

## Antigen-Specific T-Cell Responses of Leprosy Patients<sup>∇</sup>

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**The identification of human T-cell antigens of *Mycobacterium leprae* could improve treatment and help to disrupt the transmission of leprosy by directing diagnosis and vaccine programs. This study screened a panel of *M. leprae* recombinant proteins for T-cell recall responses, measured by gamma interferon (IFN- $\gamma$ ) production, among leprosy patients. After initial studies using peripheral blood mononuclear cells from leprosy patients, we transitioned our studies to simple whole-blood assays (WBA), which are more applicable in field or clinical settings. T-cell responses generated in WBA using blood from individuals in Goiânia, Brazil, demonstrated that several *M. leprae* antigens (ML0276, ML0840, ML1623, ML2044, and 46f) elicited >0.5 IU/ml IFN- $\gamma$ , and these proteins were classified as immunogenic and leprosy specific. Several of these individual antigens were recognized by cells from >60% of Brazilian paucibacillary (PB) leprosy patients, and ML0276, ML0840, ML1623, and 46f complemented each other such that 82% of PB patients had strong (>1.25 IU/ml IFN- $\gamma$ ) responses to at least one of these proteins. These proteins were also recognized by cells from a significant proportion of the household contacts of multibacillary leprosy patients, but in contrast, few responses were observed in active tuberculosis patients or healthy control groups from areas of endemicity. Our results indicate several potential candidate antigens which may be useful for either leprosy diagnosis or vaccination and demonstrate the utility of leprosy WBA that can be applied broadly in clinical or field settings.**

Leprosy is a chronic disease with a wide range of clinical outcomes. Leprosy patients are predominantly diagnosed by the appearance of disease signs, but they can also be characterized by the physical and histological attributes of skin or nerve lesions or by their immune response to crude *Mycobacterium leprae* antigens (25). Individuals infected with *M. leprae* exhibit wide-ranging immunity. One end of this immunologic spectrum comprises paucibacillary (PB) leprosy patients, who have strong cellular immunity in the form of a Th1-type response characterized by antigen-specific gamma interferon (IFN- $\gamma$ ) secretion. This response controls bacterial growth and limits dissemination, typically resulting in a few small and localized lesions. At the other end of the spectrum, multibacillary (MB) patients have poor cellular immunity but potent humoral immunity. Responses of MB patients do not control bacterial growth, and infection becomes systemic, typically resulting in disseminated lesions and significant nerve function impairment. Identifying antigens that are the target of the cellular immune response could direct the development of a defined leprosy vaccine.

Despite vigorous efforts, conditions that permit the in vitro culture of *M. leprae* bacilli have not yet been identified. This has impaired the ability to produce antigens for research purposes, and until recently, studies of antigen-specific immune responses during the course of the disease have been limited to using crude bacterial extracts or a few single *M. leprae* antigens purified from animal extracts (10, 18, 27). Partial vaccination against *M. leprae* can be achieved with other mycobacteria, but

a defined subunit vaccine for leprosy is lacking (7, 14). Following the recent completion of the *M. leprae* genome and other mycobacterial genomes (5, 12), molecular biology and bioinformatic tools have revealed *M. leprae*-specific antigens that may be used for leprosy diagnosis or vaccination.

The current study was designed to simultaneously explore several recombinant proteins for specific T-cell reactivity among leprosy patients and determine the utility of a simple whole-blood assay (WBA) for screening of leprosy patients to identify antigens with diagnostic or vaccine potential. Our data indicate that several antigens are recognized by PB leprosy patients and that this reactivity can be revealed in a simple 24-h WBA. Determining leprosy-specific targets of the immune response will facilitate diagnosis and vaccine research aimed at controlling leprosy.

### MATERIALS AND METHODS

**Subjects and samples.** For individuals from a region where leprosy is not endemic, leprosy patients and controls (purified protein derivative [PPD]-positive or -negative nonendemic controls [NEC]) were recruited at the Infectious Disease Clinic, Harborview Hospital, Seattle, WA, or the Infectious Disease Research Institute, Seattle, WA, between 2003 and 2007. For individuals from regions where leprosy is endemic, leprosy patients, healthy household contacts of MB patients (HHC), and endemic controls (EC) were recruited at Centro de Referencia em Diagnostico e Terapeutica and Hospital Anuar Auad, Goiânia, Goiás State, Brazil (from March 2006 to September 2007). All Brazilian donors had previously been immunized with *Mycobacterium bovis* BCG. With the exception of patients in Seattle, MB and PB leprosy patient blood used in this study was derived from recently diagnosed and previously untreated individuals. Patients were categorized by clinical, bacilloscopic, and histological observations (bacterial index, skin lesions, nerve involvement, and histopathology) carried out by qualified personnel. PB patients were confirmed as having either true tuberculoid or borderline tuberculoid disease by clinical and histological exams. EC were healthy individuals who had never had tuberculosis (TB), had no history of leprosy in the family, and were living in the area of leprosy endemicity. HHC were defined as adults living in the same house as an MB index case patient for

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TABLE 1. Brazilian study population

Study group (n)	Median bacterial index	No. of males/ no. of females	Mean (range) age (yr)
PB leprosy <sup>a</sup> (22)	0	13/9	36.5 (18–58)
MB leprosy <sup>b</sup> (20)	2.0	10/10	43.6 (27–72)
HHC (22)		11/11	33.6 (20–73)
EC (19)		9/10	38.5 (23–73)
TB patients (19)		10/9	38.8 (19–57)

<sup>a</sup> The PB leprosy group contained 12 true tuberculoid and 10 borderline tuberculoid patients by histopathologic exam.

<sup>b</sup> The MB group contained six lepromatous leprosy, six borderline lepromatous, and eight mid-borderline patients by histopathologic exam.

at least 6 months prior to blood collection. Blood was obtained from TB patients (*Mycobacterium tuberculosis* sputum-positive, human immunodeficiency virus-negative individuals with clinically confirmed pulmonary TB) who were undergoing treatment. All blood samples were obtained after informed consent and after local ethics committee approval in the related country. The composition of the Brazilian study population is summarized in Table 1.

**Cloning and purification of target antigens.** We previously used serologic screening of *M. leprae* genome libraries to identify *M. leprae* proteins that may be recognized by the immune responses of leprosy patients (21). To express potential antigens for further evaluation, DNAs encoding selected *M. leprae* proteins were PCR amplified from *M. leprae* Thai-53 genomic DNA, using *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). PCR primers were designed to incorporate specific restriction enzyme sites 5' and 3' of the gene of interest and excluded in the target gene for directional cloning into the expression vector pET28a (Novagen, Madison, WI). After PCR amplification, purified PCR products were digested with appropriate restriction enzymes and ligated into pET28a. Sequence-verified recombinant plasmids were transformed into the *Escherichia coli* BL21 derivative Rosetta 2(DE3)(pLysS) (Novagen). Recombinant His-tagged protein products were isolated under native (soluble recombinant proteins) or denaturing (8 M urea) conditions, using Ni-nitrilotriacetic acid metal ion affinity chromatography according to the manufacturer's instructions (QiaGen, Valencia, CA). Affinity-purified protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and quantified using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Endotoxin levels were measured by *Limulus* amoebocyte lysate QCL-1000 (Lonza Inc., Basel, Switzerland) assay and were all <100 endotoxin units/mg protein. The characteristics of each *M. leprae* protein evaluated in Brazil are summarized in Table 2.

**Determining patient reactivity by stimulation of PBMC and 24-h WBA.** Peripheral blood mononuclear cell (PBMC) were prepared from undiluted venous heparinized whole blood (Greiner). PBMC were resuspended in RPMI 1640

(Invitrogen, Carlsbad, CA) supplemented with 50,000 U penicillin-streptomycin (BioWhittaker, Walkerville, MD) and 10% heat-inactivated human serum (Valley Biomedical Inc., Winchester, VA), and  $2 \times 10^5$  cells were added to each well of a 96-well plate (200- $\mu$ l total volume/well; Corning Inc., Corning, NY) and stimulated with antigens. After 3 days of incubation at 37°C and 5% CO<sub>2</sub>, ~100  $\mu$ l of supernatant was collected and stored at -20°C until assay by enzyme-linked immunosorbent assay (ELISA). WBA were performed with undiluted venous heparinized whole blood (Greiner). Within 2 h of collection, blood was added to each well of a 24-well plate (450  $\mu$ l/well; Sigma, St. Louis, MO) and incubated with antigens for 24 h at 37°C and 5% CO<sub>2</sub>, and then ~150  $\mu$ l of plasma was collected and stored at -20°C until assay by ELISA. For each assay, stimulation was conducted with 10  $\mu$ g/ml recombinant protein, 10  $\mu$ g/ml *M. leprae* cell sonicate (provided by John Spencer, Colorado State University, Fort Collins, CO), or 1  $\mu$ g/ml phytohemagglutinin (Sigma). Spontaneous IFN- $\gamma$  secretion was observed in WBA for some TB patients, and in those cases, the expression level was subtracted to provide *M. leprae* antigen-induced values.

**Cytokine ELISA.** IFN- $\gamma$  ELISA kits (RSG [eBioscience, San Diego, CA] and Quantiferon CMI [Cellestis, Carnegie, Australia]) were used according to the manufacturers' instructions. The detection limit of the RSG ELISA kit was 20 pg/ml. For data interpretation, we assigned a positive result to concentrations above an arbitrary cutoff point of 500 pg/ml. The detection limit of the Quantiferon CMI test was 0.05 IU/ml. For data interpretation, we assigned a positive result to concentrations above an arbitrary cutoff point of 0.5 IU/ml.

**Statistical analysis.** The Mann-Whitney U test was applied for comparison between two groups. The nonparametric Kruskal-Wallis analysis of variance test was used to compare the IFN- $\gamma$  levels among all of the groups. Results were considered statistically significant when the *P* values were <0.05.

## RESULTS

**Antigen reactivity in PBMC cultures.** We first evaluated *M. leprae* antigens in PBMC stimulation assays with PBMC from blood of leprosy patients who were recruited in Seattle, WA. PBMC were cultured in the presence of antigen for 3 days, and then secreted IFN- $\gamma$  was assayed by ELISA. The antigens tested promoted no or weak IFN- $\gamma$  secretion from negative control cells (from leprosy-negative, PPD-negative individuals), indicating that these antigens do not nonspecifically activate cells and are therefore suitable for use in studies to determine the specificity of leprosy patient responses (Fig. 1A). Cells from MB leprosy patients responded with no or weak IFN- $\gamma$  responses (data not shown). Approximately one-third of the proteins tested, however, induced IFN- $\gamma$  secretion when

TABLE 2. Main characteristics of *M. leprae* antigens tested in Brazil<sup>a</sup>

Gene	Functional classification <sup>b</sup>	Protein type	Size (kDa)	<i>M. tuberculosis</i> homolog	% Identity with other mycobacterial homologs				
					<i>M. tuberculosis</i> homologs	<i>M. bovis</i> BCG homologs	<i>M. avium</i> homologs	<i>M. marinum</i> homologs	<i>M. smegmatis</i> homologs
ML0276	V	Hypothetical protein	16.2	Rv0390	79.0	78	71	70	68
ML0316	I.J.1	Transcriptional regulator	21.0	Rv0067c	68.8	38	31	39	58
ML0398	III.A	D-Ribose-binding protein	36.9	None	0	0	0	0	29
ML0541	I.F.1	Guanylate kinase	22.6	Rv1389	79.1	77	82	83	78
ML0543	I.B.7	Flavoprotein	44.2	Rv1391	87.0	84	83	82	78
ML0840	VI	Hypothetical protein	47.7	None	0	0	62	0	45
ML1011	VI	Hypothetical protein	14.6	None	0	30	31	30	28
ML1213	II.C.4	Conserved membrane protein	80.1	Rv1565c	76.5	25	28	27	54
ML1623	III.B	Hsp90 family protein	73.8	Rv2299c	80.8	77	77	77	0
ML2044	VI	Hypothetical protein	7.9	None	0	29	62	29	37
46f (ML0405-ML0568)		Artificial chimeric fusion, hypothetical protein	46						

<sup>a</sup> Annotations for gene names, functional classification, and proteins are according to the Sanger database.

<sup>b</sup> I.B.7, miscellaneous oxidoreductases and oxygenases; I.F.1, purine ribonucleotide biosynthesis proteins; I.J.1, repressors/activators; II.C.4, conserved membrane proteins; III.A, transport/binding proteins; III.B, chaperones/heat shock proteins; V, conserved hypothetical proteins; VI, unknowns.

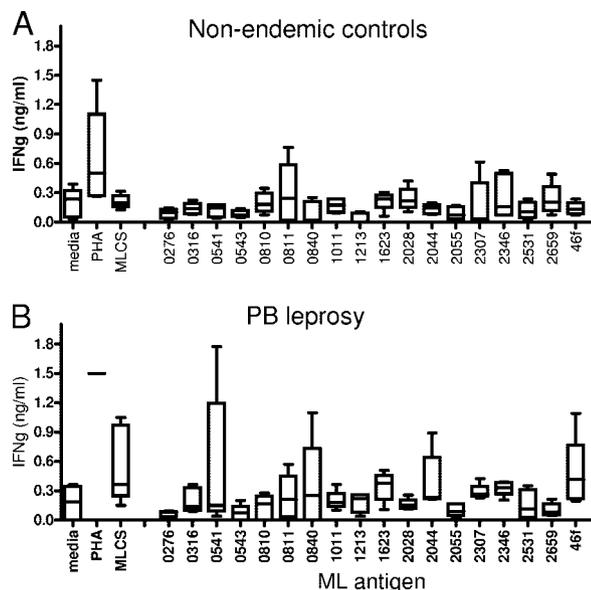


FIG. 1. IFN- $\gamma$  response of PBMC following antigen stimulation. PBMC were stimulated with recombinant antigen for 3 days, culture supernatants were collected, and IFN- $\gamma$  content was assayed by ELISA. PBMC from NEC (Seattle-based) ( $n = 5$ ) (A) and PB leprosy patients ( $n = 5$ ) (B) were stimulated. The response of each individual is shown in each panel, and results are representative of several individuals per category.

they were incubated with cells from PB leprosy patients (Fig. 1B and data not shown). The reactivity of these antigens indicates their potential as components of a defined subunit vaccine or a T-cell-based diagnostic leprosy test.

**Establishing WBA with recombinant antigens.** To establish assays that are simple and more appropriate for field application, we performed WBA. In order to exclude *M. leprae* antigens that stimulate nonspecific or cross-reactive responses in healthy individuals living in nonendemic areas, WBA were performed on 10 Seattle-based volunteers. The stimulants included phytohemagglutinin as a positive control, phosphate-buffered saline as a negative control, and for experimentation, recombinant *M. leprae* proteins and crude mycobacterial con-

trol preparations (*M. leprae* cell sonicate, *M. tuberculosis* lysate, BCG lysate, and PPD). Some of the antigens that stimulated positive responses in PBMC assays also stimulated blood from NEC to secrete IFN- $\gamma$  (Fig. 2), with >30% of NEC tested responding to ML2531. As such, this antigen will not permit leprosy diagnosis and should not be included as a component of a T-cell diagnostic test for leprosy. Other antigens, such as ML0541 and 46f, stimulated responses in some NEC ( $\leq 30\%$ ), so we decided to examine these further in a region where leprosy is endemic. Antigens that stimulated little or no IFN- $\gamma$  production from NEC samples were similarly designated for further evaluation in regions where leprosy is endemic.

**Antigen reactivity in a region of leprosy endemicity (Goiânia, Brazil).** We then tested a panel of *M. leprae* antigens for reactivity from a site with a high incidence of leprosy (Table 2). The study group from a region where leprosy is endemic consisted of newly diagnosed, previously untreated MB and PB leprosy patients in Goiânia, Goiás State, central Brazil, as well as HHC, TB patients under treatment, and healthy EC. Proteins were classified according to the pattern of IFN- $\gamma$  recall responses observed among leprosy patients and controls, as shown in Fig. 3A. Responsiveness was characterized by higher IFN- $\gamma$  production from individuals in the PB leprosy and HHC groups, distinct from the lower responses among the EC, MB leprosy, and TB groups. Among PB leprosy cases, ML0276 (19 of 22 cases), ML0840 (13 of 21 cases), ML1623 (13 of 21 cases), ML2044 (9 of 21 cases), and 46f (8 of 21 cases) induced secretion of >0.5 IU/ml IFN- $\gamma$ . Interestingly, the same antigens, ML0276 (20 of 22 cases), ML0840 (12 of 22 cases), ML1623 (17 of 22 cases), ML2044 (9 of 22 cases), and 46f (16 of 22 cases), induced IFN- $\gamma$  production among HHC.

Among the antigens tested, ML0276 was the most potent recombinant protein, inducing median values of 1.26 IU/ml IFN- $\gamma$  (interquartile range [IQR] = 3.74) among PB leprosy patients and 2.36 IU/ml IFN- $\gamma$  (IQR = 4.83) among HHC. ML0276-induced responses in the PB leprosy and HHC groups demonstrated a similar distribution ( $P$  value = 0.83). ML0276 provided the best discrimination between the PB leprosy and HHC groups compared to the other groups, with no response detected in TB patients or EC (both  $P$  values were <0.0001 compared to PB leprosy group). The ML0840 protein also provided good re-

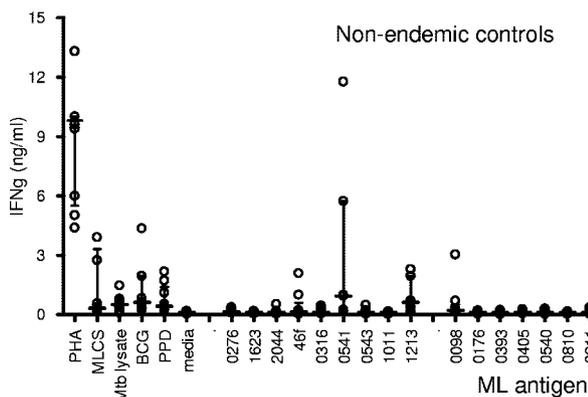


FIG. 2. WBA responses of NEC to *M. leprae* antigens. Whole blood from 10 Seattle-based healthy individuals was cultured for 24 h in the presence of stimulant, and IFN- $\gamma$  content in the plasma was measured by ELISA. Data are presented as individual data points (open circles), with the lines indicating the median and IQR.

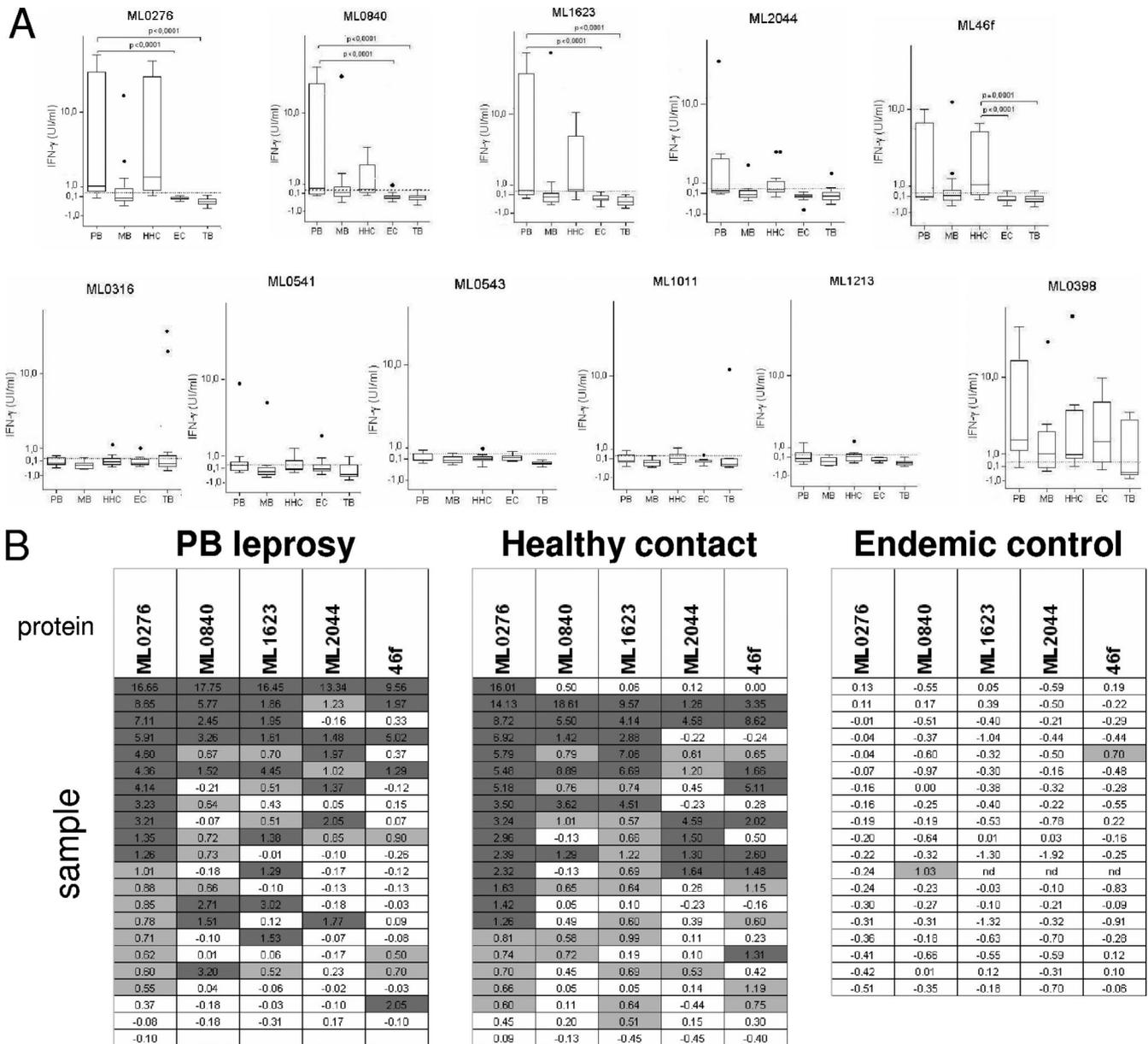


FIG. 3. IFN- $\gamma$  production in WBA with Brazilian study groups. Whole blood from PB leprosy patients, MB leprosy patients, HHC, TB patients, and EC was cultured for 24 h in the presence of antigen, and IFN- $\gamma$  content in the plasma was measured by ELISA. (A) Data are presented as box plot distributions indicating the medians and IQRs of IFN- $\gamma$  values (IU/ml). (B) IFN- $\gamma$  concentrations are depicted by dark shading (>1.25 IU/ml), light shading (>0.5 IU/ml), and no shading (<0.5 IU/ml). nd, not determined.

sponses in the test groups, although compared to those with ML0276, responses were weaker. The median IFN- $\gamma$  responses following ML0840 stimulation were 0.69 IU/ml for PB leprosy patients, 0.61 IU/ml for HHC, and 0.15 IU/ml for MB leprosy patients. Only one EC and one TB patient responded to ML0840. ML1623 provided a similar pattern of responses, inducing IFN- $\gamma$  secretion among the PB leprosy and HHC groups (0.61 IU/ml and 0.68 IU/ml, respectively). TB patients and EC did not respond to ML1623. Following stimulation by each of the individual antigens ML0276, ML0840, and ML1623, statistically significant differences between the five donor groups were observed (all Kruskal-Wallis *P* values were <0.0001).

There was an indication that other proteins could also discriminate PB leprosy patients and HHC from the other groups, albeit with weaker positive responses that resulted in median IFN- $\gamma$  concentrations below our arbitrary cutoff of 0.5 IU/ml. Following ML2044 stimulation, the median IFN- $\gamma$  response of PB patients was 0.20 IU/ml, and that of HHC was 0.33 IU/ml, whereas the median responses of the MB leprosy, TB, and EC groups were all below the limit of detection. The chimeric fusion protein 46f (comprising ML0405 and ML0568) stimulated median IFN- $\gamma$  responses of 0.12 IU/ml for PB leprosy patients, 0.82 IU/ml for HHC, and 0.20 IU/ml for MB leprosy patients, while TB and EC results were below the limit of

detection. Despite the weak responses, statistically significant differences were again observed among the five groups tested with these proteins (Kruskal-Wallis  $P$  values of  $<0.0001$ ).

When data from individuals were analyzed, we found that the ML0276, ML0840, ML1623, and 46f antigens complemented each other in combination and were capable of identifying 17 of 21 cases with IFN- $\gamma$  responses of  $>1.25$  IU/ml, well above our arbitrary cutoff for positive responses. In contrast, for the HHC group, ML0276 alone provided responses of  $>1.25$  IU/ml IFN- $\gamma$  for 15 of 22 individuals tested (Fig. 3B), and only 46f complemented ML0276 to add another individual with a strong response.

## DISCUSSION

Leprosy research has been constrained by the relative lack of tools with which to analyze the ongoing immune response. This deficiency has also impacted the clinical situation. Leprosy is diagnosed predominantly based on the appearance of clinical signs (dermal and neurological lesions), with patients classified as having PB or MB leprosy. These clinical signs may be scarce in early disease, leading to delayed diagnosis, underdiagnosis, or misdiagnosis. While MB leprosy patients can readily be diagnosed by serological antibody tests, diagnosis of PB leprosy patients may require T-cell-based assays (4, 9, 19, 22). In addition to providing data for a T-cell-based diagnostic test, the identification of antigens that are the targets of the cellular response of PB leprosy patients, who control bacterial growth, is likely to direct leprosy vaccine research. In this study, we examined the antigenicity of numerous *M. leprae* proteins to simultaneously define immune reactivity of leprosy patients with recombinant proteins and determine the utility of a simple WBA for antigen screening of leprosy patients.

In response to the immunogenic proteins, we observed more production of IFN- $\gamma$  in the PB leprosy and HHC groups. These values are distinct from the lower responses among MB leprosy patients, and these reactivity patterns are similar to those reported in other studies (1, 2, 11, 13, 26, 29). The determination of specificity in regions of leprosy endemicity may be complicated by high exposure rates to *M. leprae*, *M. tuberculosis*, and other nonpathogenic environmental mycobacteria, in addition to the routine BCG vaccination of children. In the region of leprosy endemicity examined in this study (Goiania, Brazil), some level of cross-reactivity was anticipated due to multiple exposures to mycobacteria among the study groups. We did not, however, observe responses in the EC or TB group, indicating that positive responses were related to leprosy exposure.

Interestingly, the extent of homology identified by in silico analyses against other mycobacterial genomes/proteins did not indicate the specificity of PB leprosy patient responses in a region where leprosy is highly endemic. For example, ML0276, despite having 79% identity with its *M. tuberculosis* homolog, yielded responses only in leprosy groups. In contrast, ML0398, which has no homolog in *M. tuberculosis*, induced strong IFN- $\gamma$  responses in all of the groups tested in Brazil, including TB patients. Additionally, the sequence of ML0840 is 59% identical to that of *Mycobacterium paratuberculosis* MAP2122, and stimulation with a peptide representing a predicted epitope within the ML0840 protein has

also been shown to elicit IFN- $\gamma$  production from cells of leprosy patients (26). Similar to the results we present here, the whole ML0840 protein has been shown to stimulate IFN- $\gamma$  responses in Rio de Janeiro-based leprosy patients and HHC but not EC (11; A. Geluk, personal communication). Antigen expression levels and availability may determine the immunodominant antigens of each mycobacterial infection, such that differing responses to similar proteins may distinguish each disease. Whatever the reason, our results suggest that caution should be exercised in using in silico predictions regarding disease-specific reactivity.

While we did not limit our study to *M. leprae* unique antigens, our study was limited to the examination of leprosy patient responses in the context of IFN- $\gamma$  secretion. This approach is consistent with that used for the development of T-cell-based diagnostic TB assays (20). Several studies, however, have demonstrated differences in circulating cytokine levels, such as those of interleukin-6 and tumor necrosis factor alpha, in leprosy patients undergoing reversal reactions, albeit in the absence of ex vivo antigen stimulation (15–17, 24). It is possible that other T-cell-derived cytokines, or even molecules stimulated by IFN- $\gamma$ , may provide either a more robust response for the diagnosis of leprosy patients or greater differences between the study groups.

At the population level, it is possible that diverse ethnic and genetic backgrounds, represented by different major histocompatibility complex haplotypes, contribute to the variability of responses. In partial agreement with the data generated in Brazil, however, testing conducted in the Philippines indicated that ML0276 was recognized by some, but not all, PB leprosy patients (unpublished observations). T-cell-mediated immunity (as assessed by IFN- $\gamma$  production) to some of the recombinant *M. leprae* antigens tested showed discriminatory recognition of the PB leprosy and HHC groups from the EC, MB leprosy, and TB groups in both regions, further indicating their potential as candidates for leprosy diagnosis. It is possible that multiple antigens will have to be used to provide diagnosis in a single test format across all regions where leprosy is endemic, and we are currently initiating studies to address this possibility.

Attempts to produce leprosy vaccines have been limited to the use of proteins extracted from various mycobacteria in the laboratory setting and, until relatively recently, to the use of whole bacteria in the field setting (7, 14, 18). It has been argued, paradoxically, that MB leprosy contacts are both at an increased risk of developing leprosy and protected from leprosy. HHC are at an increased risk of developing leprosy and may represent early infectious cases before or without the signs/symptoms of leprosy (3, 28). Alternatively, responses in HHC may protect these individuals by preventing infection from becoming established. For the immunogenic proteins, the absence of a response among TB patients and EC indicates specific antigen recognition by leprosy-affected individuals, and the results are compatible with exposure to *M. leprae* for prolonged periods. The identification of molecular indicators of bacterial viability would help to address if the antigen-specific cellular response of HHC is due to subclinical infection or merely to continued stimulation by bacteria expelled by the contact MB leprosy case. Long-term follow-up of individuals to determine whether T-cell responses can predict the onset of leprosy symptoms would also be beneficial. The cellular im-

mune response of PB leprosy patients inhibits bacterial growth and dissemination and, as such, is likely to provide significant insight into the important antigenic targets that determine protection. Intriguingly, in contrast with the antigen-specific responses observed in PB leprosy patients, there was essentially no complementation of responses in HHC. Antigens that yield responses in both PB leprosy patients and HHC represent candidates that might be incorporated into a defined subunit vaccine for leprosy. The identification of high IFN- $\gamma$  responses against 46f in HHC, which were even higher than those attained with PB leprosy patients, suggests that vaccination studies with this protein are warranted.

Simple and field-friendly T-cell-based assays have recently been introduced for the diagnosis of TB, and such tests could greatly facilitate leprosy research and diagnosis (6, 8, 20, 23). A leprosy diagnostic application could be developed based on comparative T-cell reactivities among leprosy patients and controls. Thirty-six percent (5 of 14 proteins) of the *M. leprae* proteins we tested in Brazil demonstrated T-cell immunogenicity, defined by an IFN- $\gamma$  response, in a simple 24-h WBA. These antigens showed discriminatory recognition patterns that indicate their potential as candidate molecules for PB leprosy diagnosis. IFN- $\gamma$  recall responses identified five immunogenic *M. leprae* proteins that have diagnostic potential for PB leprosy (ML0276, ML0840, ML1623, ML2044, and 46f). The results obtained by ML0276, ML0840, ML1623, and 46f stimulation complemented each other to provide 82% strong diagnosis (>1.25 IU/ml IFN- $\gamma$ ) of PB leprosy patients in Goiânia, Brazil. The availability of a simple, affordable, and field- and user-friendly diagnostic test for PB leprosy would significantly impact leprosy control programs in countries where this disease is endemic.

In summary, our results identified several antigens that are recognized by PB leprosy patients. Our results also demonstrate that 24-h WBA are user friendly and have good potential for the expanded screening of *M. leprae* antigens to assess their potential as either diagnostic indicators or vaccine candidates.

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