk lesions, as well as multiple mononeuropathies and polyneuropathies (33, 71–76). The relationship between NGF and TGF-β is a key determinant of the actions of NGF in patients with the lepromatous form of the disease. Coordination between NGF and TGF-β responses in inflammatory processes following tissue damage is thus fundamentally important in remodeling and tissue repair (**Figure 3**) (60).

NEED FOR FUTURE RESEARCH

Prospective cohort studies and intervention studies evaluating patients with different clinical forms of the disease and different reactive states (erythema nodosum and reverse reaction types) may help better elucidate the relationship between NGF and the immune response and the factors contributing to the protection and regeneration of nerves affected during infection. Further analysis of the relationship among tissue levels of NGF and a large panel of pro- and anti-inflammatory cytokines, blood levels of NGF, and the immune response is important for a better understanding of the involvement of NGF in the pathophysiology of chronic granulomatous peripheral nerve infections, especially leprosy.

CONCLUSION

This review demonstrates that, within lesions associated with leprosy, NGF and TGF-β respond to inflammatory processes and tissue damage while triggering tissue remodeling. Further studies are needed to elucidate the broad role of NGF in the pathogenesis of leprosy. Although our understanding of the effects of NGF on nerve damage has increased, further insights into the functional roles of NGF (and other neurotrophins) in normal skin and during disease progression are needed.

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Mycobacterium leprae-Specific Antibodies in Multibacillary Leprosy Patients Decrease During and After Treatment With Either the Regular 12 Doses Multidrug Therapy (MDT) or the Uniform 6 Doses MDT

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Leprosy serology reflects the bacillary load of patients and multidrug therapy (MDT) reduces *Mycobacterium leprae*-specific antibody titers of multibacillary (MB) patients. The *Clinical Trial for Uniform Multidrug Therapy Regimen for Leprosy Patients in Brazil* (*U-MDT/CT-BR*) compared outcomes of regular 12 doses MDT/R-MDT and the uniform 6 doses MDT/U-MDT for MB leprosy, both of regimens including rifampicin, clofazimine, and dapsone. This study investigated the impact of R-MDT and U-MDT and the kinetic of antibody responses to *M. leprae*-specific antigens in MB patients from the *U-MDT/CT-BR*. We tested 3,400 serum samples from 263 MB patients (R-MDT:121; U-MDT:142) recruited at two Brazilian reference centers (Dona Libânia, Fortaleza, Ceará; Alfredo da Matta Foundation, Manaus, Amazonas). Enzyme-linked immunosorbent assays with three *M. leprae* antigens [NT-P-BSA: trisaccharide-phenyl of phenollic glycolipid-I antigen (PGL-I); LID-1: Leprosy Infectious Disease Research Institute Diagnostic 1 di-fusion recombinant protein; and ND-O-LID: fusion complex of disaccharide-octyl of PGL-I and LID-1] were performed using around 13 samples per patient. Samples were collected at baseline/M0, during MDT (R-MDT:M1–M12 months, U-MDT:M1–M6 months) and after MDT discontinuation (first, second year). Statistical significance was assessed by the Mann–Whitney *U* test for comparison between groups (p values < 0.05). Mixed effect multilevel regression analyses were used to investigate intraindividual serological changes overtime. In R-MDT and U-MDT groups, males predominated, median age was 41 and 40.5 years, most patients were borderline lepromatous and lepromatous leprosy (R-MDT:88%, U-MDT: 90%). The bacilloscopic index at diagnosis was similar (medians: 3.6 in the R-MDT and 3.8 in the U-MDT group). In R-MDT and U-MDT groups, a significant decline in anti-PGL-I positivity was observed from M0 to M5 ($p = 0.035$, $p = 0.04$, respectively), from M6 to M12 and at the first and second year posttreatment ($p < 0.05$). Anti-LID-1 antibodies declined from M0 to M6 ($p = 0.024$), M7 to M12 in the R-MDT; from M0 to M4 ($p = 0.003$), M5 to M12 in the U-MDT and posttreatment in both groups ($p > 0.0001$). Anti-ND-O-LID antibodies decreased during and after treatment in both

groups, similarly to anti-PGL-I antibodies. Intraindividual serology results in R-MDT and U-MDT patients showed that the difference in serology decay to all three antigens was dependent upon time only. Our serology findings in MB leprosy show that regardless of the duration of the U-MDT and R-MDT, both of them reduce *M. leprae*-specific antibodies during and after treatment. In leprosy, antibody levels are considered a surrogate marker of the bacillary load; therefore, our serological results suggest that shorter U-MDT is also effective in reducing the patients' bacillary burden similarly to R-MDT.

Clinical Trial Registration: ClinicalTrials.gov, NCT00669643.

Keywords: leprosy, serology, phenollic glycolipid-I antigen, LID-1, ND-O-LID, multidrug therapy

INTRODUCTION

The infection by *Mycobacterium leprae* in humans is characterized by a wide spectrum of clinico-pathological manifestations associated with distinct bacteriologic, immunologic, and histopathologic features categorized as tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL), and lepromatous leprosy (LL) (1). In leprosy patients, the specific antibody responses depend on the bacillary load. Vigorous antibody production with low or absent *M. leprae*-specific cell-mediated immunity (CMI) are seen in multibacillary (MB) patients while paucibacillary (PB) leprosy has strong *M. leprae*-specific CMI and low or undetectable antibody levels (2). Over the years, several leprosy serologic tests using different methodologies and antigens have been reported. Lateral flow, dipstick, particle agglutination, and enzyme-linked immunosorbent assays (ELISAs) mostly employing the *M. leprae*–specific native or synthetic di- or trisaccharide epitope of the phenollic glycolipid-I antigen (PGL-I) chemically linked to bovine or human serum albumin *via* octyl or phenyl group (ND-O or NT-P) have been tested in field-based studies (3–9). These studies have shown high IgM positivity in MB patients and low positivity in PB patients (5, 10, 11). After the completion of *Mycobacterium tuberculosis* and *M. leprae* genomes (12, 13), new *M. leprae*-specific proteins have been screened for serology and CMI-tests. The ML0405 and the ML2331 proteins were shown to be highly recognized by MB patients and were later engineered as the di-fusion LID-1 antigen (Leprosy Infectious Disease Research Institute/IDRI Diagnostic-1) (14–16). Positivity to IgG ELISAs to LID-1 is also proportional to the patient's bacillary load (14, 15, 17, 18). More recently, ND-O-LID antigen, a single fusion complex of natural disaccharide-octyl epitope (ND-O) of PGL-I and LID-1 has been used for the simultaneous, detection of IgM and IgG antibodies in lateral flow test and ELISA (19–23).

Leprosy is a treatable and curable disease and for multidrug therapy (MDT) implementation, patients are classified either as MB (six or more skin lesions, LL, BL, BB forms) or PB (up to five skin lesions, TT and BT forms) (24), The standard leprosy MDT treatment comprises two different regimens: 12 months with rifampicin, dapsone, and clofazimine for MB patients and 6 months with rifampicin and dapsone for PB patients (24). In MB patients, MDT reduces *M. leprae*-specific antibody titers suggesting the application of serology to monitor treatment efficacy (25–34). In 2007, an open-label, randomized clinical trial was conducted to compare the main outcomes [relapses, leprosy reactions, bacilloscopic index (BI) decline] of patients treated with the regular WHO MDT (R-MDT) and a 6-month uniform MDT regimen (U-MDT, rifampicin, dapsone, and clofazimine) for both PB and MB leprosy, regardless of any classification [Clinical Trial for Uniform Multidrug Therapy Regimen for Leprosy Patients in Brazil (U-MDT/CT-BR)] (35–42). As part of the U-MDT/CT-BR, a bank comprising sequential serum samples collected before, during and after R-MDT and U-MDT was assembled. This study, reports the impact of the U-MDT and the R-MDT on leprosy serology to PGL-I, LID-1, and ND-O-LID antigens and the kinetics of antibody responses at different time points in both treatment groups.

MATERIALS AND METHODS

Study Population

Our study group comprises only MB patients from *U-MDT/CT-BR* (Dona Libânia, Fortaleza, Ceará state and Alfredo da Matta, Manaus, Amazonas state), recruited from 2007 to 2015 that had positive bacilloscopy and at least three serum samples collected during monitoring (36). Serum samples tested were collected before MDT (M0/month zero), from 1 to 12 months after the start of MDT (M1–M12) and at the first and second year after the conclusion of treatment (R-MDT first and second year: 24 and 36 months after treatment conclusion, respectively and U-MDT first and second year: 18 and 30 months after treatment conclusion, respectively). Details of patients' recruitment, diagnosis, and main follow-up outcomes have been previously described (35–43).

Leprosy Serology

Serologic reactivity to *M. leprae* antigens was assessed by ELISA using the following antigens: natural trisaccharide-phenyl-BSA (NT-P-BSA) a semi-synthetic analog of PGL-I (batch: Nara XVI-61; Dr. Fujiwara, Japan), Leprosy Infectious Disease Research Institute Diagnostic-1 (LID-1) (batch: ago 2012, IDRI, USA) and the single fusion complex (ND-O-LID-batch: 17 August 2012, IDRI, USA).

Detection of IgM Antibodies to PGL-I

Serum IgM antibodies to PGL-I were detected by ELISA as previously described (3). PolySorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 50 µl/well of 0.01 mg/mL of the sugar

component of NT-P-BSA or BSA and blocked with 1% BSA/PBS. Serum samples diluted 1/200 in PBS-Tween containing 10% normal goat serum/NGS (Sigma-Aldrich, St. Louis, MO, USA) were tested in NT-P-BSA and in BSA coated wells. After incubation and washings, horseradish peroxidase/HRP-conjugated anti-human IgM (Immuno Chemicals, St. Louis, MO, USA) was added. In order to control plate-to-plate and day-to-day variation, a positive reference serum was added in duplicate on each plate. After incubation and washings, peroxidase color substrate (TMB, Sigma-Aldrich, St. Louis, MO, USA, Homemade) was added and the reaction was quenched by the addition of 2.5 N H_2SO_4, when the OD at 450 nm from reference serum reached an OD value of 0.6. The OD was measured at 450 nm using a Bio-Rad micro plate reader (Life Science, Hercules, CA, USA). The final OD was calculated by subtracting the OD of BSA coated wells from OD values of NT-P-BSA coated wells. The cutoff was defined as OD > 0.25 as previously described (5).

Detection of IgG Antibodies to LID-1 and Detection of IgM and IgG Antibodies to ND-O-LID

Serum IgG antibodies to LID-1 were detected by ELISA. Polysorp 96-well plates (Corning Costar, NY, USA) were coated with 100 µl/well of 1 µg/mL LID-1 or with 100 µl/well of 0.25 µg/mL ND-O-LID. Blocking was performed with PBS-T 1% BSA. Serum samples diluted 1/200 in PBS-T-10% NGS were added in duplicate and incubated for 2 h at room temperature. Plates were washed and incubated for 1 h with HRP-conjugated anti-human IgG (Southern Biotech, Birmingham, AL, USA) for anti-LID-1 serology or for anti-ND-O-LID serology plates were incubated with anti-human IgG (Southerm Biotech, Birmingham, AL, USA) plus anti-human IgM (Immuno Chemicals, St. Louis, MO, USA). After washings, reactions were developed with peroxidase color substrate (KPL, Gaithersburg, MD, USA) and quenched by the addition of 1 N H_2SO_4. The optical density (OD) was determined (Bio-Rad microplate reader, Life Science, Hercules, CA, USA) at 450 nm. For anti-LID-1 serology, the cutoff was calculated as two times the SD of the OD of sera from healthy endemic controls, such that samples with OD > 0.3 were considered positive (15). As previously described, the anti-ND-O–LID serology threshold for positive responses was considered OD >0.923 (20). The results of serologic tests were expressed as the mean OD of duplicates.

Statistical Analyses

Antibody levels were measured taking into account the medians of the OD at different time points in each treatment group. The percentage of positive samples was calculated based on the number of samples with OD above the cutoff established for each test at each time point. The statistical analyses performed in this study aimed mainly to answer if the data supported the hypothesis that the serological results have a time trend after the beginning of treatment, reflecting the reduction in bacillary load, and if this trend differed between the two treatment groups. The first statistical analysis employed Kruskal–Wallis test one-way analysis of variance for comparison of multiple groups and the Mann–Whitney *U*-test for comparison between two groups comparing data of all patients at different time points. Results were considered statistically significant when p values <0.05 were obtained. The intraindividual decay of serology among patients from R-MDT and U-MDT was evaluated employing mixed effects hierarchical/multilevel regression analyses using STATA software (44, 45). The multilevel analyses considered the individual serological results to each different antigen during different time points of follow-up. For these analyses, the independent variable was the serological result, the dependent variables were time and treatment group, and the group variable was patient ID. These analyses allowed the investigation of the effects that vary by group (each patient) and estimate group level averages in which each patient has his own time trend where one measure is not independent of the previous one.

RESULTS

Main Characteristics of Study Population

In this study, we have assessed the serologic reactivity of 3,400 sequential serum samples collected at different time points, from 263 MB leprosy patients, with positive bacilloscopy, enrolled at U-MDT/CT-BR and treated either with the R-MDT or U-MDT regimens. Among 263 MB patients, 56 were from Amazonas State and 207 came from Ceará State. In our study group, 54% (142 out of 263) received U-MDT and 46% (121 out of 263) were treated with R-MDT. For each patient, a median of 13 sequential serum samples (range: 3–21 samples) was collected at different time points: before MDT (M0), during treatment (M1–M6 for U-MDT and M1–M12 for R-MDT) and after treatment conclusion (first and second year). The main clinical and laboratory features of MB leprosy patients included in this serological study were similar (**Table 1**). The majority of MB leprosy patients was male, and patients from R-MDT and U-MDT groups had similar age (median age: 41 and 40.5 years, respectively). The majority of patients from the R-MDT and U-MDT groups was classified as BL and LL leprosy (R-MDT: 88%, 107 out of 121; U-MDT: 90%, 128 out of 142). The median of the BI in the R-MDT group was 3.6 (0.2–5.75 range) and 3.8 (0.2–6 range) in the U-MDT group. In the R-MDT group, 61% (69 out of 113) developed a reactional episode, 67% had reversal reaction (RR) (46 out of 69) of these, 11% (5 out of 46) at diagnosis and 89% during follow-up (41 out of 46). In the R-MDT, 33% (23 out of 69) had erythema nodosum leprosum/ENL, of these 4% (1 out of 23) at diagnosis and 96% (22 out of 23) during follow-up. In the U-MDT group, 62% was reactional (82 out of 132) of these 72% had RR (59 out of 82) of these, 14% (8 out of 59) at diagnosis and 86% during follow-up (51 out of 59). In the U-MDT, 28% (23 out of 82) had ENL, of these 9% (2 out of 23) at diagnosis and 91% (21 out of 23) during follow-up.

Decline of Anti-PGL-I, Anti-LID-1, and Anti-ND-O-LID *M. leprae*-Specific Antibody Levels in the U-MDT and R-MDT Groups During Follow-Up

Compared to baseline results, in both R-MDT and U-MDT groups, a significant decline in anti-PGL-I levels was observed

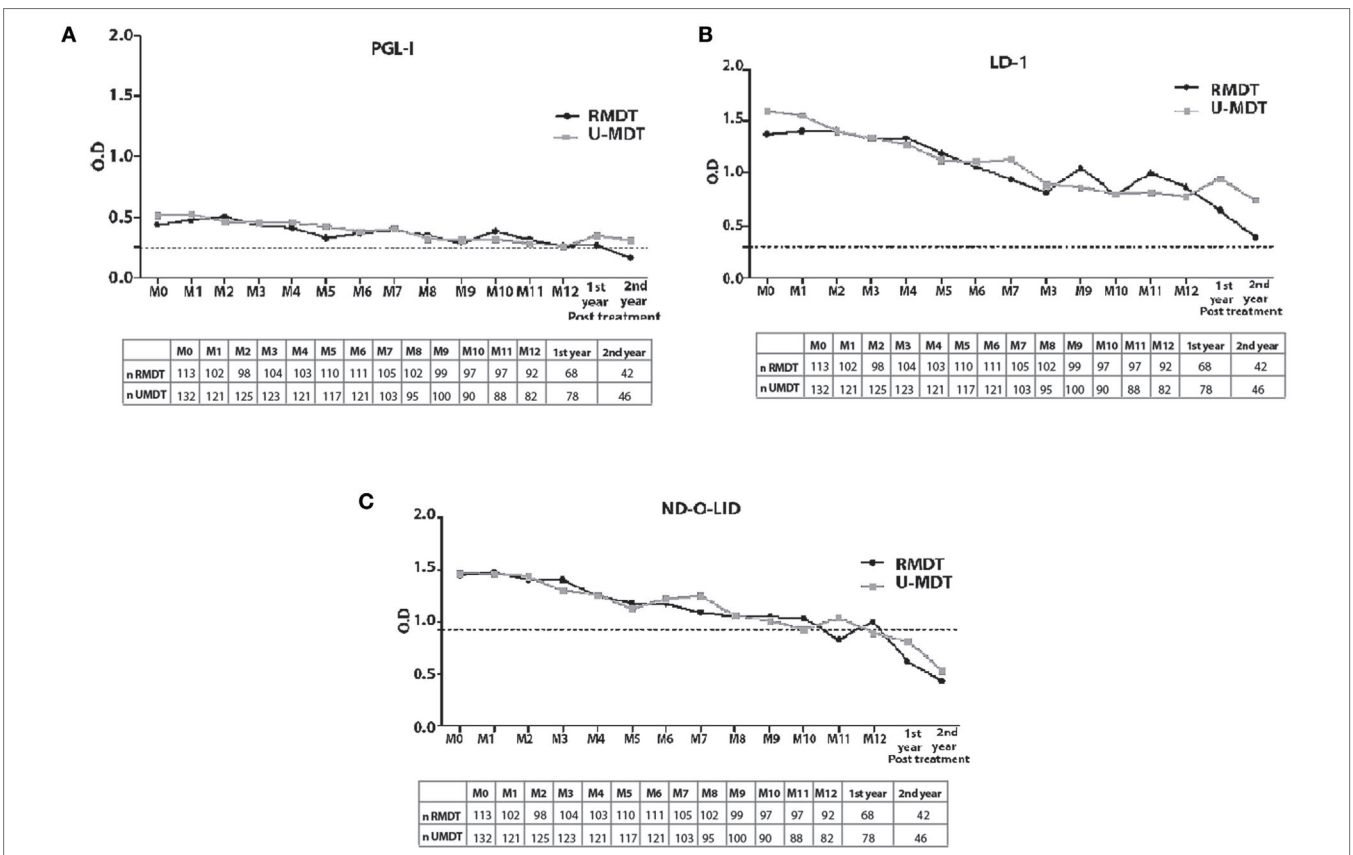
upon treatment (**Figure 1A**). At baseline, the median OD in the R-MDT group was 0.437 and in the U-MDT group, it was 0.516; after 5 months of treatment, the median OD was 0.325 in the R-MDT group and 0.424 in the U-MDT group ($p = 0.035$ and $p = 0.04$, respectively). In the R-MDT group considering baseline (M0) serology, there was a significant decline of the anti-PGL-I levels in the subsequent months (M0 vs M5, $p = 0.03$; M0 vs M7, $p = 0.09$; M0 vs M8, $p = 0.01$; M0 vs M9, $p = 0.01$; M0 vs M10, $p = 0.02$; M0 vs M11, $p = 0.007$; M0 vs M12, $p = 0.001$, M0 vs first year, $p = 0.02$; M0 vs second year, $p < 0.0001$) (**Figure 1A**). In the U-MDT group, anti-PGL-I levels at diagnosis also reduced during and after treatment (M0 vs M5, $p = 0.04$; M0 vs M6, $p = 0.02$; M0 vs M7, $p = 0.01$; M0 vs M8, $p = 0.001$; M0 vs M9, $p = 0.003$; M0 vs M10, $p = 0.0007$; M0 vs M11, $p = 0.001$; M0 vs M12, $p < 0.0001$, M0 vs first year, $p = 0.03$; M0 vs second year, $p = 0.0004$) (**Figure 1A**).

Regarding serological results in the R-MDT group, a significant decline in IgG anti-LID-1 antibodies was observed comparing baseline and M6 (median ODs at M0 = 1.386 vs M6 = 1.068; $p = 0.024$) (**Figure 1B**). In the R-MDT group, anti-LID-1 antibodies continued to decrease during subsequent months (M0 vs M7, $p = 0.0003$; M0 vs M8, $p < 0.0001$; M0 vs M9, $p = 0.0009$; M0 vs M10, $p < 0.0001$; M0 vs M11, $p = 0.0005$; M0 vs M12, $p < 0.0001$), and at the first and second year posttreatment (M0 vs first year, $p = 0.002$; M0 vs second year, $p < 0.0001$) (**Figure 1B**). In the U-MDT group, anti-LID-1 antibodies declined significantly

TABLE 1 | Main clinical and laboratory characteristics of the 263 MB leprosy patients enrolled at U-MDT/CT-BR stratified according to the treatment group.

	R-MDT (n = 121)	U-MDT (n = 142)
Gender (male/female)	86/35	105/37
Age (years) median (range)	41 (8–65)	40.5 (7–65)
R&J classification	12 BT, 2 BB, 75 BL, 32 LL	9 BT; 5 BB; 89 BL, 39 LL
BI median (range)	3.6 (0.2–5.75)	3.8 (0.2–6)
Development of reactions	73/121 (60%)	88/142 (62%)
Type and moment of development of reactions	RR: 50/73 At diagnosis: 7/50 During follow-up: 43/50 ENL: 23/73 At diagnosis: 1/23 During follow-up: 22/23	RR: 62/88 At diagnosis: 8/62 During follow-up: 54/62 ENL: 26/88 At diagnosis: 2/26 During follow-up: 24/26

R&J, Ridley and Jopling classification system; BB, borderline borderline; BL, borderline lepromatous; LL, lepromatous leprosy; BI, bacilloscopic index; R-MDT, regular multidrug therapy; U-MDT, uniform multidrug therapy; RR, reversal reaction; ENL, erythema nodosum leprosum; MDT, multidrug therapy; BT, borderline tuberculoid.



	M0	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	1st year	2nd year
n RMDT	113	102	98	104	103	110	111	105	102	99	97	97	92	68	42
n UMDT	132	121	125	123	121	117	121	103	95	100	90	88	82	78	46

FIGURE 1 | Kinetic of *Mycobacterium leprae*-specific antibody responses in multibacillary treated with regular multidrug therapy (R-MDT) (closed black circles) and uniform multidrug therapy (U-MDT) (gray closed squares) from baseline month zero (M0) to month 12 (M12) after starting treatment, and after the first and second year of treatment conclusion. **(A)** Anti-phenollic glycolipid-I antigen serology; **(B)** anti-LID-1 serology; **(C)** anti-ND-O-LID serology. Each point represents the median optical density (OD) value of each group. The dotted horizontal lines indicate cutoff points of each serological test.

from baseline to the fourth month of treatment (median ODs at M0 = 1.605, at M4 = 1.279; $p = p = 0.003$). Compared to baseline data/M0, anti-LID-1 antibodies decreased after M5 (M0 vs M5, $p < 0.0001$; M0 vs M6, $p < 0.0001$; M0 vs M7, $p = 0.0003$ and M0 vs M8, M0 vs M9, M0 vs M10, M0 vs M11 and M0 vs M12, $p < 0.0001$) and at the first and second year posttreatment (M0 vs first year and M0 vs second year, $p < 0.0001$) (**Figure 1B**).

In the R-MDT group, serology using ND-O-LID antigen showed a significant decline in antibody levels from baseline (M0) to the seventh month of treatment (M7) (median ODs at M0 = 1.449 vs M7 = 1.092; $p = 0.005$) (**Figure 1C**). Among patients treated with R-MDT, anti-ND-O-LID antibodies continued to decrease after M8 (M0 vs M8, $p = 0.003$; M0 vs M9, $p = 0.002$; M0 vs M10, $p = 0.0007$; M0 vs M11, $p = 0.0006$; M0 vs M12, $p < 0.0001$, M0 vs first year and M0 vs second year posttreatment, $p < 0.0001$) (**Figure 1C**). In the U-MDT group, a significant decline in anti-ND-O-LID antibodies was observed from baseline to the fifth month (median ODs M0 = 1.466 vs M5 = 1.126; $p = 0.006$) and antibody levels decreased after M6 (M0 vs M6, $p = 0.02$; M0 vs M7, $p = 0.01$; M0 vs M8, $p = 0.0006$; M0 vs M9, $p = 0.0003$; M0 vs M10, $p = 0.0001$; M0 vs M11, $p = 0.0006$ and M0 vs M12, $p < 0.0001$) and in the first year and second year posttreatment ($p < 0.0001$) (**Figure 1C**).

Decline in the Positivity Rates for Anti-PGL-I, LID-1, and ND-O-LID Antibodies Among U-MDT and R-MDT Groups

At baseline, 71% of MB patients who received R-MDT was anti-PGL-I positive, after 6 months MDT (M6) positivity declined to 63% ($p > 0.05$), and at the end of treatment (M12) 46% of patients remained anti-PGL positive (M0 vs M12, $p = 0.0001$) (**Figure 2A**). In the first-year posttreatment, 43.5% (27 out of 62) was positive and in the second-year posttreatment, 38% (15 out of 39) remained positive. Regarding baseline serology, the decline in anti-PGL-I positivity in the R-MDT group was statistically significant (M0 vs M12, M0 vs first year, M0 vs second year post-treatment, $p = 0.0001$).

In the U-MDT group, 74% was anti-PGL-I positive at baseline, 66% at 6 months (M0 vs M6, $p > 0.05$) and at M12, 51% remained anti-PGL-I positive (M0 vs M12, $p = 0.0002$) (**Figure 2B**). Positivity rate in the first-year posttreatment was 58% (43 out of 74) and in the second-year posttreatment 44% (18 out of 41) remained positive. Compared to serology at diagnosis, in the U-MDT group, the reduction in anti-PGL-I positivity rate was statistically significant (M0 vs first year, $p = 0.008$; M0 vs second year posttreatment, $p = 0.0001$). Anti-PGL-I positivity rates in patients from the R-MDT and the U-MDT regimens were similar at different time points: M0, M6, M12, and second year posttreatment ($p > 0.05$) (**Figures 2A,B**). In the first-year posttreatment, anti-PGL-I positivity rate was higher in the U-MDT than in the R-MDT group ($p = 0.04$).

In the R-MDT group, anti-LID-1 positivity rate at baseline was 88%, after 6 months of treatment (M6) 84% remained positive and at the end of MDT (M12), 79% had antibodies above the positivity threshold (**Figure 2C**). In the first year posttreatment, 62% (42 out of 68) of patients was seropositive and in second year 61% (26 out of 42) remained positive. In the R-MDT group, the percentage of anti-LID-1 positivity was similar at M0, M6, and M12 ($p > 0.05$). However, a statistically significant reduction in positivity rate was seen comparing M0 and first year ($p = 0.0002$) and M0 and second year posttreatment ($p = 0.0001$). In the U-MDT group, before treatment 88% of the patients was anti-LID-1 positive, after 6 months/end of treatment 86% was positive and at M12 80% remained positive (**Figure 2D**). In first year posttreatment, 78% (61 out of 78) was anti-LID-1 positive and in second year positivity was 65% (30 out of 46). The positivity rate to LID-1 antigen was similar at different time points: M0, M6, and M12 ($p > 0.05$). The reduction in the positivity rate to LID-1 serology was significant comparing M0 vs first year and M0 vs second year posttreatment ($p = 0.03$ and 0.0002, respectively). Positivity rates to LID-1 serology between U-MDT and R-MDT groups were similar at M0, M6, and M12 ($p > 0.05$) (**Figures 2C,D**). In the first year posttreatment, a statistically significant difference was observed between U-MDT and R-MDT regimens ($p = 0.01$).

Anti-ND-O-LID positivity in the R-MDT group was 73% at baseline, after 6 months MDT (M6) positivity was 63% ($p > 0.05$) and at the end of treatment (M12), 54% of patients remained positive (M0 vs M12 $p = 0.003$) (**Figure 2E**). At the first year posttreatment, 31% (21/68) of patients remained positive and in the second year 17% were seropositive (7 out of 42) (M0 vs first year, $p = 0.0001$, M0 vs second year, $p = 0.0001$). In U-MDT group, before treatment, 73% of the patients was anti-ND-O-LID positive, after 6 months/end of treatment, positivity was 62% and at M12, 47% remained positive (**Figure 2F**). In the U-MDT group, the decrease in anti-ND-O-LID positivity rate during the first 12 months of monitoring was statistically significant (M0 vs M12, $p = 0.0006$). There was a significant decline in the positivity rate to anti-ND-O-LID serology from baseline to the first year posttreatment and from baseline to the second year after treatment conclusion (M0 vs first year, $p = 0.0001$; M0 vs second year, $p = 0.0001$). Anti-ND-O-LID positivity rates between U-MDT and R-MDT were similar at M0, M6, and M12 ($p > 0.05$) (**Figures 2E,F**). In the first year after treatment conclusion, a statistically significant difference in positivity rate was observed between U-MDT and R-MDT regimens ($p = 0.03$).

Our study group of 263 MB patients included the BT, BB, BL, and LL categories, according to the adapted Ridley and Jopling classification system used. Among 263 MB patients, 28 were either BT ($n = 14$) or BB ($n = 14$), representing 12% of the R-MDT group and 10% of the U-MDT group. BT and BB have lower BI compared to BL and LL categories and as serology reflects the BI of the patient, the impact of BT/BB patients on the serology was analyzed by removing these 28 patients from the groups and by comparisons of results of the groups with and without BT/BB. Our results showed that difference in serological results obtained upon exclusion of BT and BB patients was statistically significant only for LID-1 serology at M6 while for all other antigens and time points there was no statistically significant change in the positivity rate by comparing the whole group of BT, BB, BL, LL, and the group of BL and LL patients (Table S1 in Supplementary Material).

The association between the BI at diagnosis and the serologic responses to the three antigens was evaluated 12 months after

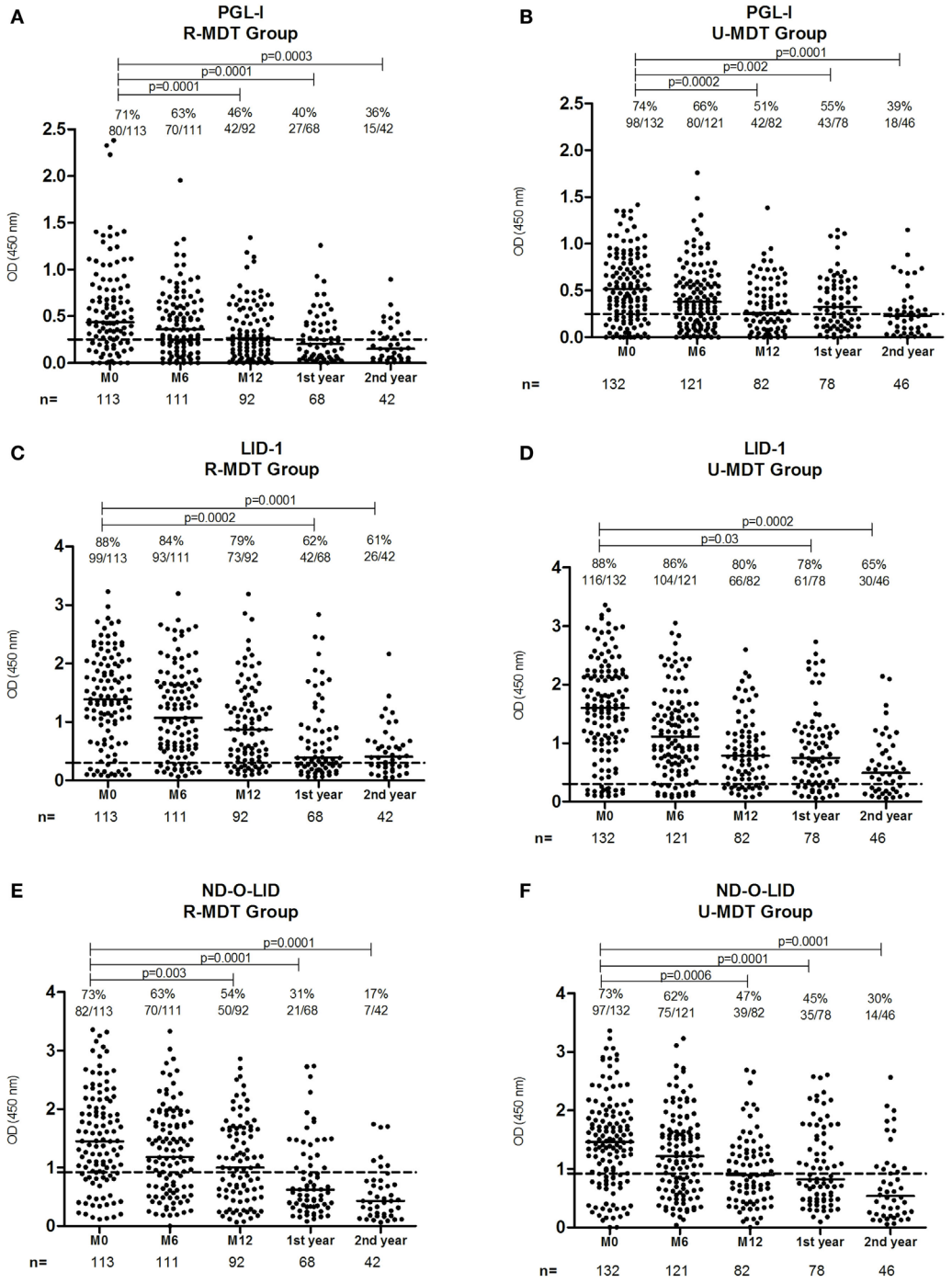


FIGURE 2 | Serologic reactivity to phenollic glycolipid-I antigen (PGL-I), LID-1, and ND-O-LID antigens among multibacillary patients from the regular multidrug therapy (R-MDT) and uniform multidrug therapy (U-MDT) groups at different time points MO, M6, M12, and first and second year posttreatment: anti-PGL-I positivity rates in **(A)** R-MDT group, **(B)** U-MDT group; anti-LID-1 positivity rates in **(C)** R-MDT group, **(D)** U-MDT group; anti-ND-O-LID positivity rates in **(E)** R-MDT group, **(F)** U-MDT group. Each point represents the mean optical density (OD) of duplicates of each individual patient. The median OD value of each group is represented by the horizontal line and the traced line represents the different cutoffs (PGL-I OD > 0.25, LID-1 OD > 0.3, and ND-O-LID OD > 0.923). The number above each dataset is the percent of positive responses. The *p* value refers to differences in positivity rates at different time points.

initiation of both regimens (**Figures 3A,B**). In the R-MDT group, these analyses included 32 patients with BI ≤ 3 (median = 2, range 0.2–3.0) and 56 patients with BI > 3 (median = 4, range 3.2–5.75). For all antigens tested, the median of OD at M12 was higher among patients with initial BI > 3 compared to patients with BI ≤ 3 ($p < 0.05$) especially for LID-1 serology ($p = 0.0002$)

(**Figure 3A**). In the U-MDT group, 28 patients with BI ≤ 3 (median BI = 1.775, range 0.2–3) and 51 patients with BI > 3 (median BI = 4, range 3.2–6) had their serologic responses compared at M12. In this group, a higher OD value to all three antigens was seen in patients with BI > 3, however, reaching statistical significance only for LID-1 antigen ($p = 0.009$) (**Figure 3B**).

Intraindividual Decline of Anti-PGL-I, Anti-LID-1, and Anti-ND-O-LID *M. leprae*-Specific Antibody Levels in the U-MDT and R-MDT Groups

Multilevel regression analyses were performed with serologic results to PGL-I (**Figure 4A**), LID-1 (**Figure 4B**), and ND-O-LID antigens (**Figure 4C**) at different time points of follow-up in 850 samples of 244 patients from both R-MDT and U-MDT groups. These analyses showed that the difference in serologic decay to all three antigens was dependent upon time only. Similar decay of serology to all three antigens was seen among patients from both R-MDT and U-MDT (**Figures 4A–C**; **Table 2**).

DISCUSSION

This study including cross sectional and intraindividual analyses showed that both shorter 6 months U-MDT and standard 12 months R-MDT using rifampicin, dapsone, and clofazimine had a similar effect on leprosy specific serology, reducing the antibodies of MB leprosy patients to three well-characterized *M. leprae* antigens: PGL-I, LID-1, and anti-ND-O-LID. Serologic responses detected at baseline declined during the course of therapy and continued to decline after discontinuation of specific treatment. Multilevel analyses of intraindividual responses showed that for both treatment regimens R-MDT and U-MDT, the decay of serologic reactivity to all three antigens tested was dependent on time only. In leprosy, serology is considered a surrogate marker of the bacillary load and a previous study has shown that MB patients from the R-MDT and the U-MDT groups had similar reduction in the bacillary load (39). In both treatment groups, despite minor oscillations, the pattern of decline in antibody levels was similar for all three *M. leprae* antigens tested. Our results indicate that, regardless of the duration of the treatment regimens for multibacillary patients, antibodies decline overtime during and after treatment interruption. This is the first description of the dynamics of antibody responses to three *M. leprae*-specific antigens among a well-characterized cohort of MB patients, mostly with high bacillary load at diagnosis, who was treated with two different MDT regimens and rigorously monitored during a clinical trial in Brazil. Our study sample contained a robust collection of 3,400 serum samples, including around 13 sequential samples per patient, which were collected since diagnosis over a 3-year period and this serologic study revealed the kinetic of specific antibody responses during this period.

The decrease in antibody levels following MDT, especially to PGL-I, has been well reported in previous studies showing longitudinal data (25–32, 34). In the U-MDT group, antibody titers to all three antigens tested, decreased during the 6-month treatment and despite some variation, anti-PGL-I antibodies continued to decline after treatment discontinuation until the first and second year after MDT conclusion. However, among MB patients most of them with high bacillary load at diagnosis, despite the decline in antibody levels, most patients remained seropositive 2 years after treatment conclusion. The decline in anti-PGL-I positivity from baseline to 2 years after treatment discontinuation was similar in both treatment groups when 38% in the R-MDT and 44% in the U-MDT showed serological responses above the cutoff for

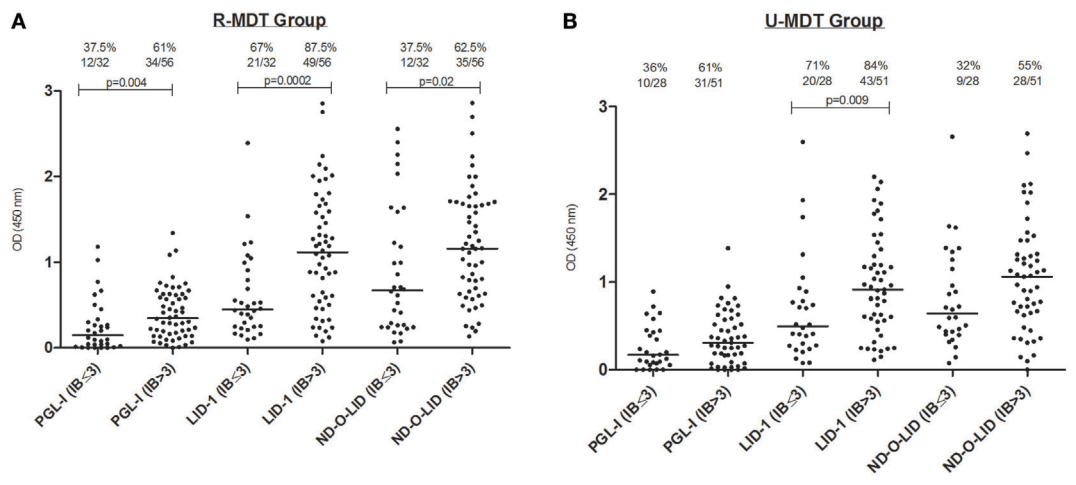


FIGURE 3 | Serologic reactivity at 12 months to phenollic glycolipid-I antigen (PGL-I), LID-1, and ND-O-LID antigens among multibacillary (MB) patients stratified by BI ≤ 3 and BI > 3 from the regular multidrug therapy (R-MDT) **(A)** and uniform multidrug therapy (U-MDT) **(B)** groups. Each point represents the mean OD of duplicates of each individual patient. The median OD value of each group is represented by the horizontal line and the traced line represents the different cutoffs (PGL-I OD > 0.25, LID-1 OD > 0.3, and ND-O-LID OD > 0.923). The number above each dataset is the percent of positive responses. The *p* value refers to differences in medians at different time points. OD, optical density; BI, bacilloscopic index.

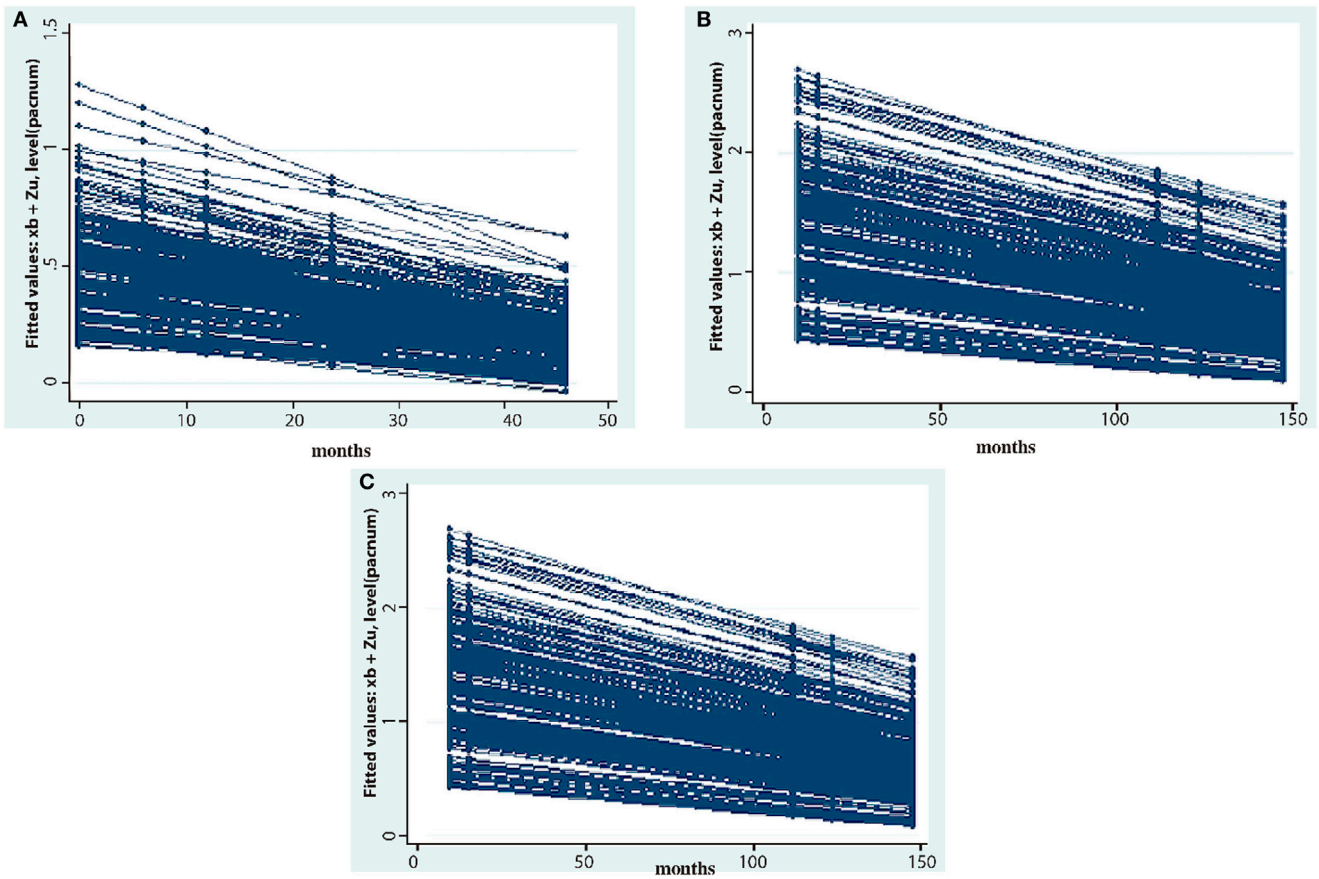


FIGURE 4 | Mixed effect multilevel analyses of 244 patients treated either with regular multidrug therapy (R-MDT) or the uniform multidrug therapy (U-MDT) showing the decay of antibody responses to different *Mycobacterium leprae* antigens (optical density, *y* axis) overtime (months, *x* axis): **(A)** phenollic glycolipid-I antigen, **(B)** LID-1, **(C)** ND-O-LID.

TABLE 2 | Results of adjusted mixed multilevel regression analyses of anti-PGL-I, anti-LID-1, and anti-NDO-LID serology overtime in leprosy patients treated with R-MDT and U-MDT.

	Coefficient	SE	*z*	*p* > *z*	95% Confidence interval	
Anti-PGL-I						
Month	−0.006871	0.0007285	−9.43	0	−0.0082988	−0.0054431
Group	−0.0035163	0.0333417	−0.11	0.916	−0.0688648	0.0618322
_Cons	0.5085021	0.1160942	4.38	0	0.2809617	0.7360426
Anti-LID-1						
Month	−0.0051023	0.0003361	−15.18	0	−0.005761	−0.0044436
Group	−0.0571696	0.0719866	−0.79	0.427	−0.1982608	0.0839216
_Cons	1.618.408	0.2525812	6.41	0	1.123.358	2.113.458
Anti-NDO-LID						
Month	−0.0164264	0.0012887	−12.75	0	−0.0189521	−0.0139006
Group	0.0016501	0.074866	0.02	0.982	−0.1450847	0.1483848
_Cons	1.379.876	0.2617582	5.27	0	0.866839	1.892.912

The mixed effects multilevel regression analyses evaluated the individual results of anti-PGL-I, anti-LID-1, and anti-ND-O-LID serology during follow-up considering the serological result as the independent variable (constant), the dependent variables were time (month) and treatment group (R-MDT and U-MDT) and the group variable was the patient ID. These analyses showed that the difference in serologic decay to all three antigens was dependent upon time only.
PGL-I, phenollic glycolipid-I antigen; R-MDT, regular multidrug therapy; U-MDT, uniform multidrug therapy.

positivity. A study among MB Venezuelan patients with high BI showed that the levels of anti-PGL-I antibodies dropped by 57% over 2 years (30). In general, previous studies have shown 50–90% drop in anti-PGL-I levels 2 years after conclusion of treatment (31, 46, 47). Analyses of antibody responses to different antigens in three MB patients showed little, if any, decline in anti-PGL-I serology after MDT (33). Overall, our results show that the gradual drop in antibody levels seen in both U-MDT and R-MDT groups is consistent with a slow reduction in the bacillary load in both treatment groups. In fact, the decline in BI in MB patients is known to occur slowly (0.5–1 log U/year), so that a significant proportion of patients with very high BI at diagnosis, independently of therapy duration, may remain slit skin smear positive for years after treatment (24), therefore stimulating antibody production.

In our study, the percentage of seropositivity varied among tests and also at different time points probably reflecting the sensitivity of the test and the initial bacillary load of the patients. Our data showed that anti-LID-1 serology provided the highest positivity rate at baseline, but as discussed, the decrease in seropositivity was gradual. Among MB patients, the positivity rates remained high 2 years posttreatment, especially to LID-1

antigen. Our results differ from earlier studies that have shown a faster decline in anti-LID-1 titers compared to anti-PGL-I (32, 33). These previous results have suggested that protein antigen was cleared faster than glycolipid/carbohydrate PGL-I antigen. A study showed that a single dose of rifampicin caused a rapid fall in the PGL-I antigen in serum of untreated MB patients (29) and the extent of the reduction of the PGL-I antigen in antibody production is suggested by decreased serology. However, in LL Brazilian patients, the anti-PGL-I antibody levels pre and posttreatment showed a small drop in positivity (from 100 to 90%) and also in BL patients (from 100 to 80%) (32). Results obtained among Venezuelan cured patients who were evaluated 10 years after treatment showed very low anti-LID-1 levels (32). Also, similar to our findings, in MB patients with high bacillary load, the anti-LID-1 positivity rates pre and posttreatment were either unchanged or slightly reduced: from 87 to 91% in LL and from 100 to 79% in BL patients (32). In another study among Brazilian patients, antibody levels to PGL-I and LID-1 dropped after MDT conclusion, however, some patients remained positive around 2 years after MDT (34). In the current study, non-compliance to MDT can be excluded among the causes for persistent antibody production as all patients were fully compliant to R-MDT and U-MDT; however, the high bacillary burden at diagnosis is compatible with a longer time required for the complete clearance of bacillary antigens. In fact, our results showed that despite the consistent reduction in antibody levels to all three antigens investigated, this decrease was gradual over time, so that 2 years after the conclusion of treatment, a significant percentage of patients continued to be seropositive. In conclusion, for MB patients with high BI at diagnosis, 2 years follow-up after treatment conclusion seems a short period for a significant clearance of antigens and also for a significant decrease in seropositivity. Therefore, the applicability of serology to monitor treatment efficacy seems limited for MB patients with high bacillary load at diagnosis evaluated in a short-term follow-up after treatment conclusion, such as 2 years.

The loss to follow-up in the first and second year posttreatment represents a limitation of the current study, however, the consistent and gradual decline in antibody levels seen overtime for all three antigens investigated indicates the validity of our data. Our results showed that the decline patterns seen in anti-PGL-I and anti-ND-O-LID serology were very similar. The simultaneous detection of IgM and IgG to the ND-O-LID conjugate which contains the disaccharide epitope of PGL-I and the diffusion LID-1 protein did not enhance sensitivity. A recent study on leprosy patients showed similar high positivity with these three antigens in BL and LL patients while the proportion of seropositivity to PGL-I and anti-ND-O-LID antigens was similar but lower than anti-LID-1 positivity (48). Another recent study which evaluated the diagnostic potential of two rapid tests using different antigens (PGL-I and ND-O-LID) and technologies (immunochromatographic lateral flow and luminescent-up-converting phosphor UCP-LFA) showed that both tests corresponded to BI but the UCP-LFA showed higher sensitivity (49). The use of ND-O-LID conjugate antigen in leprosy serology, which is expected to enhance sensitivity is recent and results reported so far are not conclusive if detection of IgM and IgG antibodies to the antigens contained in ND-O-LID effectively leads to higher sensitivity than observed using individual antigens and this topic deserves further investigations.

Previous studies have investigated the potential use of leprosy serology as a marker of reactional episodes (50–52). Two previous publications from our group using the U-MDT database and sera bank (40, 41) have described the impact of baseline serology on the development of leprosy reactions. The baseline ML flow test results showed limited sensitivity and specificity as prognostic markers for the development of leprosy reactions during subsequent follow-up (40). Also, the analyses of anti-PGL-I, anti-LID-1, anti-ND-O-LID antibodies at diagnosis showed low sensitivity and specificity for predicting reversal reaction while anti-LID-1 serology at diagnosis showed prognostic value for the development of ENL in BI positive patients (41). We acknowledge the importance of the analysis of the impact of reactional episodes on longitudinal serology data, however, these analyses are out of the scope of the current study which focused on the effect of different treatment regimens on leprosy serology to three antigens. Leprosy serology reflects the bacillary load of the patient and several studies have reported that MB patients are more vulnerable to develop leprosy reactions. In this sense, slit skin smears at diagnosis can indicate patients at higher risk of developing leprosy reactions, but these tests are not part of diagnostic routine and are not used to monitor reactions or relapse. Compared to slit skin smears, serology can be considered a simpler test that could indicate the risk for the development of reactions, especially ENL.

In conclusion, our study on MB leprosy patients, the majority with high bacillary load at diagnosis, indicated a similar decrease in *M. leprae* antibody production to PGL-I, LID-1, and ND-O-LID in patients treated with R-MDT and U-MDT for 12 and 6 months, respectively. The slow reduction in seropositivity rates seen in MB patients treated with both R-MDT and U-MDT is compatible with the slow decline of bacillary load, regardless of the duration of the treatment. This slow reduction indicates that the applicability of serological monitoring to evaluate MDT efficacy or track the effectiveness of MDT is limited at least in short term period of 2 years posttreatment as within this time period, a significant rate of patients remains positive.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Resolution 466/2012 from the National Health Council/Ministry of Health, with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the National Committee for Ethics in Research (CONEP) (protocol number 001/06). Data confidentiality was strictly guaranteed and all patients were free to leave the study and opt for the R-MDT regimen outside the study (ClinicalTrials.gov identifier: NCT00669643).

AUTHOR CONTRIBUTIONS

EH, SB-S, RO, GOP, and MMAS conceived and designed the experiments. EH, SB-S, RO, and MMAS performed the experiments. EH, SB-S, MLFP, GOP, and MMAS analyzed the data. SB-S, LA, MAAP, RC, HG, MLFP, GOP, and MMAS contributed with reagents/materials/analysis tools. EH, SB-S, and MMAS wrote the paper. EH, SB-S, and MLFP were in charge of data bank preparation and quality control of data. EH, SB-S, LA, RO, MAAP, HG, MLFP, GOP, and MMAS participated in the interpretation of data, critical reading and approval of the final manuscript. We thank Carlos Sarina, FIOCRUZ, Brasilia for providing high resolution figures.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00915/full#supplementary-material.

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Anti-Lipoarabinomannan-Specific Salivary IgA as Prognostic Marker for Leprosy Reactions in Patients and Cellular Immunity in Contacts

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Leprosy causes the most common peripheral neuropathy of infectious etiology, posing an important public health problem worldwide. Understanding the molecular and immunological mechanisms of nerve damage induced by *M. leprae* is mandatory to develop tools for early diagnosis and preventive measures. The phenolic glycolipid 1 (PGL-1) and lipoarabinomannan (LAM) antigens are major components of the bacterial surface and are implicated on leprosy immunopathogenesis and neural damage. Although the anti-PGL-1 serum IgM is highly used for operational classification of patients, the anti-LAM salivary IgA (sIgA) has not been investigated as diagnostic or prognostic marker in leprosy. Our aim was to assess the presence of anti-LAM sIgA in leprosy patients and their contacts in order to demonstrate whether such expression was associated with leprosy reactions. Distinct patterns of anti-LAM sIgA were observed among groups, which were stratified into treatment-naïve patients (116), patients who completed multidrug therapy—MDT (39), household contacts (111), and endemic controls (11). Both anti-LAM sIgA and anti-PGL-I serum IgM presented similar prognostic odds toward leprosy reactions [(odds ratio) OR = 2.33 and 2.78, respectively]. Furthermore, the anti-LAM sIgA was highly correlated with multibacillary (MB) forms (OR = 4.15). Contrarily, among contacts the positive anti-LAM sIgA was highly correlated with those with positive Mitsuda test, suggesting that the presence of anti-LAM sIgA may act as an indicator of cellular immunity conferred to contacts. Our data suggest that anti-LAM sIgA may be used as a tool to monitor patients undergoing treatment to predict reactional episodes and may also be used in contacts to evaluate their cellular immunity without the need of Mitsuda tests.

Keywords: salivary IgA, leprosy reactions, prognostic marker, lipoarabinomannan, household contacts

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INTRODUCTION

Leprosy continues to be the major cause of neuropathies and disabilities worldwide. Despite effective multidrug therapy (MDT), it is still endemic in many regions of the world, especially in Brazil and in India. Most of the infected population remains free of the disease, while a subset of infected individuals develops clinical symptoms, which are associated with the immunity of the host (1). Difficulties persist in clinical conduct, treatment of patients, and monitoring of leprosy reactions, which may lead to nerve damage (2, 3).

Disability in patients with recent diagnosis of leprosy and those who completed MDT treatment continues to be challenging. There is a consensus that the development and installation of neuromotor functional deficiencies and disabilities in leprosy patients are associated with morbidity and chronicity of the disease as pertains to social exclusion and stigma (4).

The major surface antigens of *M. leprae*, lipoarabinomannan (LAM), and phenolic glycolipid 1 (PGL-1), may be detected in saliva, and their participation in mucosal immunity has been under investigation. LAM is exposed on the bacterial surface and is directly implied on the immunopathogenesis of tuberculosis and leprosy (5). The membrane attack complex (MAC) co-localized with LAM in axons has pointed toward the role of this *M. leprae* antigen in the activation of the complement and neural damage in leprosy patients (1, 6).

It has been suggested that IgA may play a role in the protection against infections by mycobacteria of the respiratory tract through the blockage of pathogen entry and/or modulating the pro-inflammatory responses (7). Knockout mice for IgA (−/−) presented greater susceptibility to infection by BCG, compared to normal mice (+/+), as revealed by high bacterial load in the lungs. This result was also followed by an important reduction in IFN-γ and TNF-α in the lungs of IgA (−/−) when compared with IgA (+/+) mice. The detection of antibodies in saliva represents the expression of local immunity (8, 9), but its presence is not sufficient to block the infection process by *M. leprae* (10, 11), although it's local effect should be considered. Nevertheless, *M. leprae* has been identified in buccal mucosa (12–15).

The presence of salivary IgA (sIgA) against the native LAM antigen in leprosy patients and their contacts has not been investigated yet. Based on prior evidences of the association of LAM with neural damage, and the lack of information of sIgA in patients and contacts, we hypothesized that this response could be used as tool for prognosis of leprosy reactions due to its link with cellular immunity. Therefore, we have performed an investigation on the specific anti-LAM sIgA response and associated outcomes in patients (treatment naïve and treated), contacts and endemic controls, which are discussed herein.

MATERIALS AND METHODS

Studied Population and Group Stratification

Saliva samples were obtained from patients and controls, which were stratified into four groups: group 1: 116 treatment naïve leprosy patients (72 men and 44 women); group 2: 39 leprosy patients (22 men and 17 women) who had completed MDT and were evaluated at discharge (release from treatment), and among them 16 were evaluated at both diagnosis and discharge; group 3: 111 household contacts (40 men and 71 women); and group 4: 11 (11) healthy endemic controls (three men and eight women) were recruited in the population with the following criteria: absence of active leprosy or leprosy in the past, no contact with leprosy patients (family, friend, or colleague), live in the same endemic area, older than 18 years of age, not pregnant or using immunosuppressive medication. All patients and controls were attended at the National Reference Center for Sanitary Dermatology and Leprosy (CREDESH) of the Federal University of Uberlândia (UFU), MG, Brazil, and leprosy reactions were recorded for 3 years, from 2011 to 2014. This study was carried out in accordance with the recommendations of the "Guidelines of the National Board on Human Research Ethics" (CONEP) and with the Declaration of Helsinki, with written informed consent obtained from all subjects. The protocol was approved by UFU Research Ethics Committee under the number 643/11.

Clinical Data

The operational classification of patients into paucibacillary (PB) and multibacillary (MB) forms were performed for treatment purpose (16), and the clinical classification was done according to Ridley & Jopling (17). Patients' clinical classification was: 8 tuberculoid (TT); 58 borderline-tuberculoid (BT), in which 29 cases were BT/PB and 29 were BT/MB; 11 borderline–borderline (BB); 17 borderline-lepromatous (BL); and 19 lepromatous form (LL). Additionally, three patients presented the indeterminate form (I).

All patients were submitted to a clinical-laboratorial protocol for the leprosy diagnosis and clinical classification, considering the histopathology of skin lesions, bacilloscopy (18), Mitsuda test results (16, 19), and indirect anti-PGL-1 IgM enzyme-linked immunosorbent assay (ELISA) test (20, 21).

The Mitsuda test was performed on patients to measure the levels of specific cellular immune response for *M. leprae*. Results were obtained 4 weeks after intradermal application of 0.1 mL of the antigen in the right forearm by measurement in millimeters (mm) of the diameter of the local induration. The Mitsuda test results were classified as follows: 0–3 mm—negative; 4–7 mm—weakly positive; 8–10 mm—positive; and greater than 10—strongly positive (16), and previously employed by our group with minor modifications (19), where results were stratified into two categorical groups: "negative" for readings up to 7 mm (0–7 mm), which consisted of negative to weakly positive results, and the "positive" for readings greater than 7 mm (>7 mm), which includes results that are positive and strongly positive with or without ulcerations.

From household contacts, data collection consisted of ELISA anti-PGL-1 serology test and Mitsuda test. The immunization data were assessed according to the presence and number of BCG scars (sBCG 0, 1, or 2 scars). Contacts were further classified according to clinical form (CF) and operational classification of their index case.

Clinical Characterization of Leprosy Reactions

Leprosy reactions (type 1, type 2, and mixed) were categorized based on clinical and immunological criteria described elsewhere (22). Briefly, type 1 (reversal) reactions occur in the group borderline (BT, BB, and BL) and consisted of acute inflammation in skin lesions or nerves or both. Type 2 reactions occur in LL and BL CFs and cause acute inflammation in any organ or tissue where *M. leprae* are found. Type 2 reactions are also known as erythema nodosum leprosum (ENL). The skin lesions of ENL were characterized by the presence of cutaneous erythematous inflamed nodules and papules that may turn into pustules, then become ulcerated and necrotic. Type 2 reactions often cause

neuritis in the form of painful enlarged nerves, nerve function impairment and at systemic level, present high fever, prostration, orchitis, lymphadenopathy, organomegaly, joint involvement, dactylitis, and bone tenderness.

Saliva Collection

Non-stimulated saliva collection was done by using "Salivette" (Sarstedt, Germany), according to the manufacturer's instruction. Patients independently collect the sample material using a plain cotton swab. The swab was removed from the Salivette tube and placed in the mouth for chewing for about 60 s to stimulate salivation then the swab was returned with the absorbed saliva to the conical tube. After centrifugation at 5,000 rpm at 4°C for 10 min, a clear saliva sample was obtained, aliquoted, transferred to 0.5 mL microtubes, and frozen at −20°C. Sample volumes varied from 0.5 to 1.5 mL.

Indirect ELISA for Detection of Anti-LAM Salivary IgA and Anti-PGL-1 Serum IgM

High affinity plates (Maxsorp—Nunc®) with 96 wells were sensitized with 50 µL of native LAM (BEI RESOURCES)[1] diluted in carbonate/bicarbonate buffer (50 µL of native LAM 100 µg/mL diluted in 4,950 µL of carbonate/bicarbonate buffer, pH 9.6). The plates were incubated overnight in a cold chamber at 4°C. Four washings were done with PBS_T 0.05% (200 µL/well) and saliva samples diluted in 5% PBS/BSA (1:5) were added in triplicate. The plates were incubated for 1 h at 37°C, and after five washings with PBS_T 0.05%, 50 µL of anti-IgA were added (CALBIOCHEM®, USA; 1.0 mg/mL) labeled with diluted peroxidase 1:1,000 in PBS/BSA and incubated for 1 h at 37°C. After six washings with PBS_T 0.05%, reactions were developed by adding 50 µL of OPD solution for 5 min (2 mg OPD + 5,000 µL citrate buffer + 2 µL H_2O_2), and the reaction was then stopped with 20 µL/well of sulfuric acid (H_2SO_4 2N). ELISA readings were performed in a microplate reader (TP—READER, THERMO PLATE) at 492 nm.

The anti-PGL-1 ELISA was also an indirect test to detect circulating IgM antibodies in serum against the *M. leprae* native PGL-1, and it was performed as previously described (23).

ELISA Index (EI)

Saliva samples were processed in triplicate. Results were converted into an EI, in which a value of 1.1 was considered a positive threshold. For the EI calculation, the absorbance mean value was divided by the cutoff, considering the values greater than 1 as positive. The cutoff value was obtained with absorbance readings of negative controls, and three SDs were added to the mean (23).

Statistical Analysis

A descriptive analysis was used for all patients, contacts, and controls. The normality of samples was verified by the Shapiro–Wilk test. The variables did not present normal distribution. The Mann–Whitney *U* and Kruskal–Wallis tests were performed to test whether medians between groups were different, under the assumption that the shapes of the underlying distributions were the same. The non-parametric tests were performed with GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA), and the odds ratios (OR) were calculated through the MedCalc server.[2] Significant values were considered when $P \leq 0.05$.

RESULTS

The frequency distribution of treatment naïve leprosy patients with or without reactions (during or soon after MDT treatment) was stratified according to the operational classification, gender, Mitsuda test, ELISAs anti-LAM sIgA, and anti-PGL-1 IgM (**Table 1**). Patients with MB leprosy presented higher chances of developing leprosy reactions (OR = 4.15; $P < 0.001$) without gender preference. A significant positive correlation was observed between anti-LAM slgA+ and leprosy reactions. Among reactional patients, 69.4% (34/49) were also anti-LAM positive at diagnosis, with a 2.33-fold higher chance of developing reactions. Similarly, the positive IgM serology also showed a significant correlation with leprosy reactions (OR = 2.78; $P < 0.008$).

The frequency distribution of leprosy patients by CF, type of leprosy reactions, and positivity for secretory anti-LAM IgA is shown in **Table 2**. The BT form was the only form that presented significant correlation with type 1 leprosy reaction, with a 6.9-fold higher chance of having reactions ($P < 0.006$). In group 1, 12 patients (10.3%) were household contacts that became ill (6 BT/PB; 3 BT/MB; 1 I/PB; 1 TT/PB; 1 BL/MB), and among them, one developed a type 1 reaction after discharge (female, BT/MB with positive salivary anti-LAM sIgA at the time of diagnosis). Regarding the small ORs for the MB forms (BB, BL, and LL), it is important to emphasize that the sample size collected for these forms was very small, so data should be carefully interpreted. Our data corroborate the notion that salivary sIgA+ is associated with type 1 (reversal) reaction, since only PB CFs presented very large odds, followed by small ORs with lack of significance in MB forms.

Thirty-nine patients were evaluated at the time of discharge from MDT, in which 35.9% (14/39) presented positive salivary anti-LAM slgA. Among them, 16 patients were also evaluated at diagnosis, in which 9 were males and 7 females, 10 MB and 6 PB. In this group, nine patients (56.2%) developed leprosy reactions, in which one was PB and eight were MB. At discharge from MDT, the mean EI anti-LAM slgA of patients with reaction was 0.91, while patients without reactions presented an EI mean of 0.53 ($p = 0.29$). Considering the stratification of patients' groups into those with reactions and without reactions, the EI kinetics from diagnosis (D) to discharge (A) displayed a different profile, in which the group without reaction had decreasing values or remained low (data not shown).

Table 3 shows the ELISA results using salivary anti-LAM sIgA from 111 contacts, which were correlated with the Mitsuda test, and the presence/absence of the sBCG. For the Mitsuda test, values of 0–3 mm (0) were considered as the worst prognosis (−), and values ≥4 as the best prognosis (+). For sBCG, negative (−) was considered as absence of scar (0) and positive (+) was considered

[1] https://www.beiresources.org/ (Accessed: May 19, 2018).

[2] www.medcalc.org/calc/odds_ratio.php (Accessed: May 19, 2018).

TABLE 1 | Frequencies of treatment naïve leprosy patients with or without reactions during or after MDT, divided according to their operational classification, gender, Mitsuda test result, anti-phenolic glycolipid 1 (PGL-1) IgM serology, and anti-lipoarabinomannan (LAM) sIgA in saliva, obtained at diagnosis.

Variables			Leprosy reactions			Odds ratio	Confidence interval (95%)	*P*
			Yes		Total (n)			
			n	(%)				
Operational classification								
Multibacillary (MB)			41	(52.6)	78	**4.15**	**1.69–10.19**	**0.001**
Paucibacillary (PB)			8	(21.1)	38			
Gender/operational classification								
Male	72	MB	24	(45.3)	53	**4.41**	**1.14–16.96**	**0.030**
		PB	3	(15.8)	19			
Female	44	MB	17	(68)	25	**5.95**	**1.58–22.32**	**0.008**
		PB	5	(26.3)	19			
Total			49	(42.2)	116			
Mitsuda								
0–7 mm			16	(35.6)	45	2.34	0.67–8.17	0.181
>7 mm			4	(19.0)	21			
Anti-PGL-1 IgM								
PGL-1+			32	(54.2)	59	**2.78**	**1.29–5.98**	**0.008**
PGL-1–			17	(29.8)	57			
Anti-LAM sIgA								
LAM+			**34**	**(50.7)**	**67**	**2.33**	**1.07–5.06**	**0.031**
LAM–			15	(30.6)	49			

Mitsuda test reading system: "negative" for readings up to 7 mm (0–7 mm) and "positive" for readings greater than 7 mm (>7 mm) (17). P, probability value.
Bold fonts were used to emphasize the significant values.

TABLE 2 | Frequencies of leprosy patients by CF, type of leprosy reactions, and positivity for salivary anti-lipoarabinomannan (LAM) sIgA, followed by odds ratios (OR), confidence interval at 95% ($CI_{95\%}$), and probability levels (P) toward the occurrence of reactions.

CF	n	Anti-LAM sIgA	Total, n(%)	Reaction (n)			Total (n)		OR	Confidence interval (95%)	P
				T1	T2	M	R	No			
TT	8	+	4 (50)	2	0	0	2	2	9.00	0.29–271.67	0.20
		–	4 (50)	0	0	0	0	4			
BT	58	+	33 (56.9)	16	0	0	16	17	**6.90**[a]	**1.72–27.60**	**0.006**
		–	25 (43.1)	3	0	0	3	22			
BB	11	+	7 (63.6)	3	0	0	3	4	0.08	0.003–2.20	0.138
		–	4 (36.4)	4	0	0	4	0			
BL	17	+	10 (58.8)	2	1	3	6	4	1.12	0.15–7.98	0.906
		–	7 (41.2)	3	0	1	4	3			
LL	19	+	12 (63.2)	0	6	1	7	5	1.05	0.15–6.92	0.959
		–	7 (36.8)	0	4	0	4	3			
Total	116			33	11	5	49	67			

Bold fonts were used to emphasize the significant values.
[a]*Statistically significant.*
CF, clinical form; TT, tuberculoid; BT, borderline-tuberculoid; BB, borderline–borderline; BL, borderline-lepromatous; LL, lepromatous.
Reaction (R): T1 = reaction type 1 (RR, reversal reaction); T2 = reaction type 2 (ENR, erythema nodosum reaction); M = mixed reaction (T1/T2); No = no reaction.

with the presence of 1 or 2 scars (**Table 3**). There was a positive correlation between anti-LAM slgA+ and positive Mitsuda test, with a significant OR (OR = 0.29; $p = 0.011$), suggesting that positivity for anti-LAM in the saliva of contacts may be an indicator of natural resistance to leprosy, due to the greater frequency of positive sIgA in Mitsuda-positive individuals (OR = 3.41; $p = 0.011$). Significant differences of salivary sIgA were observed among patients between groups 1 and 4 ($p = 0.0329$) and between groups 1 and 2 ($p = 0.0003$) (**Figure 1**), suggesting that treatment reduces the bacillary load, which is reflected by detecting reduced anti-LAM sIgA in saliva in most patients, except in those that presented leprosy reactions. The Figure S1 in Supplementary Material is presented with raw ELISA data in saliva to demonstrate the range of original values found in each group before transformation to ELISA indices.

Among contacts that presented 0 (no scar), 1, and 2 sBCGs, 63.6% (14/22), 51.4% (37/72), and 58.8% (10/17) were positive for salivary LAM, respectively. Among those without sBCG, a significant positive association was observed between sIgA+ and positive Mitsuda test (OR = 10.00; $CI_{95\%}$=1.26–79.3; $p = 0.02$). Contacts without BCG scar were vaccinated with BCG as soon as they entered the CREDESH's monitoring program.

The endemic control (EC) group consisted of 11 volunteers without any personal or familial history of leprosy, and the very small sample size in this group may seem a weakness of the paper if one considers the marker for diagnostics, which is not the case. The aim was to demonstrate the validity of the anti-LAM sIgA as a prognostic marker in patients and contacts, so endemic controls did not contribute for the calculation of odds, although it provided additional support to the data, since only one individual

TABLE 3 | Prognostic analyses through odds ratio (OR) calculations for household contacts considering the interactions of the three prognostic factors: Mitsuda test, presence of BCG scar (sBCG), and salivary anti-lipoarabinomannan (LAM) sIgA.

Markers interactions		sBCG*		OR	Confidence interval (95%)	P
		–	+			
Salivary IgA	+	14	47	1.56	0.59–4.09	0.362
	–	8	42			
		Mitsuda*				
		–	+			
	+	8	53	**0.29**	**0.11–0.75**	**0.011**
	–	17	33			
		Mitsuda/sBCG*				
		–	+			
	+	2	41	0.29	0.05–1.61	0.15
	–	5	30			

Bold fonts were used to emphasize the significant values.
sBCG (–) = worse prognosis (0 cBCG) and (+) = better prognosis (1 and 2 sBCG).
Mitsuda (–) = worse prognosis (0–3 mm) and (+) = better prognosis (≥4 mm).
Mitsuda/sBCG (–) = worse prognosis (Mit < 4/sBCG-ID = 0) and (+) = better prognosis (Mitsuda ≥ 4/sBCG ≥ 1).

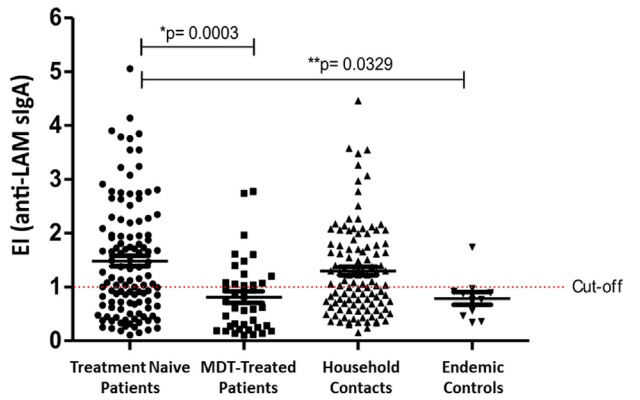

FIGURE 1 | Enzyme-linked immunosorbent assay (ELISA) index values of salivary anti-lipoarabinomannan (LAM) sIgA detection in patients at diagnosis, patients at MDT discharge, household contacts, and endemic controls. Cutoff value of ELISA index is ≥1. Median comparisons performed with the Kruskal–Wallis test.

presented a positive ELISA. It is possible that this individual has been exposed to *M. leprae* through a contact or patient without knowing this condition.

DISCUSSION

The present study characterized the salivary anti-LAM secretory IgA response in leprosy patients and their contacts and suggests its use as a prognostic tool for leprosy reactions in patients, and as immunity status in contacts by associating sIgA values with laboratorial and clinical parameters. Saliva has been the study subject as diagnostic or as supplementary tool for diagnosis or for monitoring of oral and systemic diseases (24, 25). The simple sample collection, the minimum invasiveness, and diminished contamination risk for healthcare professionals represent important aspects to support the choice of saliva as a promising tool for diagnosis and for monitoring of clinical evolution of patients during treatment and post-discharged.

Besides being the primary surface antigen and one of the dominant virulence factors of *M. leprae*, LAM also shows a close relationship with leprosy reactions, since it has promoted neural damage in a mouse model by activating the complement system *via* MAC (1, 6). Our data corroborate this immune response by showing a positive correlation between positive anti-LAM sIgA with the occurrence of leprosy reactions, suggesting the involvement of exacerbated cellular response against LAM of *M. leprae*. Our results are also supported by the evidence that deposition of complement is associated with LAM of *M. leprae* in leprosy lesions, and positivity for LAM in the nerves is also associated with deposition of MAC (1). Additional support for the involvement of LAM with the occurrence of leprosy reactions comes from the fact that even after finishing treatment, LAM can still be detected in skin and nerve biopsies, with a clearance that is even slower than that of PGL-1 (26).

Our data also point out toward a greater occurrence of leprosy reactions in patients with MB leprosy, which are also corroborated by other studies elsewhere (3, 27), and interestingly, we also showed that this association is highly linked with detection of anti-LAM salivary sIgA. Patients with MB leprosy and with positive anti-LAM sIgA presented chances fourfold higher toward having leprosy reactions than those with negative results. Nevertheless, considering the positivity of anti-LAM salivary sIgA in all patients, the chances are at least twofold greater toward the development of leprosy reactions.

The distinctive behavior of salivary anti-LAM sIgA, especially when we compared patients with endemic controls, suggests that salivary anti-LAM can be a marker of exposure to *M. leprae*. This information is even strengthened when patients groups (1 and 2) are compared, in which treated patients (group 2) displayed a significant lower positivity for anti-LAM sIgA than that of the treatment naïve patients (group 1), suggesting that MDT monitoring with this marker is possible. The differences in immune salivary response between the contact and the endemic controls reinforces the role of anti-LAM IgA as an indicator of exposure to *M. leprae*, as proposed elsewhere (28). Interestingly, we have also evaluated patients for anti-LAM sIgA both at diagnosis and at discharge from MDT, and those who maintained or presented elevated their levels during treatment had greater chances of developing leprosy reactions than those whose levels of anti-LAM sIgA had declined, further supporting the results obtained for all leprosy patients, and demonstrating the importance of anti-LAM sIgA as a predictive biomarker of leprosy reactions in patients.

Our results with contacts also demonstrated that anti-LAM sIgA significantly correlated with the positive Mitsuda test, suggesting that positivity for anti-LAM in saliva may be used as an indicator of resistance to leprosy, either conferred by prior exposure or by natural resistance. These results are corroborated elsewhere, in which IgG positivity to LAM was significantly increased in patients vaccinated with BCG and in patients with active tuberculosis. Oral vaccination with BCG induced a significant increase of secretory IgA to LAM as well. These authors suggested that trials with immunoglobulins reactive to LAM may serve as markers of humoral and cellular response in future vaccinations with

BCG and/or with attenuated mycobacteria (29). These results were also corroborated in another study that showed significant increases in specific anti-LAM IgGs after primary vaccinations and in booster doses of BCG (30). Although prior exposure to *M. leprae* can also lead to humoral and cellular responses, it is likely that the presence of sIgA in the saliva of household contacts may also suggest oral immunization, correlating with a cellular response characterized by the positive Mitsuda test, performed before the saliva collection during the first analysis of the contact. Prior studies have indicated generalized subclinical transmission of *M. leprae* with transient infection of the nose, and possibly in the oral cavity (29), resulting in the development of a mucosal immune response that can be protective (31). It remains to be verified whether contacts with negative anti-LAM slgA in saliva along with other parameters, such as absence of BCG scar and presence of serum PGL-1, represent a greater risk of disease development.

Our data indicate that there is no benefit in testing individuals of unknown leprosy status, because salivary anti-LAM sIgA cannot be used as a diagnostics marker, due to its absence in more than 40% of patients at diagnosis and persistence of detection in more than 35% of patients at discharge. However, monitoring anti-LAM slgA in saliva of leprosy patients undergoing treatment may become an important tool in detecting groups at risk for the development of leprosy reactions, especially type 1, and positivity in household contacts suggests greater resistance to leprosy; however, the possibility of resistance to exposure to *M. leprae* should be further investigated. Importantly, the worldwide absence of Mitsuda tests to evaluate contacts and patients' cellular immunity poses an important issue in leprosy monitoring programs, in which the salivary anti-LAM slgA may become a good substitute tool of the Mitsuda test due to its high correlation with it. Besides, saliva avoids de use of this invasive procedure with intradermal injection of standardized extract of inactivated bacilli, and ELISA takes just a few hours instead of 21 days for reaction evaluation.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Guidelines of the National Board on Research Ethics (CONEP)" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by UFU Research Ethics Committee/CEP under the number 643/11.

AUTHOR CONTRIBUTIONS

LG: senior author, conceived the concept and the experimental design, performed biological assays, statistical analyses, manuscript writing and revision. IG: co-senior author, sample collection, data interpretation, manuscript writing and revision. AN: performed biological assays, statistical analyses and interpretation, and manuscript writing. ML: performed biological assays and manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01205/full#supplementary-material.

FIGURE S1 | Enzyme-linked immunosorbent assay values (absorbances at 492 nm) of anti-lipoarabinomannan (LAM) sIgA detection in patients at diagnosis, patients at MDT discharge, household contacts, and endemic controls. Median comparisons performed with the Kruskal–Wallis test.

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Autophagy Impairment Is Associated With Increased Inflammasome Activation and Reversal Reaction Development in Multibacillary Leprosy

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Leprosy reactions are responsible for incapacities in leprosy and represent the major cause of permanent neuropathy. The identification of biomarkers able to identify patients more prone to develop reaction could contribute to adequate clinical management and the prevention of disability. Reversal reaction may occur in unstable borderline patients and also in lepromatous patients. To identify biomarker signature profiles related with the reversal reaction onset, multibacillary patients were recruited and classified accordingly the occurrence or not of reversal reaction during or after multidrugtherapy. Analysis of skin lesion cells at diagnosis of multibacillary leprosy demonstrated that in the group that developed reaction (T1R) in the future there was a downregulation of autophagy associated with the overexpression of *TLR2* and *MLST8*. The autophagy impairment in T1R group was associated with increased expression of *NLRP3*, caspase-1 (p10) and IL-1β production. In addition, analysis of IL-1β production in serum from multibacillary patients demonstrated that patients who developed reversal reaction have significantly increased concentrations of IL-1β at diagnosis, suggesting that the pattern of innate immune responses could predict the reactional episode outcome. *In vitro* analysis demonstrated that the blockade of autophagy with 3-methyladenine (3-MA) in *Mycobacterium leprae*-stimulated human primary monocytes increased the assembly of NLRP3 specks assembly, and it was associated with an increase of IL-1β and IL-6 production. Together, our data suggest an important role for autophagy in multibacillary leprosy patients to avoid exacerbated inflammasome activation and the onset of reversal reaction.

Keywords: leprosy, multibacillary patients, reversal reaction, autophagy, inflammasome, *Mycobacterium leprae*

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INTRODUCTION

Mycobacterium leprae infection results in a chronic disease denominated leprosy (1). The disease presents different clinical forms accordingly the host cellular immune response against mycobacterial antigens and histopathological features (2). *M. leprae* infection can modulate several pathways and modify the microenvironment to favors its survival inside the host cells, including the increase

in iron uptake and storage (3, 4), lipids uptake (5, 6), and deactivation of antimicrobial pathways (5, 7).

Our recent study has demonstrated that live *M. leprae* is able to impair the autophagic flux in host cells as an escape immune mechanism (7). Analysis of skin lesion cells demonstrated an upregulation of autophagy genes in paucibacillary tuberculoid and lepromatous patients with reversal reaction when compared with lepromatous patients, which present the progressive form of leprosy (7). Macroautophagy (hereafter termed autophagy) is an evolutionarily conserved mechanism that engulfs targets (e.g., organelles, proteins, and bacteria) through double-membrane vesicles called autophagosomes and directs them for lysosomal degradation [reviewed by Feng et al. (8)].

Basal autophagy is important for the prevention of protein aggregation and the control of reactive oxygen species production (9, 10). Previous studies described autophagy as an important regulatory mechanism controlling unappropriated and potentially deleterious inflammatory responses [reviewed by Harris (11)]. Autophagosomes can sequestrate and degrade inflammasome components including the adaptor ASC, AIM2, NLRP3 (12), and also pro-IL-1β (13). Autophagy inhibition was also described as a potent inflammasome activator, since in the absence of autophagy there is an accumulation of endogenous stimuli (second signal) to inflammasome activation (12–15). Although autophagy machinery is partially impaired in lepromatous patients, the analysis of autophagy genes and proteins expressions during the occurrence of reversal reaction in this group demonstrated that during the reactional episode it was restored (7), maybe mediated by inflammatory mediators, since previous studies have indicated CXCL-10, IL-6, and IFN-γ as biomarkers of reversal reaction (16–19).

Reversal reaction in multibacillary patients is distinguished by the sudden change in the immunological response to mycobacterial antigens and is the leading cause of the leprosy-related morbidity (20–24). The early identification of those episodes is of paramount importance to prevent the neural damage associated with the reactional states. Reversal reaction or Type 1 reaction is an inflammatory exacerbation of skin lesions that may comprise the appearance of new lesions and/or the reactivation of old ones. Reversal reaction may occur across the leprosy clinical spectrum (25). In multibacillary patients, the development of the reversal reaction has been associated with a shift for a Th1 response (26) and although the mechanisms related to the reaction onset are less understood in multibacillary patients, the study of this group permits to identify the role of innate mechanisms related to immunopathogenesis before any previous specific cellular immune response. In addition, due the potential severity of reversal reaction, it is a priority to identify biomarkers of leprosy reaction that may be used to aid the clinicians in patient's management.

The use of cytokines and chemokines as biomarkers has limitations since they are implicated in various disease states and are not so specific. So, in this study we assessed the pattern of gene expression in skin cells from lepromatous patients who developed or not reversal reaction during or after treatment. We observed that during *M. leprae* infection in monocytes autophagy is important to control inflammasome activation, and that cells from multibacillary patients who did not develop reaction (WR) have increased autophagy when compared with cells from patients who developed reversal reaction (T1R) in the future. The blockade of autophagy in the T1R group cells is accompanied by enhanced NLRP3 inflammasome activation. Therefore, the data presented here suggest that autophagy is important to control of the excessive activation of inflammasome and the possible involvement of NLRP3 inflammasome in the onset of reversal reaction in multibacillary leprosy patients.

MATERIALS AND METHODS

Patients and Clinical Specimens

The participants enrolled in this study, recruited from the Souza Araújo Outpatient Unit (FIOCRUZ), were categorized according to Ridley and Jopling's classification scale (2). Skin lesion fragments used were obtained *via* 3–6 mm punch, taken from multibacillary leprosy (MB) patients [borderline lepromatous and lepromatous polar (LL)] at diagnosis, prior to treatment, that did not exhibit any signs of leprosy reactions. Blood without anticoagulants was also collected for the obtainment of serum. The patients were monitored for 2 years after the leprosy diagnosis. The patients who developed reversal reaction during this period were included in the T1R group, while the ones that did not develop leprosy reactions were classified as without reaction (WR) (**Table 1**; Table S1 in Supplementary Material). All the samples used in this study were collected at the multibacillary leprosy diagnosis, no reactional samples were used. The study was endorsed by the Oswaldo Cruz Foundation Human Ethics Committee (CAAE 34239814.7.0000.5248). All the study participants provided informed written consent.

RNA Isolation, Reverse Transcription, and qPCR Analysis

RNA was extracted from the patients skin lesions fragments and blood-derived monocyte cultures by the TRIzol method (Life Technologies, 15596-018) following the manufacturer's instructions. In order to avoid genomic DNA contamination, the RNA was treated with DNAse (RTS DNase Kit, MO BIO Laboratories); integrity was analyzed *via* 1.2% agarose gel electrophoresis. SuperScript III

TABLE 1 | Baseline characteristics of the multibacillary patients included in the study.

	WR	T1R
Characteristic		
Male/female, *n*	5/5	8/4
Age, mean (range)	42.9 (25–65)	44.8 (28–66)
BI, mean (range)	4.19 (1.75–5.85)	3.67 (1–5.50)
LBI, mean (range)	4.84 (3.5–5.85)	4.68 (3.5–5.95)
Ridley and Jopling clinical form of leprosy, *n*		
BL	2	6
LL	8	6
Leprosy treatment status, *n*		
Pretreatment	10	12

WR, without reaction; T1R, type 1 reaction; BI, bacillary index; LBI, logarithmic bacillary index of skin lesion; BL, borderline lepromatous; LL, lepromatous leprosy.

First-Strand Synthesis System (Life Technologies, 18080-051) was used to perform the reverse transcription. Real-time gene expression was performed using human innate and adaptative immunity (Real-Time Primers, HAIIR-I), and autophagy (Real-Time Primers, HATPL-I) PCR arrays composed of 88 process-related targets and 8 reference genes. The qPCR arrays were performed using the manufacturer-recommended conditions using Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659).

Alternatively, mRNA expression of *NLRP3*, *IL33*, *IL18*, *IL1B*, and *CASP1* was evaluated using TaqMan Fast Universal PCR Master Mix (2×) (Applied Biosystems, 4352042) in a StepOnePlus Real-Time PCR System (Applied Biosystems, MA, USA). All primers were acquired from ThermoFisher Scientific (4331182).

The $2^{-\Delta CT}$ method was used to analyze the gene-expression data, using β-2-microglobulin (*B2M*; Real-Time Primers) as reference gene for the innate and adaptative immunity PCR array, hypoxanthine phosphoribosyltransferase 1 (*HPRT1*; Real-Time Primers) for the autophagy PCR array, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Hs02758991_g1, ThermoFisher Scientific) for the TaqMan assays.

Pathway Analysis

The RT-qPCR innate and adaptative immunity, and autophagy arrays were used to define the gene-expression profiles of leprosy skin lesions. The disparity in gene-expression profiles between the studied groups were assessed by Linear Model for Series of Arrays (lmFit) and Empirical Bayes Statistics for Differential Expression (ebayes) functions from "limma" (Bioconductor) R package. The differentially expressed genes were identified by log2-fold change ≥ 1.5-fold and moderated by *t*-test P value < 0.05 thresholds (5, 7, 27). The Enhanced Heat Map (heatmap.2) function from the "gplots" R package was used to generate the heat maps, displayed in a *z*-scores scaling. Jegga et al. (28) set list of human gene symbols associated to autophagy and lysosomal pathways was used to sub-categorize the differentially regulated autophagy processes-related genes in four functional subgroups (autophagy, autophagy regulators, lysosome, and lysosome regulators).

Gene Interaction and Enrichment Analysis

The differentially modulated genes in the pathway analyses were evaluated *via* Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) 10.0 database (http://string-db.org/) (29). STRING action and confidence views were used to generate network maps of gene–gene interactions. Gene ontology (GO) and KEGG pathways functional enrichment analysis was generated using the "Enrichment" tool of STRING with false discovery rate and Bonferroni corrections for specific annotations. The $P < 0.05$ threshold was adopted for statistical significance.

Immunoperoxidase

Frozen skin lesion sections 4-µm thick from LL patients who developed (T1R) or not (WR) episodes of reversal reaction were made in a Leica LM3000 cryostat (Leica, Wetzlar, Germany) and analyzed by the immunoperoxidase technique. The skin sections were fixed with acetone, hydrated with 0.01 M $Ca^{2+}Mg^{2+}$-free phosphate-buffered saline (PBS), and the endogenous peroxidase activity was quenched by a 10-min incubation with hydrogen peroxide 0.3% in PBS. Normal horse serum (VECTASTAIN Elite ABC-HRP Kit Mouse IgG, Vector Laboratories, PK-6102) for 30 min at room temperature was used to block unspecific binding sites. The sections were incubated for 1 h at room temperature with 1:50 mouse anti-human LC3 mAb antibody (MBL International, M152-3) diluted in PBS 0.25% Triton X-100 (Sigma-Aldrich, 9002-93-1). After three washes with PBS 0.25% Triton X-100, the slides were incubated for 1 h at room temperature with biotinylated horse anti-mouse IgG (VECTASTAIN Elite ABC-HRP Kit). Next, the sections were washed and incubated with avidin DH-biotinylated horseradish peroxidase (HRP) H complex (VECTASTAIN Elite ABC-HRP Kit) for 40 min for signal amplification. 3-amino-9-ethylcarbazole (AEC Peroxidase HRP Substrate Kit, Vector Laboratories, SK-4200) was used for 10 min at room temperature to develop the reaction. Mayer's hematoxylin (Dako) was used to counterstain the skin lesion sections. Slides were mounted with aqueous Faramount mounting medium (Dako), and analyzed *via* a Nikon Eclipse E400 microscope with a plan-apochromat 40×/0.65 objective (Nikon Instruments Inc., NY, USA). INFINITYX-32C camera and Infinity Capture software 6.1.0 (Lumenera Corporation, ON, Canada) were used to capture images. LC3-positive area was calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) by the ratio between labeled and total tissue areas.

Skin Lesion Macrophages Isolation

Skin lesion macrophages were isolated as described by Moura et al. (30). Briefly, the dermis was cleaved into small sections and digested overnight at 37°C with 4 mg/mL dispase II (GIBCO, 17105041), and 0.5 mg/mL collagenase type I (GIBCO, 17018029) in RPMI 1640, 10% fetal bovine serum (FBS, GIBCO, 10437028) in a 5% CO_2 atmosphere. The cell suspension was passed through a 70-µm nylon mesh cell strainer and washed three times with RPMI 1640 by centrifugation at 500 × g for 10 min at 4°C. The cells were resuspended in RPMI 1640 supplemented with 10% FBS, 2 mM L-alanyl-L-glutamine (GlutaMAX I, GIBCO, 35050061), and 100 µg/mL ampicillin (Sigma-Aldrich, A8351), plated at 1×10^5 cells/mL on 15-mm sterile circular coverslips and cultured for 7 days at 37°C in a 5% CO_2 atmosphere.

Peripheral Blood Mononuclear Cells (PBMCs) Isolation and Monocyte Cultures

Peripheral blood mononuclear cells from healthy donors were isolated *via* Ficoll-Paque PLUS method (GE Healthcare, 17-1440-03) under endotoxin-free conditions. Cells were ressuspended in RPMI 1640 supplemented with 10% FBS, 2 mM L-alanyl-L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, P4333) and plated at 5×10^5 cells/mL on 15-mm sterile circular coverslips and cultured for 2 h at 37°C in a 5% CO_2 atmosphere. Alternatively, cells were plated at 1×10^6 cells/mL in 24-well plates for qPCR assays. The supernatant was discarded and coverslips were rinsed with PBS to remove non-adherent cells. The media was replaced and the monocytes were stimulated with armadillo γ-irradiated *M. leprae* at 10 µg/mL (~10:1) in the presence or absence of the following stimuli for 18 h at 37°C in a 5% CO_2 atmosphere. Autophagy was triggered with 200 ng/mL

rapamycin (RP) (Sigma-Aldrich, R0395), 100 μM chloroquine (CQ) (Invitrogen, P36235) was used as an autophagic flux blocker, and 10 μM 3-methyladenine (3-MA) (Sigma-Aldrich, M9281) as an autophagy inhibitor.

Immunofluorescence Assay

After 7 days of culture, non-adherent cells were removed and the skin lesion macrophages were fixed for 20 min at 4°C using 4% paraformaldehyde (Sigma-Aldrich, 158127). After three washes with PBS 0.05% saponin, cells were blocked for 1 h at room temperature with PBS 10% FBS, 10% normal goat serum (NGS; Sigma-Aldrich, S26-100ML), and 1% bovine serum albumin (BSA; Sigma-Aldrich, 05470-25G). Mouse IgG1 anti-human LC3 antibody (1:50; MBL International, M152-3) was added and incubated overnight at 4°C. Next, the coverslips were washed and 1:500 Alexa Fluor 633 goat anti-mouse IgG1 (Invitrogen, A21126) secondary antibody was added for 2 h at room temperature. DAPI (1:10,000, Molecular Probes) was used to stain the nuclei and the coverslips were mounted in glass slides with Lab Vision PermaFluor Aqueous Mounting Medium (Thermo Scientific, TA-030-FM).

Alternatively, blood-derived monocytes were washed three times with PBS 0.05% saponin and blocked with PBS 10% FBS, 10% NGS, and 1% BSA for 1 h at room temperature. The buffer was then removed and the following primary antibodies were added: rabbit IgG anti-human LC3B (1:100; Novus Biologicals, NB100-2220) and mouse IgG1 anti-human NLRP3 (1:100; abcam, ab17267); and incubated overnight at 4°C. Afterward, the cells were washed and incubated with the secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG (1:500, Invitrogen, A11008) and Alexa Fluor 568 goat anti-mouse IgG1 (1:500; Invitrogen, A21124) for 2 h at room temperature. Finally, nuclei were stained with DAPI. The coverslips were mounted with PermaFluor Aqueous Mounting Medium.

An Axio Observer.Z1 microscope equipped with a Colibri.2 and ApoTome.2 illumination systems (Carl Zeiss, Oberkochen, Germany), the EC Plan-Neofluar 100×/1.30 oil objective, a digital camera AxioCam HRm and its coupled computer equipped with AxioVision Rel. 4.8.2.0 software (Carl Zeiss) were used to image the cells. The Particle Analyzer plugin from ImageJ software was used to assess the numbers of LC3 fluorescent puncta and NLRP3 specks after image thresholding (7). For both analysis, a minimum of 100 cells per sample were scored for each experiment.

Protein Dialysis and Immunoblotting

After RNA and DNA extraction by the TRIzol method, the protein phases of the skin lesion fragments were obtained *via* protein dialysis as instructed by the manufacturer. 12% polyacrylamide gel electrophoresis was performed with 10 μg protein extracts. After electrophoresis, the resolved proteins were transferred to Hybond-C Extra nitrocellulose membranes (Amersham Biosciences, RPN303E) *via* an electrophoretic transfer system with cold-block (Bio-Rad, CA, USA). Blocking was made with 5% BSA (Sigma-Aldrich, A2153) in PBS 0.1% Tween-20 for 1 h at room temperature. Next, the primary antibodies rabbit anti-human Caspase-1 p10 (1:200, Santa Cruz Biotechnology, sc-515) and mouse IgG1 anti-human GAPDH (1:500, Santa Cruz Biotechnology, sc-47724) were incubated sequentially in the membranes overnight at 4°C. After washing, appropriate HRP-conjugated secondary antibodies goat anti-mouse IgG-HRP (1:2,000; DakoCytomation, P0447) or goat anti-rabbit IgG-HRP (1:2,000; DakoCytomation, P0448) were added for 1 h at room temperature. Chemoluminescent substrate Western Blotting Luminol reagent (Santa Cruz Biotechnology, sc-2048) was added to detect immuno-reactive band. Blottings were revealed using medical X-ray film (Carestream Kodak X-Omat LS film, Amersham Biosciences, F1149). Densitometric analysis was performed using Adobe Photoshop CC software version 14.2.1.x64 (Adobe Systems Incorporated, USA).

ELISA

To determine the concentration of IL-1β in the patient's serum, the Human IL-1 beta ELISA Ready-SET-Go! Kit (Affymetrix, eBioscience, 88-7261-77) was used according to the manufacturer's directions. Alternativelly, IL-1β, IL-6, and TNF concentrations were evaluated in the monocyte culture supernatants accordingly the instructions of the fabricant (Affymetrix, eBioscience, respectively 88-7261-77, 88-7066-77, 88-7346-77).

Statistical Analysis

Statistical significance was calculated by Mann–Whitney test or Kruskal–Wallis with Dunn's multiple comparison post-test *via* GraphPad Prism 5.00.288 software (GraphPad, La Jolla, CA, USA). A $p < 0.05$ was deemed statistically significant.

RESULTS

Innate Immunity Is Differentially Regulated in WR versus T1R Patients

To identify possible markers of future development of reversal reaction in multibacillary patients, isolated mRNAs of skin lesions obtained at diagnosis were evaluated by RT-qPCR array for innate and adaptative immunity genes.

The gene-expression profiles of multibacillary patients showed a differential regulation of innate and adaptative immunity between patients who developed (T1R) or not (WR) reversal reaction episodes in the future. Patients of the group T1R showed a significative increase in *TLR2* expression (**Figure 1A**; Table S2 in Supplementary Material), as well as a predominance of genes related to pro-inflammatory responses, as *CRP*, *IL36B*, *IL36G*, *IL36RN*, *IL6*, *IFNG*, and *LY96*, type 1 interferon pathway, as *IRF1*, *IFN1*, *IFNA1*, *IFNB1*, TLR activation, as *TLR1*, *TLR6*, *TLR9*, *TLR10*, inflammasome activation, as *IL1RAP* and *IL1RAPL2*, and antimicrobial responses, as *DEFB4A* and *LYZ* (**Figure 1A**; Table S2 in Supplementary Material).

Conversely, WR patients lesions present an increased expression of the genes inhibitors of NFκB, *IKBKB*, and *CHUK*, the immunoregulator *TGFB1*, the antimicrobial peptide codifier *CAMP*, *TLR8,* extracellular matrix protein, *FN1, NFKB2,* the scavenger receptor of oxidatively modified low-density lipoprotein *COLEC12,* the inflammatory caspase *CASP4,* and the cytokine receptors *IL1RL2* and *IFNGR1* (**Figure 1A**; Table S2 in Supplementary Material).

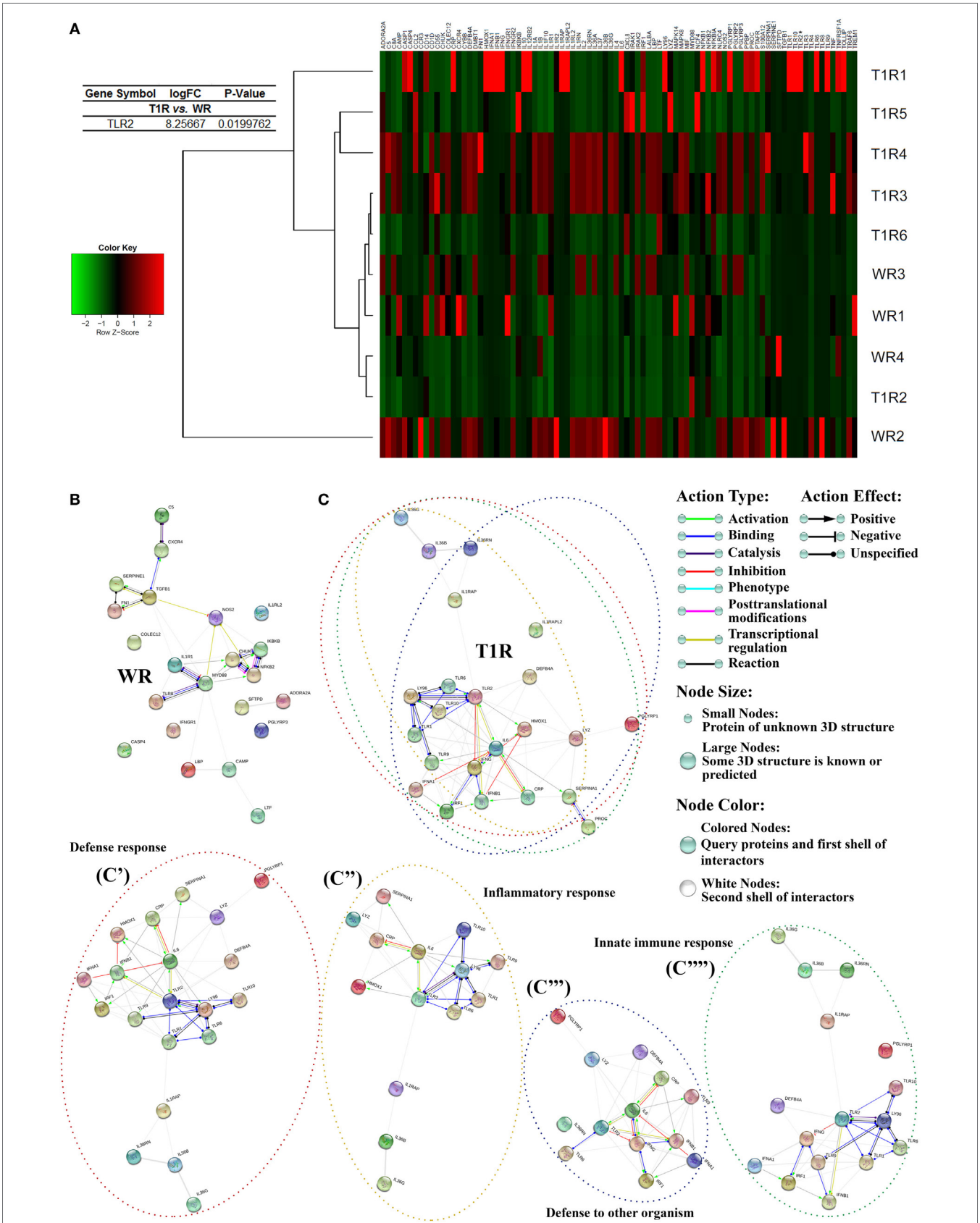


FIGURE 1 | Continued

FIGURE 1 | Gene-expression profile of leprosy lesions showed a modulation of innate and adaptative immunity-associated genes between multibacillary patients who developed (T1R) or not (WR) reversal reactional episodes in the future. Purified mRNAs from skin lesions of multibacillary patients who developed or not reversal reaction episodes were analyzed by RT-qPCR innate and adaptative immunity array. The expression fold values of the significantly upregulated genes in WR and T1R lesions were tabulated (full data are available in Table S2 in Supplementary Material). The threshold for statistical significance was $p < 0.05$. **(A)** Heat map showing analysis of differential expression of innate and adaptative immunity-related genes in leprosy patients. Each row represents one donor. Asterisks indicate genes with differential expression. Heat map data are representative of four WR and six T1R samples. **(B,C)** Innate and adaptative immunity gene interaction networks in WR and T1R skin lesions. Genes with a differential expression in leprosy lesions according to autophagy PCR array analysis were visualized by STRING. The action network view. In this view, colored lines and arrow styles between genes indicate the various types of interactions. Network nodes represent genes. Edges represent gene–gene associations. **(C)** Interactions in genes annotated to defense response (C′), inflammatory response (C″), defense to other organism (C‴), and innate immune response (C⁗) ontology terms in T1R group patients are shown. Interaction maps are representative of four WR and six T1R samples.

The genes differentially expressed between the two groups of multibacillary patients were submitted to gene–gene interactions and enrichment analysis using the STRING database. Network maps of upregulated genes in leprosy skin lesions showed more interactions between innate and adaptative immunity-associated genes in T1R than WR patients (**Figures 1B,C**; Figure S1 in Supplementary Material). GO enrichment analysis of canonical pathways showed that T1R lesions were predominantly enriched for innate immunity-associated GO terms such as defense response, inflammatory response, defense to other organism, innate immune response, and so on, as compared to WR lesions (**Figure 1C**).

There were not significantly changes in TLR2 gene and protein expression in *M. leprae*-stimulated primary monocytes from T1R and WR patients (data not shown), suggesting the existence of a specific immune response in skin. Taken together, those data indicate a predominance of TLR and inflammasome activation in skin cells, as well as of pro-inflammatory responses in patients who developed reversal reactional episodes in the future rather than the WR group.

Autophagy Is Differentially Regulated in WR versus T1R Patients

The prior results indicated that the innate immune response gene activation was upregulated in skin lesions of multibacillary patients who developed reversal reactional episodes (**Figure 1**). To further identify the host pathways involved in leprosy immune response, we analyzed the transcriptional regulation of the autophagic pathway, an innate mechanism recently described to be implicated in leprosy polarization (7). In this order, the ATG (autophagy-related) gene-expression profile of multibacillary skin lesions mRNAs was analyzed by RT-qPCR using a human autophagy pathway PCR array.

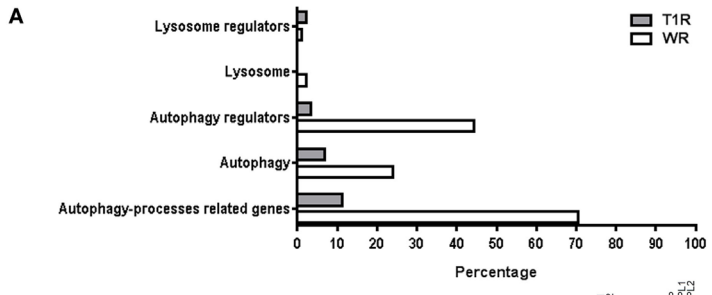
Surprisingly, multibacillary patients that did not develop reversal reactional episodes (WR, 70% of genes) presented a strong upregulation of several autophagy processes-related genes versus those who developed (T1R, 11%) by fold-change analysis. Upregulated genes in multibacillary lesions are involved in regulation of autophagy (44% of WR versus 3% of T1R), autophagosome formation (24% of WR genes versus 6% of T1R genes), lysosomal function or pathways (2% of WR versus 0% of T1R), and regulation of the lysosome (1% of WR versus 2% of T1R) by using Jegga et al. (28) functional autophagy-lysosomal gene classification (**Figure 2A**; Table S3 in Supplementary Material).

Furthermore, WR lesions displayed a significantly higher expression of eight genes, most involved in autophagy regulation, when compared to just one in T1R lesions. WR lesions presented a highly significant expression of *FRS3*, *GPSM3*, *SEC24C*, *LETM1*, *LAMP2*, *ULK4*, *APOL1*, and *HSPA5* (**Figures 2A,B**; Table S3 in Supplementary Material). Many other genes of the core autophagic machinery were also upregulated in the WR skin lesions, as members of the Atg1/ULK complex (*ULK1* and *3*), the first complex to regulate autophagosome assembly, Atg9 and its cycling system (*ATG2A/B*, *ATG9A*, and *WIPI1/2*), which has a role in supplying membranes for phagophore expansion, the PIK3 complex (*PIK3C3* and *PIK3R4*) that participates in the vesicle nucleation stage and promotes the recruitment of PI3P-binding proteins to the site of phagophore biogenesis, the Atg8 (*ATG4A*, *ATG7*, and *MAP1LC3A*) and Atg12 conjugation systems (*ATG7*, *ATG10*, *ATG12*, *ATG16L1*, and *L2*) two ubiquitin-like conjugation systems involved in vesicle expansion, and the lysosomal components (*LAMP1* and *2*) required for autophagosome-lysosome fusion step (**Figure 2B**; Table S3 in Supplementary Material) [reviewed by Feng et al. (8)].

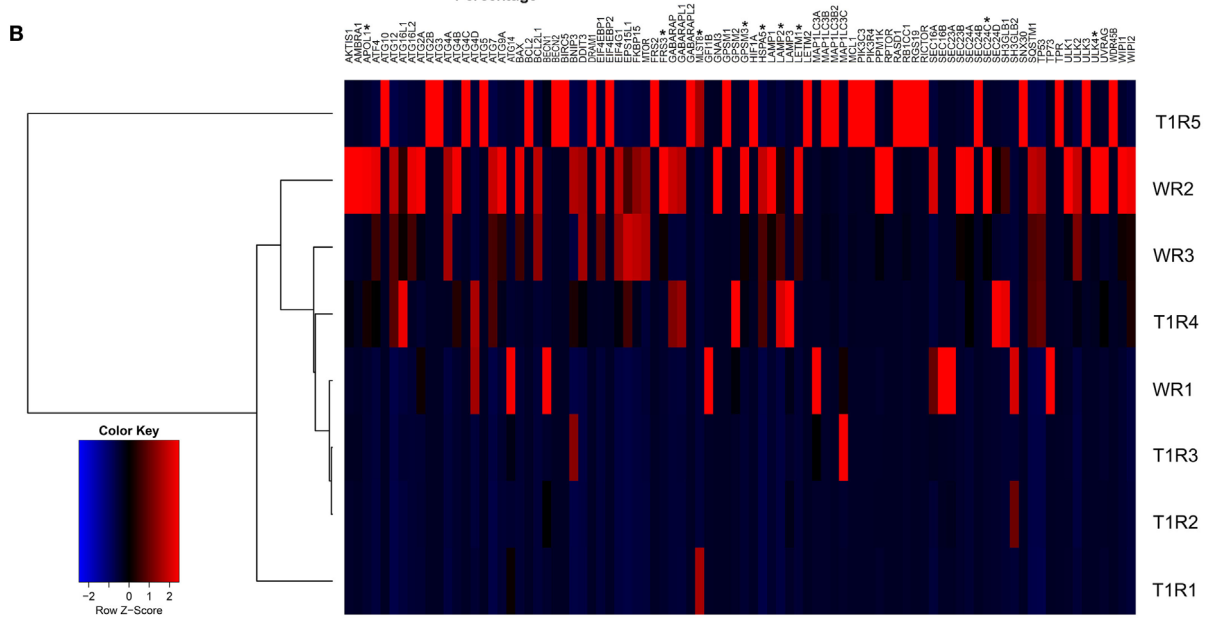
On the other hand, in T1R lesions a significant expression of the MTOR complex gene *MLST8* was found. Fold change analysis showed an increased expression of a subset of genes also implicated in autophagy activation, such as *WDR45B*, a component of Atg9 and its cycling system, *LAMP3*, a lysosomal system constituent, *ATG4D*, *GABARAP*, *MAP1LC3B2*, members of the Atg8 conjugation system, *ATG5*, a component of the Atg12 conjugation system, *BIRC5*, a member of the inhibitors of apoptosis gene family, *SH3GLB2*, a component of the phagophore membrane curvature complex, and *BECN2*, a mammal-specific homolog of the PIK3 complex gene *BECN1* (**Figure 2B**; Table S3 in Supplementary Material) [reviewed by Feng et al. (8)].

Gene–gene interactions and enrichment analysis were made *via* the STRING database in the differentially regulated genes in multibacillary skin lesions. The superexpressed gene network maps of leprosy lesions displayed an increased number of interactions among autophagy-associated genes in WR when compared to T1R patients (**Figures 2C,D**; Figure S2 in Supplementary Material). GO enrichment analysis of canonical pathways showed that WR lesions were predominantly enriched for autophagy-associated GO terms such as autophagy assembly, mitophagy, macroautophagy, nucleophagy, and so on, as compared to T1R lesions (**Figures 2C,D**).

PCR array data analysis supplied correlative results regarding increased autophagy in WR as opposed to T1R lesions (**Figure 2**).



Gene Symbol	logFC	P-Value
WR *vs.* T1R		
FRS3	16.64127	0.008101898
GPSM3	14.83407	0.018183722
SEC24C	14.48627	0.021068142
LETM1	13.25687	0.034698704
LAMP2	12.69767	0.04306004
ULK4	12.57027	0.045187401
APOL1	12.56220	0.045325041
HSPA5	12.55500	0.045448192
T1R *vs.* WR		
MLST8	12.95913	0.03895891



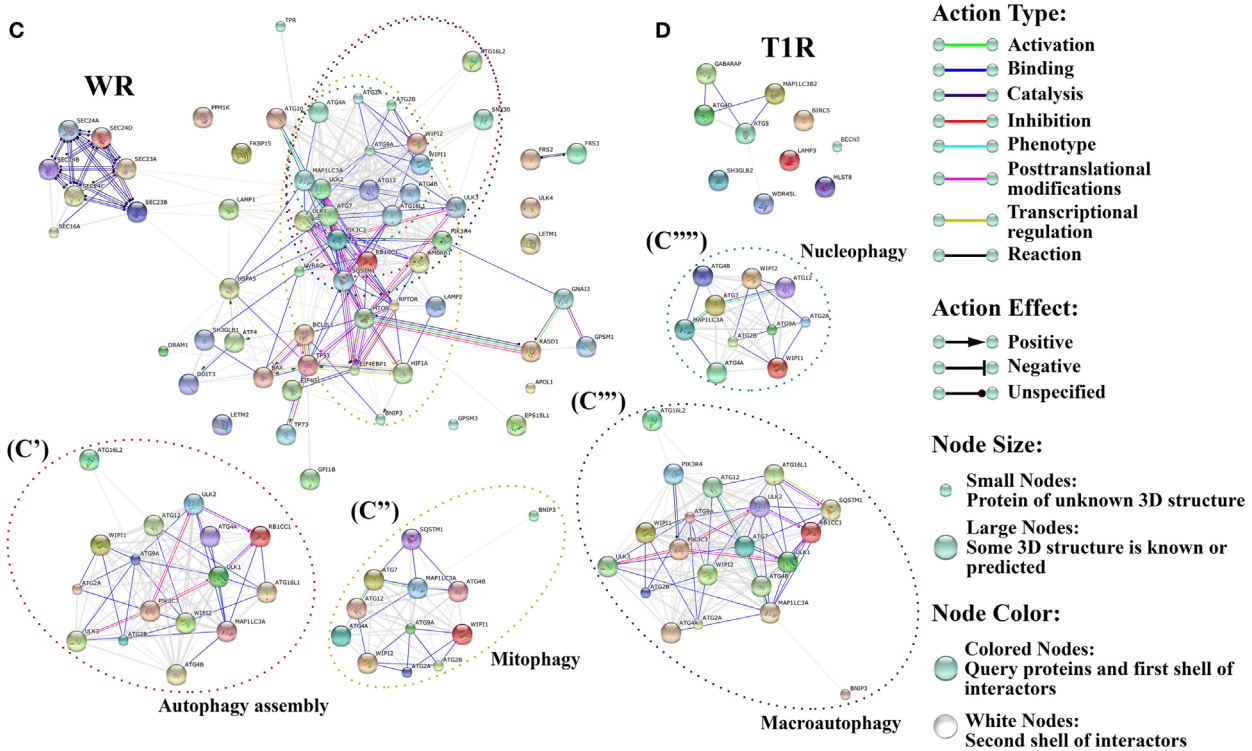


FIGURE 2 | Continued

FIGURE 2 | Gene-expression profile of leprosy lesions showed a modulation of autophagy-associated genes between multibacillary patients who developed (T1R) or not (WR) reversal reactional episodes in the future. Purified mRNAs from skin lesions of multibacillary patients who developed (T1R) or not (WR) reversal reactional episodes were analyzed by RT-qPCR autophagy array. **(A)** Differentially expressed autophagy processes-related genes were sub-categorized. The expression fold values of the significantly upregulated genes in WR and T1R lesions were tabulated (full data are available in Table S3 in Supplementary Material). The threshold for statistical significance was $p < 0.05$. **(B)** Heat map showing analysis of differential expression of autophagy processes-related genes in leprosy patients. Each row represents one donor. Asterisks indicate genes with differential expression. Heat map data are representative of three WR and five T1R samples. **(C,D)** Autophagy gene interaction network in WR and T1R skin lesions. Genes with a differential expression in leprosy lesions according to autophagy PCR array analysis were visualized by STRING. The action network view. In this view, colored lines and arrow styles between genes indicate the various types of interactions. Network nodes represent genes. Edges represent gene–gene associations. Interactions among autophagy processes-related genes were more evident in WR than T1R patients. **(C)** Interactions in genes annotated to autophagy assembly (C′), mitophagy (C″), macroautophagy (C‴), and nucleophagy (C⁗) ontology terms in WR group patients are shown. Interaction maps are representative of three WR and five T1R samples.

Thus, to confirm that there is increased autophagy in multibacillary patients that not undergo reversal reactional episodes (WR), the protein expression of LC3 in WR and T1R lesions was measured. Immunohistochemistry analysis showed a higher presence of endogenous LC3 in non-reactional multibacillary patients (WR) when compared to those who developed reversal reaction lesions (T1R) in the future (**Figure 3A**). Additionally, skin lesion-derived WR macrophages exhibited higher autophagic puncta formation than macrophages of T1R patients through immunofluorescence staining of the autophagy marker LC3 (**Figure 3B**). Taken together, our results indicate that the blockade of autophagy may be associated with the occurrence of reversal reaction episodes in multibacillary patients.

Inflammasome NLRP3-IL-1β Pathway Is Differentially Regulated in WR versus T1R Patients

Since our previous data showed increased expression of IL-1 receptor accessory proteins and a blockage of the autophagic pathway in the multibacillary patients from the group T1R, and several studies report that autophagy blockade can potentialize the inflammasome activation (7, 12–15), we evaluated the expression of the NLRP3 pathway genes, *NLRP3*, *IL1B*, *IL18*, *IL33*, and *CASP1*, in the skin lesion cells by RT-qPCR.

The T1R samples presented an increased expression of *NLRP3*, *IL1B*, and *CASP1* (**Figure 4A**).

Inflammasomes catalyze pro-caspase-1 in caspase-1, an enzyme that in turn proteolytically activates IL-1β [reviewed by Harris (11)].

The increase in the gene expression of the T1R samples was accompanied by increased caspase-1 (p10) and ratio caspase-1-pro-caspase-1 (p10/p45), indicating increased caspase-1 activation and activity in those samples (**Figure 4B**). On the other hand, samples of the WR group showed accumulation of pro-caspase-1 (p45) confirming our hypothesis (**Figure 4B**). We also observed increased IL-1β amounts in the sera of patients who developed reversal reactional episodes in the future (**Figure 4C**).

Taken together, our data indicate increased autophagy in multibacillary patients that did not develop reversal reaction episodes (WR), with accumulation of pro-caspase-1 in the tissue. On the other hand, multibacillary patients who developed reversal reactional episodes (T1R) in the future presented a blockage of autophagy and increased inflammasome activation and consequent IL-1β secretion already at diagnosis time point, 2–20 months before the reactional episode occurrence.

Autophagy Regulates Inflammasome Activation in *M. leprae*-Stimulated Primary Human Monocytes

To determine whether autophagy affected inflammasome activation during *M. leprae* stimulation, we examined the effect of autophagy activation or blockade on NLRP3 activation in human blood-derived monocytes from healthy donors stimulated with γ-irradiated *M. leprae*. Upon inflammasome activation, NLRP3 recruits the adapter protein ASC and assembles large protein scaffold complexes, which are termed "specks," which causes caspase-1 activation, resulting in the maturation of IL-1β. Hence, due to the significantly large size of these structures, NLRP3/ASC specks can be effortlessly visualized by fluorescence microscopy as a simple upstream readout for inflammasome activation (31).

Immunofluorescence analysis revealed that induction of autophagy by dead *M. leprae* or RP treatment was able to increase LC3 puncta formation in monocytes (**Figure 5A**). Exposure to these stimuli also triggered a degradation of inflammasomes, as observed by the reduced numbers of NLRP3 specks, similar to levels of unstimulated cells (**Figure 5A**). Autophagy was sensitive to inhibition by 3-MA leading to blockage of the autophagosome generation, as well as to the inhibitor of autophagosome-lysosome fusion CQ, which led to accumulation of LC3-positive dots (**Figure 5A**). However, we found that the NLRP3 activation was insensitive to neutralization of autophagy by CQ, since only 3-MA treatment promoted the increase of NLRP3 specks, although there is no significant difference in the number of NLRP3 specks by themselves in comparison with control cells, which was already expected since in these cells there is not much stimulus (e.g., first signal) for inflamassome activation (**Figure 5A**).

By treating *M. leprae*-stimulated monocytes with RP, which trigger autophagy by inhibiting the nutrient and energy sensor mTOR, we showed an even higher increase in the number of LC3 puncta per cell with reduced amounts of NLRP3 specks (**Figure 5A**). In contrast, addition of CQ or 3-MA together with *M. leprae* was able to increase the number of NLRP3 specks observed per cell, suggesting an increase in inflammasome activation, which was followed by autophagy deactivation at the same time, as seen through the increase or decrease of LC3-positive vesicles in CQ- or 3-MA-treated *M. leprae*-stimulated monocytes, respectively (**Figure 5A**).

In order to confirm this observation, the mRNA expression of *LC3B* and *IL1B* was evaluated in blood-derived monocytes cultures stimulated with γ-irradiated *M. leprae* and the autophagy blocker

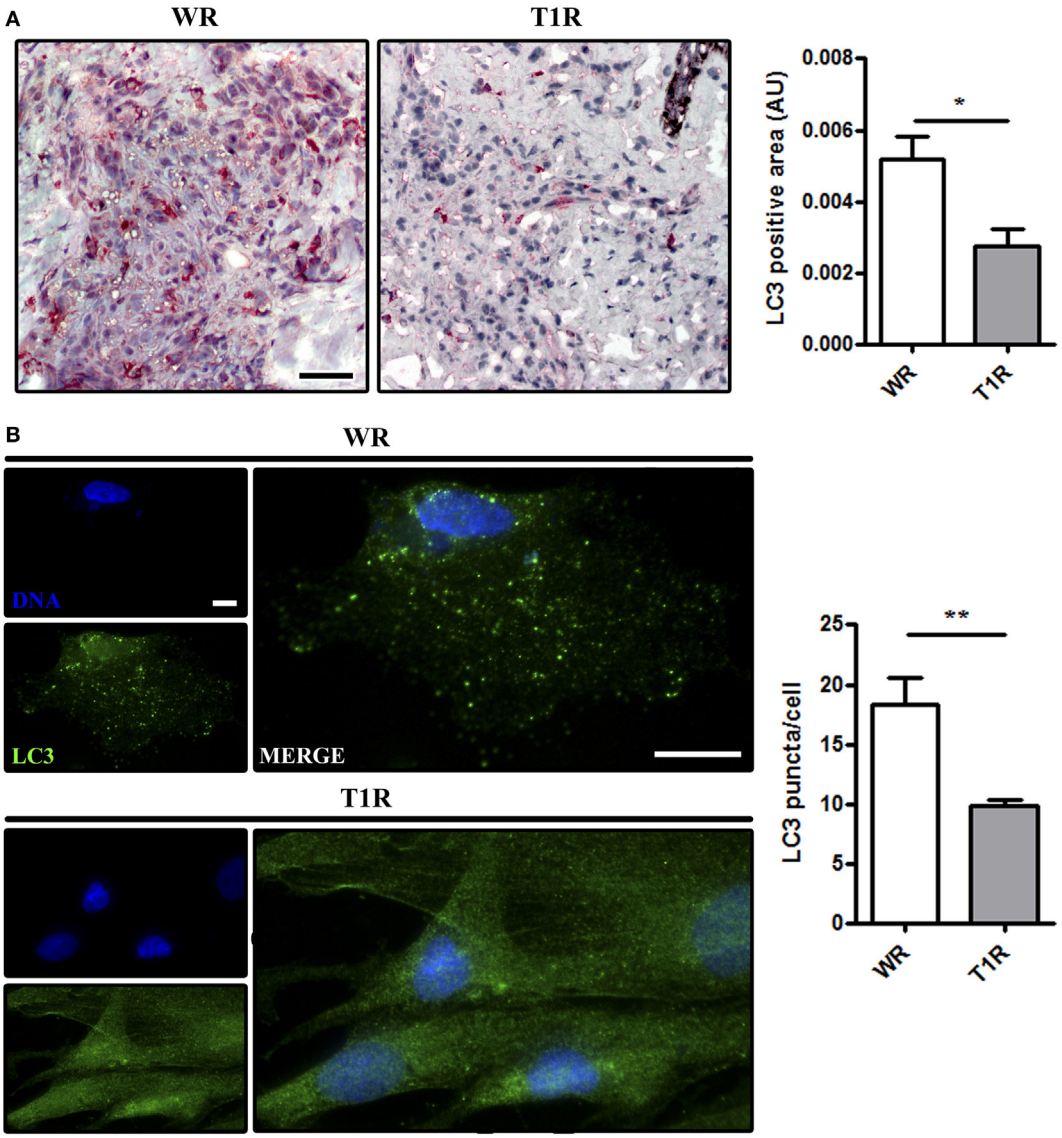


FIGURE 3 | Increase of autophagy levels in skin lesions of multibacillary patients that did not develop reversal reactional episodes (WR). Skin lesion samples were obtained from multibacillary patients who developed (T1R) or not (WR) reversal reactional episodes and analyzed as indicated. **(A,B)** Increased LC3 expression in skin lesion cells of WR patients. **(A)** Immunohistochemical (IHC) analysis of endogenous LC3. Representative micrographs from WR ($n = 3$) and T1R ($n = 4$) patients are shown. IHC images were quantified and data are expressed as arbitrary units (AU). Bars represent the mean values ± SEM. *$p < 0.05$. Scale bar: 50 μm. **(B)** Macrophages were isolated from skin lesions of multibacillary patients who developed (T1R) or not (WR) episodes of reversal reaction in the future, and cultured for 18 h. Cells were fixed and stained with the anti-LC3 antibody (green) and DAPI (blue). Macrophages of WR skin lesions showed enhanced LC3 puncta formation as compared to T1R macrophages. Immunofluorescence images were quantified and bars represent the mean values of the number of LC3 puncta per cell ± SEM (WR, $n = 3$; T1R, $n = 3$) (**$p < 0.01$). Scale bar: 20 μm.

3-MA. RP, used as a positive control for autophagy activation, was able to induce 57.6-fold increase in *LC3B* expression as compared to the non-stimulated control (**Figure 5B**). The *M. leprae* stimuli was able to increase in two-fold the gene expression of *LC3B* (**Figure 5B**). This increase was reverted 16.3-fold by the treatment with 3-MA (−14.3-fold in relation to non-stimulated) (**Figure 5B**). Conversely, autophagy induction was able to decrease in −7.3-fold the transcription of *IL1B* in comparison with non-stimulated monocytes (**Figure 5B**), and the inhibition of autophagy with 3-MA was able to increase the *IL1B* gene expression in 364.4-fold in relation to *M. leprae*-stimulated monocytes (369.4-fold change in relation to non-stimulated monocytes) (**Figure 5B**).

Next, we evaluated the secretion of IL-1β, and the pro-inflammatory cytokines IL-6 and TNF in the *M. leprae* and 3-MA-stimulated monocyte cultures supernatants. The stimuli with *M. leprae* was able to increase the production of IL-1β and the other pro-inflammatory cytokines evaluated in comparison to non-stimulated cultures (**Figure 5C**). The autophagy blockade by

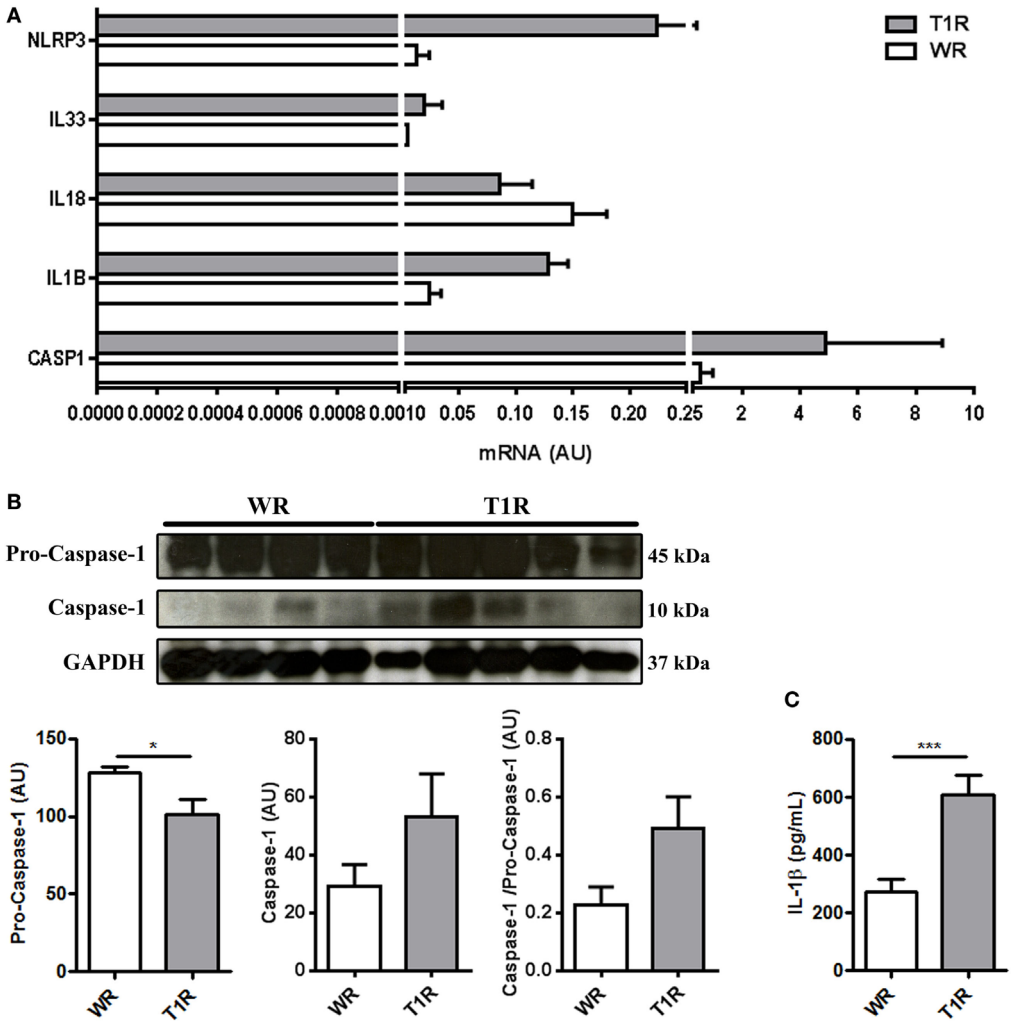


FIGURE 4 | Leprosy lesions present a modulation of inflammasome-associated genes, the subunits of caspase-1 protein, and IL-1 β secretion between multibacillary patients who developed (T1R) or not (WR) reversal reactional episodes in the future. **(A,B)** Skin lesion samples were obtained from multibacillary patients who developed (T1R) or not (WR) reversal reactional episodes and analyzed as indicated. **(A)** Purified mRNAs from skin lesions of multibacillary patients who developed or not T1R episodes were analyzed by RT-qPCR for *NLRP3*, *IL33*, *IL18*, *IL1B*, and *CASP1*. Bars represent the mean values ± SEM of three patients of each group. **(B)** Increased activity of caspase-1 in T1R patients skin lesion. Protein contents from leprosy lesions were analyzed by immunoblotting with anti-caspase-1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to verify protein amount loading. Blots are shown (WR, $n = 4$; T1R, $n = 5$). Densitometric analysis of the blots was performed and the caspase-1 (p10 subunit) and pro-caspase-1 (p45 subunit)/GAPDH ratios are expressed as arbitrary units (AU). Data are presented as mean ± SEM. $^{*}p < 0.05$. **(C)** The IL-1β levels were assessed in the sera of multibacillary patients who developed (T1R) or not (WR) reversal reaction episodes by ELISA. Bars represent the mean values ± SEM (WR, $n = 10$; T1R, $n = 12$). $^{***}p < 0.001$.

3-MA treatment in conjunct with *M. leprae* was able to increase the secretion of IL-1β and IL-6, but not TNF as compared to the wells treated just with the mycobacteria (**Figure 5C**).

Together, these data indicate that autophagy is an important regulator of inflammasome activation during *M. leprae* infection and helps to control the exacerbated inflammation that leads to reversal reaction episodes onset.

DISCUSSION

The identification of biomarkers of leprosy reaction is an urgent demand. Leprosy reactions are responsible for nerve lesions and physical incapacities caused by leprosy (1). Reversal reaction may occur in borderline patients and also in subpolar lepromatous patients. It is described as an exacerbation of pre-occurring lesions in the skin and nerves (16, 32). Several studies have described possible biomarkers of reverse reaction (16, 18, 19, 33–36).

The outcome of reversal reaction is attributed to a sudden shift of the immune system, with an increase in the cell-mediated response against the bacilli (37). In the present study, we recruited multibacillary patients at the diagnosis and realized a 24-month follow-up to classify them accordingly the occurrence or not of reversal reaction. Leprosy reactions may occur in any time, before, during, and even after completing the multidrug therapy.

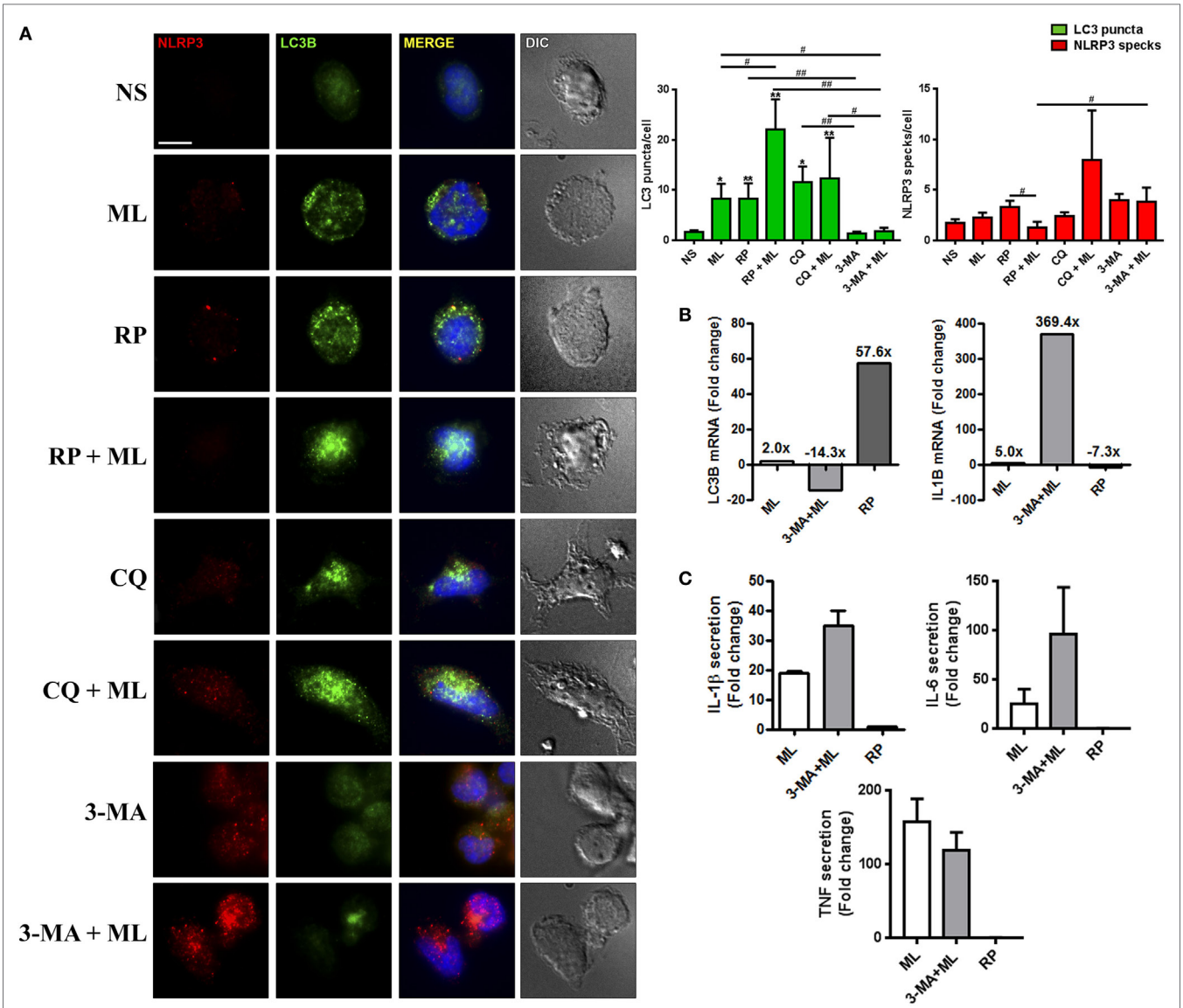


FIGURE 5 | Autophagic pathway regulates inflammasome activation in blood-derived monocytes stimulated with *M. leprae*. **(A–C)** Monocytes were purified from healthy donors' peripheral blood mononuclear cells by 2-h adherence and stimulated with 10 μg/mL *M. leprae* (ML) in the presence or absence of autophagy regulators for 18 h. Autophagy was triggered with 200 ng/mL rapamycin (RP), and inhibited with 100 μM chloroquine (CQ, autophagic flux blocker) and 10 μM 3-methyladenine (3-MA). **(A)** Monocytes stimulated with *M. leprae*, RP, CQ, and 3-MA were fixed and stained with anti-NLRP3 (red), anti-LC3B (green) antibodies and DAPI (blue). Monocytes stimulated with *M. leprae* increase the number of LC3-II-decorated autophagosomes (puncta) per cell, which is reforced by RP addition, and reverted by 3-MA. Autophagy blocking with CQ and 3-MA was able to increase NLRP3 specks numbers per cell in relation to the well stimulated with *M. leprae* and RP. Immunofluorescence images were quantified and bars represent the mean values of the number of LC3 puncta and NLRP3 specks per cell ± SEM ($n = 3$). *(in relation to NS) or #(indicated by the dashes) $p < 0.05$. Scale bar: 10 μm. **(B)** Purified mRNAs from monocyte cultures stimulated with *M. leprae*, the autophagy blocker 3-MA, and RP, as a control of autophagy activation, were analyzed by RT-qPCR for *LC3B* and *IL1B*. Bars represent the mean of the fold change values in relation to the non-stimulated controls of three independent experiments with similar results. **(C)** The IL-1β, IL-6, and TNF levels in the monocyte cultures supernatants were assessed by ELISA. Bars represent the fold change values in relation to the non-stimulated controls ± SEM ($n = 3$).

One limitation of our study was short period follow-up, of only 1 year after the end of treatment. Besides that, our strategy was sufficient to identify the involvement of autophagy impairment and inflammasome activation as the mechanisms responsible for reversal reaction outcome in multibacillary patients.

Cellular immune mechanisms are associated with the development of reversal reaction. To identify the immune pathways related to reversal reaction we evaluated the expression of a total of 88 innate and adaptative immune genes in skin lesions from multibacillary patients. Analysis of innate and adaptive immune pathways demonstrated that *TLR2* expression was significantly different when comparing the group that did not develop reaction during treatment (WR) and the group that develop reversal reaction (T1R). Although TLR2 expression was significantly increased

in cells from skin lesions, analysis of *M. leprae*-stimulated monocytes did not shown differences between cells from WR and T1R groups (not shown), suggesting that there is a modulation of local immune response. A previous study has described *TLR2* polymorphisms associated with reversal reaction (38) and analysis of skin cells from patients with reversal reaction demonstrated that corticosteroids may reduce both gene and protein expression of TLR2 (39). Our present study demonstrated that in patients that develop reversal reaction in the future (T1R), *TLR2* is modulated differentially even before the appearance of the clinical symptoms of the reactional episode.

Several studies have demonstrated that TLR2 is associated with autophagy (40–43). However, more recently, it was demonstrated that both microRNAs miR0125a and miR-23a-5p inhibit autophagy activation during *M. tuberculosis* infection by mechanisms related to increased TLR2 expression (40, 44). Future studies will determine if the increased TLR2 expression in T1R group is related to the activation of pro-inflammatory signals associated to the initial stages of the development of reversal reaction or if it is contributing for the blockade of autophagy in multibacillary group.

Gene-expression analysis demonstrated an upregulation of autophagic genes in WR group when compared with T1R. PCR array analysis demonstrated that those genes which had their expressions significantly upregulated in WR group are associated with fibrosis (*FRS3*), vesicle trafficking (*SEC24C*), cellular viability (*LETM1*), protection, maintenance and adhesion of lysosomes (*LAMP2*), neuronal migration and neurite branching and elongation (*ULK4*), folding and assembly of proteins in the endoplasmic reticulum (*HSPA5*), and regulation of inflammation (*GPSM3*). In addition, *APOL1*, a gene that codifies a protein that is part of IFN-γ inducible host defense against *M. leprae*, is upregulated in WR group (45). It is possible that mechanisms related to basal autophagy may be important to sustain *M. leprae* infection in host cells, since autophagy is observed in patients in distinct regulation pattern in the different clinical forms of the disease, being downregulated in cells from multibacillary patients that have predisposition to develop reversal reaction.

MLST8 was the unique gene significantly upregulated in T1R group. mLST8 is a subunit of both mTORC1 and mTORC2 being necessary for the mTOR kinase activation (46). The increase of mLST8 could partially explain the autophagy impairment in T1R since autophagy is negatively regulated by mTOR (47, 48).

Previous study comparing gene-expression pattern in patients with the different polar forms of leprosy demonstrated an increase in type I IFN in lepromatous patients (49) and that type 1 IFN may negatively regulate the responses of type II IFN (49). More recently, our group demonstrated that the gene encoding 2′-5′ oligoadenylate synthetase-like is upregulated in *M. leprae*-infected human macrophage cell lineages, primary monocytes, and skin lesion specimens from patients with lepromatous leprosy (50). Type I IFNs is important for the suppression of inflammasome and it was reported to inhibit NLRP3-activated inflammasome *via* STAT1 (51, 52). In the present study, the downregulation of autophagy pathways in cells from T1R group was accompanied by the increase in NLRP3 inflammasome-IL-1β pathway.

Inflammasomes are multiprotein oligomers that controls the maturation of IL-1β-related cytokines through activation of caspase-1 (53, 54). Pro-inflammatory cytokines were previously associated with reversal reaction occurrence, including IL-1β (17, 55–57) but mechanisms of the reactional episode outcome have not been elucidated. In the present work, it was not possible to determine if the bacilli or the inflammatory environment were responsible for mTOR activation in T1R group, however, the data presented here clearly suggest that the axis autophagy-inflammasome is crucial in the development or not of the reversal reaction in multibacillary patients. The strongest evidence of the involvement of inflammasome activation in the development of reversal reaction is the fact that in WR group *GPSM3* was overexpressed and a previous study demonstrated that GPSM3 is a negative regulator of IL-1β production triggered by NLRP3-dependent inflammasome activators (54), as well as the increase of the protein caspase-1 and secretion of IL-1β in the T1R group samples.

Wang et al. (58) demonstrated that upon inflammasome activation, inflammatory caspases cleave cGAS and render it inactive dampening of IFN activating pathways. It is possible that in the T1R group, the increase in inflammasome activation decreases type I IFN responses, contributing for the increase of type II IFN and other pro-inflammatory cytokines that could be associated with the reversal reaction onset.

In vitro experiments demonstrated that the blockade of autophagy pathway by 3-MA was able to increase NLRP3 expression in *M. leprae*-stimulated human monocytes, which was accompanied by an increase in *IL1B* mRNA. Previous studies have demonstrated that 3-MA leads to the accumulation of damaged-mitochondria-producing-ROS, and the activation of NLRP3 inflammasome, and consequently, causes IL-1β secretion (59). Analysis of cytokine production in *M. leprae*-stimulated cultures demonstrated that the blockade of autophagy by 3-MA did not affect the concentrations of TNF, but increased IL-1β and IL-6 secretion in *M. leprae*-stimulated cells. Previous studies have demonstrated that SNPs in *IL6* are associated with the outcome of reactional episodes (60) and IL-6 has been determined as a plasma marker for type 1 reaction (16).

In conclusion, the overall results of the present work demonstrated that in multibacillary patients more prone to develop reversal reaction there is an overexpression of both TLR2 and NLRP3 inflammasome-IL-1β pathway that is consequence of downregulation of autophagy. The data suggest that inappropriate inflammasome activation may contribute for the development of reversal reaction and open the perspective for the use of pro-autophagic drugs in the control not only of the bacillary load, as previously shown (7), but also the outcome of reversal reaction in multibacillary patients.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Human Ethics Committee of the Oswaldo Cruz Foundation (approved protocol CAAE 34239814.7.0000.5248).

All subjects gave their written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

MGMB, and ROP designed research and wrote the original draft; MGMB, BJAS, TQA, RBSP, HF, PRA, JAPO, and JACN performed research; MGMB, BJAS, GMSS, and ROP analyzed and interpreted the data; MGMB, BJAS, ENS, and ROP edited and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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Genetic Susceptibility to Leprosy—From Classic Immune-Related Candidate Genes to Hypothesis-Free, Whole Genome Approaches

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Genetics plays a crucial role in controlling susceptibility to infectious diseases by modulating the interplay between humans and pathogens. This is particularly evident in leprosy, since the etiological agent, *Mycobacterium leprae*, displays semiclonal characteristics not compatible with the wide spectrum of disease phenotypes. Over the past decades, genetic studies have unraveled several gene variants as risk factors for leprosy *per se*, disease clinical forms and the occurrence of leprosy reactions. As expected, several of these genes are immune-related; yet, hypothesis-free approaches have led to genes not classically linked to immune response. The *PARK2,* originally described as a Parkinson's disease gene, illustrates the case: Parkin—the protein coded by *PARK2*—was defined as an important player regulating innate and adaptive immune responses only years after its description as a leprosy susceptibility gene. Interestingly, even with the use of powerful hypothesis-free study designs such as genome-wide association studies, most of the major gene effect controlling leprosy susceptibility remains elusive. One hypothesis to explain this "hidden heritability" is that rare variants not captured by classic association studies are of critical importance. To address this question, massively parallel sequencing of large segments of the human genome—even whole exomes/genomes—is an alternative to properly identify rare, disease-causing mutations. These mutations may then be investigated through sophisticated approaches such as cell reprogramming and genome editing applied to create *in vitro* models for functional leprosy studies.

Keywords: leprosy, genetics, association studies, *PARK2*, next-generation sequencing, disease modeling

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INTRODUCTION

Infectious diseases are essentially caused by pathogens capable to transpose the immunological barrier and colonize the host organism. Exposure to an infectious agent is necessary but not sufficient to determine disease; exposed organisms need to be naturally susceptible and even then, clinical disease outcomes often display marked interindividual variation (1). The explanation for such variability can be addressed to different reasons, including environmental factors, divergence in virulence of pathogen strains and particularly, to the complex interplay between host and pathogens. A remarkable demonstration of this variability was observed in the Lübeck disaster occurred in the late 1920s: 251 neonates were accidentally infected by virulent *Mycobacterium*

tuberculosis contaminating a batch of Bacille Calmette-Guérin (BCG) vaccine. Twenty-three infants (9.2%) did not show any clinical signs of tuberculosis and the mortality rate was 29%; 68% of neonates who presented clinical disease spontaneously progressed to cure (2).

Robust evidence that the host–pathogen interplay is largely influenced by the genetic make-up of the host has been brilliantly demonstrated in an adoptee study: predisposition to infectious disease was predominantly inherited, in an interesting contrast with cancer that was found to be much more dependent of non-genetic factors (3). Innate predisposition to infection seems to be particularly crucial for leprosy: it is estimated that only a small fraction (from 5 to 12%) of individuals exposed to *Mycobacterium leprae* are successfully infected (4, 5). Although leprosy is treatable by an efficient multidrug regimen available for free around the world, latest reports from 143 countries show 214,783 new cases, with India (63.08%), Brazil (11.74%), and Indonesia (7.83%) presenting the highest percentage of registered cases (6). Patients display a wide spectrum of clinical phenotypes that are related to individual differences in immune response and distributes between two poles: in one extreme, tuberculoid patients presents a strong cellular (Th1) immune response with increased production of pro-inflammatory cytokines such as interleukin-2 and interferon-γ and a low or inexistent bacillary load in lesions; on the other extreme of the spectrum, lepromatous leprosy is characterized by a predominantly antibody-based (Th2) immune response with predominant expression of interleukin-10 and interleukin-4 and a high number of *M. leprae* in skin smears. Borderline disease displays a gradient of immune features depending on the proximity to one of the poles (7, 8). During the course of the disease, treatment or even after cure, up to 50% of patients develop one of the two types of an aggressive, sudden inflammatory response known as leprosy reaction, the major cause of permanent neural damage with consequent disabilities today (9, 10).

The *M. leprae* is an acid-fast, Gram-positive bacillus incapable of growing in axenic media, thus strongly dependent on the host cellular environment. The bacterium presents a reduced genome and semi-clonal characteristics across strains distributed worldwide (11), reinforcing the impact of host genetics over control of disease *per se*, its clinical forms, and the occurrence of leprosy reactions. Decades of extensive research positions host genetics as a major player controlling susceptibility to leprosy (12, 13). Early evidence comes from genetic descriptive, DNA-free studies: leprosy occurrence displays strong familial aggregation (14) and concordance of infection is higher in monozygotic (59.7%) as compared to dizygotic twins (20%) (15). Several complex segregation analysis (CSA) consistently revealed the presence of a major gene effect controlling susceptibility to leprosy *per se* in different population samples of distinct genetic backgrounds, although no consensus on the exact model of inheritance has been achieved (16, 17). Later, hypothesis-free genome-wide linkage scans have identified chromosomal regions such as 10p13, 6q25-27, and 6p21 as positional candidates to harbor leprosy susceptibility genes (18, 19), and the first Genome-Wide Association Study (GWAS) on leprosy has been performed using a large Han Chinese sample set: a total of 491,883 single nucleotide polymorphisms (SNPs) spanned over the genome were first genotyped in 706 patients and 1,225 controls; the 93 markers associated with the smallest *p*-values were later tested for replication in two additional independent population samples (20). Combined, these molecular strategies have led to the description of a multitude of genes associated to leprosy (**Figure 1**; **Table 1**), several of them participating in host immune response and/or bacterial routes of infection and evasion from the immunological barrier.

A natural functional and positional candidate genomic region has been the major histocompatibility complex (MHC)/human leukocyte antigen (HLA) located in the highly polymorphic 4 Mb interval at chromosome 6p21. The complex is essential for recognition, processing, and presentation of antigens during immune response. Genes located in all three MHC/HLA classes have been exhaustively studied in leprosy and haplotypes have been associated with both susceptibility and protection against the disease in distinct populations (13, 48). Killer immunoglobin-like receptors genes—*KIR2DS1*, *2DS2*, and *3DS1*—and their HLA ligands were associated with leprosy in a Brazilian population (32, 33) and *HLA-C*, a classical ligand for KIRs, was observed as a risk factor for leprosy in Vietnamese family based and Indian case-control populations (23). Genetic variants in the class-II *HLA-DR–DQ* locus have been consistently associated with protection against leprosy (20, 24). In the MHC class-III region, linkage disequilibrium mapping of the 6p21 region identified the low-producing A allele of the variant + 80 of Lymphotoxin-α (*LTA + 80*) as a risk factor for infection: association was reported in a Vietnamese, family-based sample and validated in a Brazilian case-control sample (39).

Receptors for pathogen-associated molecular patterns, classic molecules of the innate immune response, have been also consistently associated with leprosy. The non-synonymous

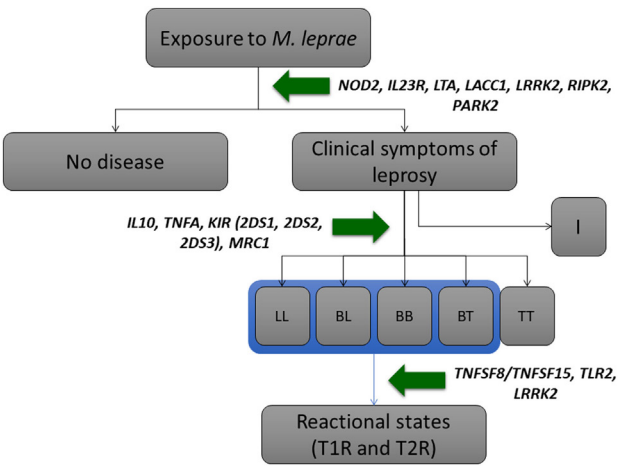

FIGURE 1 | Pathogenesis of leprosy with selected genes impacting on its phenotypes. Adapted from Mira (12) and Sauer et al. (13). For a more detailed list of genes, please refer to **Table 1**. Abbreviations: I, indeterminate; LL, lepromatous leprosy; BL, borderline-lepromatous; BB, borderline–borderline; BT, borderline-tuberculoid; TT, tuberculoid–tuberculoid; T1R, type-1 reaction; T2R, type-2 reaction.

TABLE 1 | Leprosy-associated genes with functional evidence or replicated status.

Gene	Name	Identification strategy	Population sample (Reference)	Gene function/pathway
CARD9	Caspase recruitment domain family member 9	GWAS—protein-coding variants	Chinese case-control (21)	Regulatory function in cell apoptosis and induction of NF-kB
FLG	Filaggrin	GWAS—protein-coding variants	Chinese case-control (21)	The structural component of the epidermis
HIF1A	Hypoxia-inducible factor 1 alpha subunit	GWAS—protein-coding variants	Chinese case-control (22)	Regulator of cellular and systemic homeostatic response to hypoxia; inflammation, autophagy, and immune reactions
HLA-C	Major histocompatibility complex (MHC), class-I. Human leukocyte antigen (HLA)	Association scan of the HLA *locus*	Vietnamese family-based (23) Indian case-control (23)	Immune recognition and antigen presentation
HLA-DR-DQ	MHC, class-II. HLA	GWAS Association scan Candidate gene analysis	Chinese case-control (20) Indian case-control (24) Vietnamese family-based (25)	Immune recognition and antigen presentation
IL10	Interleukin 10	Candidate gene analysis	Brazilian case-control (26) Brazilian case-control (27) Indian case-control (28) Brazilian family-based; meta-analysis (29)	Immunoregulation; downregulates Th1 response and induces B-cell survival, proliferation, and antibody production
IL12B	Interleukin 12B	GWAS	Chinese case-control (30)	Activator of NK and T-cells. Inducer of Th1 immune response
IL18RAP/ IL18R1	Interleukin 18 receptor accessory protein/interleukin 18 receptor 1	GWAS	Chinese case-control (30)	The receptor of IL18, a proinflammatory cytokine that induces cell-mediated immune response
IL23R	Interleukin 23 receptor	GWAS—protein-coding variants GWAS	Chinese case-control (21) Chinese case-control (31)	Binds to IL23 activating NK and T-cells; pro-inflammatory receptor
IL27	Interleukin 27	GWAS—protein-coding variants	Chinese case-control (21)	Modulator of T-cell differentiation
KIR (*2DS1, 2DS2, 2DS3*)	Killer immunoglobulin-like receptor (KIR)	Candidate gene analysis	Brazilian case-control (32) Brazilian case-control (33)	Regulatory molecules of NK cells surface; mediates NK reactivity against target cells; depending on HLA-I ligands
LACC1—CCDC122	Laccase domain containing—coiled-coil domain containing 122	GWAS Candidate gene analysis GWAS—protein-coding variants	Chinese case-control (20) Chinese case-control (34) Brazilian family-based (35) Brazilian case-control (35) Vietnamese family-based (25) Chinese case-control (22)	LACC1 is involved in fatty-acid oxidation with inflammasome activation, ROS production, and modulation of bactericidal activity of macrophages. CCDC122 function is presently unknown
LRRK2	Leucine rich repeat kinase 2/Dardarin	GWAS Candidate gene analysis	Chinese case-control (20) Indian case-control (36) Chinese case-control (37) Vietnamese family-based (38)	Regulation of autophagy, inflammasome activity, and production of ROS and inflammatory cytokines
LTA	Lymphotoxin-α	Genome-wide linkage analysis	2 Vietnamese family-based (39) Brazilian case-control (39) Indian case-control (39)	Pro-inflammatory cytokine, it mediates inflammatory response
MRC1	Mannose receptor C-type 1	Candidate gene analysis	Vietnamese family-based (40) Brazilian case-control (40)	Membrane receptor that mediates carbohydrate recognition
NCKIPSD	NCK interacting protein with SH3 domain	GWAS—protein-coding variants	Chinese case-control (21)	Signal transduction; regulation of cytoskeleton
NOD2	Nucleotide-binding oligomerization domain containing 2	GWAS Candidate gene analysis	Chinese case-control (20) Brazilian family-based (35) Brazilian case-control (35) Vietnamese family-based (25)	Recognition of LPS bacterial structure and activation of NF-kB

(*Continued*)

TABLE 1 | Continued

Gene	Name	Identification strategy	Population sample (Reference)	Gene function/pathway
PARK2	Parkin RBR E3 ubiquitin protein ligase	Genome-wide linkage analysis Candidate gene analysis	Vietnamese family-based (41) Brazilian case-control (41) 2 Indian case-control (42) Vietnamese family-based (43)	E3 ubiquitin-protein ligase with a role on proteasome function, mitophagy, intracellular bacterial clearance, and mitochondrial antigen presentation
RAB32	RAB32, member RAS oncogene family	GWAS—protein-coding variants GWAS	Chinese case-control (21) Chinese case-control (31)	Protein metabolism, vesicle-mediated transport and autophagy
RIPK2	Receptor-interacting serine/threonine kinase 2	GWAS Candidate gene analysis	Chinese case-control (20) Chinese case-control (34) Vietnamese family-based (25)	Signaling, innate and adaptive immune response; NF-kB inducer
SLC29A3	Solute carrier family 29 member 3	GWAS—protein-coding variants	Chinese case-control (21)	Nucleoside transporter
TLR1	Toll-like receptor 1	GWAS Candidate gene analysis	Indian case-control (24) Brazilian case-control (44) Brazilian family-based (44)	Pathogen recognition and activation of innate immunity
TLR2	Toll-like receptor 2	Candidate gene analysis	Ethiopian case-control (45)	Pathogen recognition and activation of innate immunity
TNFA	Tumor necrosis factor alfa	Candidate-gene analysis	Brazilian family-based and case-control; meta-analysis (46)	Pro-inflammatory cytokine
TNFSF8/ TNFSF15	Tumor necrosis factor (Ligand) Superfamily, Member 8/Member 15	GWAS Candidate gene analysis	Chinese case-control (20) Vietnamese family-based (47) Brazilian case-control (47)	Pro-inflammatory cytokine
TYK2	Tyrosine kinase 2	GWAS—protein-coding variants	Chinese case-control (21)	Cytokine modulator, interferon signaling pathway

GWAS, Genome-wide association study; ROS, reactive oxygen species; LPS, lipopolysaccharides; NK, natural killer.

single-nucleotide polymorphism G396S located at the Mannose Receptor C-type lectin (*MRC1*) gene located in region 10p13 was described as a risk factor for leprosy susceptibility in different populations (40). Polymorphisms in the toll-like receptor (TLR) family were repeatedly associated with leprosy and its phenotypes. Amino acid substitutions N248S and I602S in the *TLR1* gene have been associated with susceptibility (44) and protection (24) against leprosy, respectively. SNP markers 597 C/T (rs3804099) and a 280 bp-length microsatellite of *TLR2* have been associated with protection and increased risk of leprosy reactions, respectively (45) Another sensing molecule consistently associated with leprosy is the nucleotide-binding oligomerization domain 2 (NOD2), a cytoplasmic receptor responsible for recognizing intracellular pathogens *via* their peptidoglycan components of the bacterial cell wall. *NOD2* involvement in leprosy was first identified in a GWAS (20) and later replicated (35); in addition, association of *NOD2* variants with leprosy reaction has been detected (49). Functionally analysis has demonstrated that a structurally unique muramyl dipeptide of *M. leprae* is recognized by NOD2, triggering expression of interleukin-32 and monocytes differentiation into dendritic cells (50).

Cytokines regulating the Th1/Th2 immune responses have also been described associated with leprosy phenotypes. *TNFA* and *IL10* genetic variants are classic risk factors for leprosy (29, 46); gene products TNF-α and IL-10 are major signature cytokines for the tuberculoid and lepromatous pole, respectively (51). More recently, GWAS have suggested a role in leprosy susceptibility control of *IL12B*, *IL27*, and pro-inflammatory receptors *IL23R* and *IL18RAP/IL18R1* that regulates the adaptive immune response (21, 30). Functional assays indicate regulation of IL10 expression by IL27, inhibiting host defense through IFN-γ-induced antimicrobial activity (52).

A more comprehensive analysis of leprosy genetic studies reveals a complex network of interactions among associated genes. This is well exemplified by *LRRK2*, initially identified in the first leprosy GWAS (20) and later replicated in an Indian population (36); *LRRK2* participates in the control of autophagy with involvement of the small GTPase RAB32 (53), which gene is associated with leprosy in two unrelated GWAS (21, 31). Interestingly, LRRK2 is also correlated to bacterial survival and co-localization as observed in RAW 264.7 cells infected by *Salmonella typhimurium* (54); although, the increase of LRRK2-kinase activity increases *M. tuberculosis* survival through reduction of phagosome maturation (55). Recently, *LRRK2* has been associated with leprosy type-1-reaction, a pathological inflammatory response event (38). Finally, *LRRK2* is a negative regulator of inflammasome activation (56, 57) and an inducer of ROS production (54, 57), two known mechanisms of immune defense against bacterial infections also modified by variants of *LACC1*, a gene consistently associated with leprosy (20, 25, 34, 35). Recently, the *LACC1* contribution to leprosy risk has been reinforced: a GWAS-based analysis focusing on functional variants detected association between leprosy and a *LACC1* missense variant (rs3764147, c.760A > G, p.Ile254Val) (22).

Several other genes enrolled in immune-response pathways have been associated to leprosy and its phenotypes; however, a full description of these studies goes beyond the scope of this paper, for more detailed data, please refer to **Table 1** and Ref. (12, 13, 58, 59).

An intriguing aspect revealed by leprosy hypothesis-free genetic studies is the often identification of genes not classically related to immune response pathways—genetics studies on leprosy have contributed to the description of unsuspected immune-related roles for these genes; Parkin, the protein coded by *PARK2*, illustrates the case.

THE PARK2/PARKIN CASE

The *PARK2* gene was originally described in 1998 as a result of an investigation of microdeletions in patients carrying autosomal recessive juvenile parkinsonism (AR-JP): the authors isolated a 2,960 bp DNA sequence containing an open reading frame coding for a 465 amino acid protein. Characterization of the sequence by alignment and screening of DNA libraries led to the discovery of a ubiquitin-like protein, named Parkin due to its impact on Parkinson disease (60). Two years later, the *PARK2* gene product was defined as a ubiquitin-protein ligase and its loss of function reputed as causal of AR-JP (61).

First evidence of a role for *PARK2* in leprosy control came from a genome-wide linkage analysis. Genotyping of 388 microsatellite markers covering the whole genome (10 cM interval) was conducted in 86 Vietnamese families displaying 205 affect siblings; 11 chromosome regions were initially linked to leprosy. In a second-round of genotyping, all 11 regions were saturated with additional 89 markers and results evidenced strong co-segregation of the 6q25-q27 region and leprosy (maximum likelihood binomial LOD score 4.31; $P = 5 \times 10^{-6}$) (19). In a follow-up study, association fine mapping of the 6q25-27 genomic region using 208 independent simplex Vietnamese families lead to the discovery of SNPs clustered in the shared promoter region of *PARK2* and *PACRG* genes, associated with increased risk of leprosy in two ethnically independent population, Vietnamese and Brazilian. Linkage-disequilibrium analysis evidenced two tag-SNPs—common allele "T" from PARK2_e01(-2599) and rare allele "C" from rs1040079—capturing the complete association information (41). Interestingly, *PARK2*, a non-immune related gene by the time of the study, was the first gene described and validated as having an impact on susceptibility to leprosy by a hypothesis-free, positional cloning strategy.

PARK2 association with leprosy was further replicated in an Indian population; however, association signal did not pass a conservative Bonferroni correction for multiple testing (62). Polymorphisms in *PARK2*/*PACRG* co-regulatory region were also found associated with leprosy risk in Croatian (63) and two unrelated Indians population samples (42). Moreover, the association was confirmed in independent Vietnamese and Indian samples with a remarkable contribution of age-at-diagnosis to the association signal (43). *PARK2's* impact over susceptibility to infection was also demonstrated by association of the T allele-2599 to typhi and paratyphoid fever, diseases caused by *Salmonella*, an intracellular pathogen (64).

Parkin is an E3 ubiquitin-ligase involved in the proteasome pathway, in particular, the autophagy cellular mechanism of turnover of damaged biomolecules (lipids and proteins) and organelles. Parkin targets are marked and delivered to autophagosomes that are fused with lysosomes and consequently degraded. Of particular importance is the role of Parkin in the mitophagy pathway of mitochondrial recycling: along with PTEN-induced putative kinase protein 1 (PINK1), a mitochondrial kinase, Parkin modulates mitochondrial quality control by mediating the ubiquitination of mitochondrial proteins when the organelle is depolarized (65).

Autophagy has been described as an important defense mechanism aiming to destroy intracellular pathogens, an innate immune response process named xenophagy (66). Through this mechanism, invading microbes are labeled with ubiquitin and adaptor proteins (e.g., p62, NDP52, and optineurin) to be presented to autophagy protein LC3 and initialize assembly of the autophagosome (67). Bacterial degradation by xenophagy has been described against mycobacteria, including *M. tuberculosis* (68). More recently, Parkin has been described to participate in this pathway mediating resistance against *M. tuberculosis* and *Salmonella enterica* serovar Typhi. Parkin is essential for colocalization of ubiquitin along phagosomes markers within *M. tuberculosis*; murine bone-marrow-derived-macrophages bearing double knockouts for *PARK2* are more susceptible to *M. tuberculosis* or *S. enterica* growth and present a decrease in survival rate after infection (69). Parkin role in the clearance of intracellular bacteria is corroborated by functional assays performed using dendritic cells infected by Chlamydia: autophagosome degradation of chlamydial infections and MHC-I antigen presentation are increased in presence of Parkin (70).

The influence of Parkin in T-cell stimulation has been also demonstrated in the mitochondrial antigen presentation (MitAP) pathway, based on the generation and trafficking of mitochondrial-derived vesicles (MDV) and mediated by Parkin and PINK1. Under stress, Sorting Nexin-9 (Snx9) and the GTPase Rab9 are recruited to mitochondria and triggers MDV formation; Parkin modulates this process by regulating the level of Snx9 in the cytosol in a proteasome-dependent manner, consequently repressing MitAP in antigen-presenting cells and impacting over immune tolerance (71). It is worth to note that MitAP is not mediated by mitophagy; also, Parkin has an effect upon the production of interleukin-6 and monocyte chemoattractant protein-1 (MCP-1/*CCL2*) (72), suggesting an impact of Parkin in multiple pathways related to immunity. Interestingly, this impact seems to be conserved among species since impairment of autophagic activity and lifespan after infection is observed in *Drosophila melanogaster* in the absence of *PARK2* expression (69, 73).

In summary, genetic and functional studies have provided strong evidence of *PARK2* as a key player in the pathogenesis of leprosy and other infectious diseases. However, these exciting findings are not enough to explain the strong genetic effect observed and estimated through CSA and twin studies—causal variants with high penetrance, able to explain the major gene effect, are yet to be evidenced. The strategies presented next might be powerful to contribute to the advance of the complete dissection of the molecular basis of susceptibility to infection.

STRATEGIES AND FUTURE PERSPECTIVES

A main genetic assumption underlying classic genetic epidemiology studies is that common diseases are caused by common variants [i.e., nucleotide changes with Minor Allelic Frequency (MAF) > 1%]. Thus, genetic study designs, including GWAS, have been focusing on identifying these common variants and several have been associated to leprosy, some with consistent replication/validation across populations of distinct genetic backgrounds. This positive scenario led to the expectation that these powerful studies would reveal most—if not all—of the genetic variation impacting on susceptibility to common diseases in general and leprosy in particular. However, the picture that emerges today is distinct: GWAS have been revealing a large number of common variants associated with complex traits with very low odds ratios, and the combined effects explain ~5% or less of genetic variance to a given trait (74). These observations led to the term "missing heritability," which can be at least partially explained by rare variants (MAF < 1%) with larger effects on phenotype or variants other than SNPs, such as copy number variants, both poorly represented in typical genotyping arrays (75). To address this hypothesis, massive deep sequencing technology has been proved to be a powerful tool. In recent years, advances in DNA sequencing chemistry and platforms have allowed an enormous improvement in data generation with a reduced cost (76). Therefore, human whole-genome sequencing (WGS) or whole-exome sequencing (WES) have become feasible and these approaches, especially WES has been proven effective to the identification of genes underlying several Mendelian diseases (77–79). Alternative designs might be used to reduce costs, improve power of detection, and increase individual sample sizes for sequencing; an insightful approach for leprosy might be to submit genes consistently associated with the disease to deep sequencing and search for new/rare variants as causal candidates. Moreover, exons can be preferentially targeted, as variants that cause amino acid change are more likely to have an impact on the phenotypes (80). This rationale was recently used to identify a common and a rare missense functional variant of *LACC1* and *HIF1A*, respectively, as risk factors for leprosy, using WES and targeted second-generation sequencing (22). Another powerful approach has been the use of WGS/WES on the investigation of families or patients displaying extreme or atypical phenotypes; for example, individuals who do not present clinical disease albeit being exposed to an infectious microorganism, as it has been demonstrated for HIV (81, 82). As variants will be likely enriched in such cases, the discovery of causal mutations could be performed in smaller samples size (77).

A natural step further following genetic variant discovery is functional testing. Advances in genome edition technology and cell reprogramming have been allowing isogenic models ideal for functional studies on complex diseases. Such approaches have been proven useful to study neurological diseases such as Huntington's (83), Parkinson's (84), and Alzheimer's disease (85). Genomic variants can be edited by CRISPR/Cas9 in the presence of a donor DNA harboring the nucleotide change; after DNA cleavage by Cas9 nuclease, the homology-directed repair machinery is activated and the donor-DNA is inserted (86), creating a feasible strategy to perform *in vitro* disease modeling with isogenic controls. In a potentially powerful combination, genome editing strategies could be applied to modify induced pluripotent stem cells (iPSC) (87) that could be differentiated into cell types, for example, targets of a specific pathogen. Although, Cas9 edition system might display off-targets, tools to reduce off-targets mutations have been developed, such as the use of Cas9 in a ribonucleoprotein complex (88) and nickases-Cas9 (89), which cleaves a single strand of DNA, thus a complementary pair of anti-sense gRNAs is necessary to induce mutation (90).

CONCLUSION

Genetics studies have significantly contributed to the understanding of the molecular basis of leprosy susceptibility and the pathophysiology of the disease. Interestingly, genome-wide, hypothesis-free studies led to the discovery of unsuspected immune-related genes such as *PARK2* in the past and, more recently, *LACC1* (22, 57, 91). Yet, the impact of rare variants upon disease mechanisms is largely unknown and causal variants that could explain the major gene effects are yet to be described. Advances in genome sequencing technology and functional studies approaches might contribute substantially to further advances in leprosy and other infectious/common diseases.

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GC designed the manuscript and performed major writing. MM contributed to the writing and provided senior supervision.

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