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New Biomarkers with Relevance to Leprosy Diagnosis Applicable in Areas Hyperendemic for Leprosy


Leprosy is not eradicable with currently available diagnostics or interventions, as evidenced by its stable incidence. Early diagnosis of Mycobacterium leprae infection should therefore be emphasized in leprosy research. It remains challenging to develop tests based on immunological biomarkers that distinguish individuals controlling bacterial replication from those developing disease. To identify biomarkers for field-applicable diagnostics, we determined cytokines/chemokines induced by M. leprae proteins in blood of leprosy patients and endemic controls (EC) from high leprosy-prevalence areas (Bangladesh, Brazil, Ethiopia) and from South Korea, where leprosy is not endemic anymore. M. leprae-sonicate–induced IFN-γ was similar for all groups, excluding M. leprae/IFN-γ as a diagnostic readout. By contrast, ML2478 and ML0840 induced high IFN-γ concentrations in Bangladeshi EC, which were completely absent for South Korean controls. Importantly, ML2478/IFN-γ could indicate distinct degrees of M. leprae exposure, and thereby the risk of infection and transmission, in different parts of Brazilian and Ethiopian cities. Notwithstanding these discriminatory responses, M. leprae proteins did not distinguish patients from EC in one leprosy-endemic area based on IFN-γ. Analyses of additional cytokines/chemokines showed that M. leprae and ML2478 induced significantly higher concentrations of MCP-1, MIP-1β, and IL-1β in patients compared with EC, whereas IFN-inducible protein-10, like IFN-γ, differed between EC from areas with dissimilar leprosy prevalence. This study identifies M. leprae-unique Ags, particularly ML2478, as biomarker tools to measure M. leprae exposure using IFN-γ or IFN-inducible protein-10, and also shows that MCP-1, MIP-1β, and IL-1β can potentially distinguish pathogenic immune responses from those induced during asymptomatic exposure to M. leprae.

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of nerve damage, disability, and deformity, the hallmarks of leprosy. To design new diagnostic tests for early diagnosis, various studies have focused on identifying genes encoding *M. leprae*-unique Ags since the availability of the *M. leprae* genome sequence about one decade ago (6). Subsequently, these (hypothetical) Ags were used as recombinant proteins or synthetic peptides in vitro T cell stimulation assays, mostly assessing IFN-γ production (7–12). Although it is not an immunological correlate of protection, the number of IFN-γ–releasing Ag-specific T cells and the amount of total IFN-γ released remain widely used as surrogate markers for the proinflammatory immune response against *M. leprae* and *M. tuberculosis* (13). A pitfall of the use of IFN-γ for leprosy diagnosis in a leprosy-endemic area, however, is that not only infected individuals, but also individuals with adequate immunity against *M. leprae* produce substantial concentrations of IFN-γ in response to *M. leprae* Ags.

In a previous study, we tested recombinant proteins that had been selected based on their unique sequence in *M. leprae* (10). Notwithstanding this selection, IFN-γ production by endemic control (EC)-derived PBMC or whole blood was observed in response to most of these *M. leprae* proteins. Because these EC were living in areas with pockets of high leprosy prevalence (e.g., Dhaka and Karachi) and also responded to *M. leprae* whole-cell sonicate (WCS) in vitro, the observed cellular responses toward the *M. leprae*-unique proteins may still have indicated *M. leprae* specificity. The inclusion in the current study of groups of individuals with distinct degrees of exposure to *M. leprae* allowed us to investigate whether and to what extent the level of leprosy endemcity in a certain community influences the cellular immunity to *M. leprae*-unique Ags.

Because host immunity and immunopathogenicity in response to *M. leprae* involve complex interactions between a variety of cells expressing different effector and regulatory molecules, assessment of multiple rather than single biomarkers may be more representative of the immune status of the host and may identify patterns predisposing to leprosy. Therefore, in this study, we have analyzed the concentrations of multiple cytokines, besides IFN-γ, after 24-h whole-blood stimulation with 17 *M. leprae* Ags in various cohorts from leprosy-endemic areas in Bangladesh, Brazil, and Ethiopia. To our knowledge, this study describes the first identification of cellular host biomarkers, other than IFN-γ, that differ between leprosy patients and EC in one endemic area, and thus could have value for early diagnosing leprosy and monitoring the response to multidrug therapy.

**Materials and Methods**

**General procedure of the study**

Patients and controls were recruited at International Center for Diarrheal Disease Research Bangladesh (Dhaka, Bangladesh), Yonsei University (Seoul, South Korea), Fiocruz Fortaleza (Brazil), and the Armauer Hansen Research Institute (Addis Ababa, Ethiopia) (Table I). To ensure reproducibility of data throughout the study at each site, all experiments carried out by the laboratories involved were performed according to standard operating procedures, and each site was provided with identical reagents. Multiplex analyses were performed in one laboratory.

**Recombinant proteins**

*M. leprae* candidate genes were amplified by PCR from genomic DNA of *M. leprae* and cloned using the Gateway technology platform (Invitrogen, Carlsbad, CA) with pDEST17 expression vector containing an N-terminal histidine tag (Invitrogen) (14). Sequencing was performed on selected clones to confirm identity of all cloned DNA fragments. Recombinant proteins were overexpressed in *Escherichia coli* BL21(DE3) and purified, as described, to remove any traces of endotoxin (14). Each purified recombinant protein was analyzed by 12% SDS-PAGE, followed by Coomassie Brilliant Blue staining and Western blotting with an anti-His Ab (Invitrogen) to confirm size and purity. Endotoxin contents were <50 endotoxin unit per mg of recombinant protein, as tested using a *Limulus* amebocyte lysate QCL-1000 assay (Lonza, Basel, Switzerland). Recombinant proteins tested in this study (n = 17) included the following: ML0009, ML0091, ML0755, ML0811, ML0840, ML0953, ML0957, ML1601, ML1976, ML2044, ML2055, ML2307, ML2313, ML2478, ML2531, ML2532, and ML2666. ML0091, ML0811, ML2044, and ML2055 were provided by M. Duthie (Seattle, WA).

Recombinant proteins were tested to exclude protein-nonspecific T cell stimulation and cellular toxicity in IFN-γ release assays using PBMC of in vitro purified protein derivative of *M. tuberculosis* (PPD)-negative, healthy Dutch donors recruited at the Blood Bank Sanquin (Leiden, The Netherlands). None of these controls had experienced any known prior contact with leprosy or TB patients.

**M. leprae** WCS

Irradiated armadillo-derived *M. leprae* whole cells were probe sonicated with a Sanyo sonicator to >95% breakage. This material was provided through the National Institutes of Health/National Institute of Allergy and Infectious Diseases Leprosy Research Support Contract N01 AI-25469 from Colorado State University (now available through the Biodefense and Emerging Infections Research Resources Repository listed at http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx).

**Study participants**

The following HIV-negative individuals were recruited between August 2008 and February 2011: in Bangladesh (prevalence = 2.45/10,000), 10 tuberculoid leprosy/borderline tuberculoid leprosy (TT/BT) leprosy patients (Leprosy Control Institute and Hospital, Dhaka), 10 healthy household contacts of borderline lepromatous leprosy/lepromatous leprosy patients (HHC), and 10 healthy individuals from the same endemic area (EC); in South Korea (prevalence <1/10,000), 10 smear-positive, pulmonary TB patients and 10 healthy controls (EC); in Brazil, 10 TT/BT leprosy patients, 10 HHC, 10 EC living in an area of Fortaleza with low prevalence (Mericles; prevalence <0.2/10,000; EC10), and 10 healthy controls living in an area of Fortaleza with high prevalence (Bom Jardin; prevalence ≥4/10,000; EC10-high). In Ethiopia, 35 healthy controls were tested, 18 EC-high were derived from a subcity of Addis Ababa (Kolfe Keranio) with a prevalence rate of 1.5 per 10,000 (72 in 465,811), whereas 17 EC-low, were derived from areas with a prevalence rate of 0.36 per 10,000 (10 in 273,310). Leprosy endemcity for each Ethiopian EC was based on the number of new cases and leprosy prevalence in nearby health centers per area.

Leprosy was diagnosed based on clinical, bacteriological, and histological observations and classified by a skin biopsy evaluated according to the Ridley and Jopling classification (15) by qualified personnel. Patients were treated with chemotherapy for <3 mo, with no signs of leprosy reactions. HHC were defined as adults living in the same house as a borderline lepromatous leprosy/lepromatous leprosy index patient for at least the preceding 6 mo. TB patients were diagnosed based on a positive culture of *M. tuberculosis* in sputum; were recruited at the outpatient clinic of the Pulmonary Division, Severans Hospital, Yonsei University Health System; and had been on chemotherapy for at least 3 mo to enable recovery of T cell function. EC were assessed for the absence of signs and symptoms of TB and leprosy. Staff members working in the leprosy centers or TB clinics were excluded as EC. Ethical approval of the study protocol was obtained through the appropriate local and national or institutional ethics committees, namely, in Bangladesh, Ethical Review Committee of International Center for Diarrheal Disease Research Bangladesh; in South Korea, Institutional Review Board for the Protection of Human Subjects at Yonsei University Health System; in Brazil, Brazilian National Council of Ethics in Research; and in Ethiopia, National Health Research Ethical Review Committee. Informed consent was obtained from all individuals before venepuncture.

**Whole-blood assays**

Within 3 h of collection, venous heparinized blood (450 µl/well) was incubated in 48-well plates at 37°C at 5% CO2, 90% relative humidity, with 50 µl Ag solution (100 µg/ml). After 24 h, 150 µl supernatants were removed from each well and frozen in aliquots at −20°C until further analysis.

**Lymphocyte stimulation tests**

PBMC were isolated from Ficoll density centrifugation from venous, heparinized blood and plated in triplicate cultures (2 × 105 cells/well) in 96-well round-bottom plates (Costar, Cambridge, MA) in 200 µl/well serum-free adoptive immunotherapy medium (AIM-V; Invitrogen). Recombinant protein, *M. leprae* WCS, or PPD (Statens Serum Institut, Copenhagen, Denmark) was added at final concentrations of 10 µg/ml. As a positive control, 1 µg/ml PHA (Remel, Oioxid, Haarlem, The Netherlands) was used. After 6 d of culture at 37°C at 5% CO2, 90% relative humidity, 75 µl supernatant was removed from each well, triplicates were pooled, and frozen in aliquots at −20°C until further analysis.

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The concentrations of 19 analytes (IL-1β, Multicytokine and multichemokine assay, Recombinant protein ML2028 (Serum Ab ELISA, buffycot (Sanquin, Leiden, The Netherlands) was provided to all laboratories as a reference positive control supernatant. Lyophilized supernatant of PHA cultures of PBMC from an anonymous individual (South Korea) was used to determine the cutoff value to define positive IFN-γ (Serum Ab ELISA, buffycot, PHA, IFN-γ) activity was considered to be 3 times the background OD average for the non-reactive control sera (Table I). Participating study sites and study groups

<table>
<thead>
<tr>
<th>Site</th>
<th>Prevalence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Category</th>
<th>Bi&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sex Ratio (M/F)</th>
<th>Mean Age (y)</th>
<th>Age Range (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh (Dhaka)</td>
<td>0.28</td>
<td>TT/BT</td>
<td>0</td>
<td>7/3</td>
<td>38.5</td>
<td>22–65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HHC</td>
<td>NA</td>
<td>6/4</td>
<td>35.7</td>
<td>20–70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC</td>
<td>NA</td>
<td>7/3</td>
<td>28.1</td>
<td>24–35</td>
</tr>
<tr>
<td>South Korea (Seoul)</td>
<td>&lt;0.1</td>
<td>EC</td>
<td>NA</td>
<td>9/1</td>
<td>23</td>
<td>21–25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC</td>
<td>NA</td>
<td>4/6</td>
<td>51.2</td>
<td>24–77</td>
</tr>
<tr>
<td>Ethiopia (Addis Ababa)</td>
<td>0.36</td>
<td>EC&lt;sub&gt;low&lt;/sub&gt;</td>
<td>NA</td>
<td>5/13</td>
<td>27.6</td>
<td>18–40</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>EC&lt;sub&gt;high&lt;/sub&gt;</td>
<td>NA</td>
<td>8/9</td>
<td>33.6</td>
<td>18–38</td>
</tr>
<tr>
<td>Brazil (Fortaleza)</td>
<td>&lt;0.2</td>
<td>EC&lt;sub&gt;low&lt;/sub&gt;</td>
<td>NA</td>
<td>5/5</td>
<td>34.7</td>
<td>22–60</td>
</tr>
<tr>
<td></td>
<td>&gt;4</td>
<td>EC&lt;sub&gt;high&lt;/sub&gt;</td>
<td>NA</td>
<td>5/5</td>
<td>36.6</td>
<td>18–58</td>
</tr>
</tbody>
</table>

<sup>a</sup>Prevalence per 10,000 individuals at the end of 2010.  
<sup>b</sup>Bi, bacterial index (mean).  
NA, Not applicable.

**IFN-γ ELISA**

IFN-γ concentrations were determined by ELISA (U-Cyt, U-Tech, U-Tech, The Netherlands) as described (16). The cutoff value to define positive responses was set beforehand at 100 pg/ml. The assay sensitivity level was 40 pg/ml. Values for unstimulated cell cultures were typically <20 pg/ml. Lyophilized supernatant of PHA cultures of PBMC from an anonymous buffycot (Sanquin, Leiden, The Netherlands) was provided to all laboratories as a reference positive control supernatant.

**Serum Ab ELISA**

Recombinant protein ML2028 (*M. leprae* Ag85B), a synthetic analog of the *M. leprae*-specific phenolic glycolipid-I (PGL-I; ND-O-BSA), and *M. leprae* lipoparabinomannan were coated onto high-affinity polystyrene Immulon IV 96-well ELISA plates (Dynex Technologies, Chantilly, VA) using 50 ng/well in 100 μl M carbonate buffer (pH 9.0) at 4°C overnight. Unbound Ag was washed away using PBS (pH 7.4) containing 1% BSA and 0.05% Tween 80 (blocking buffer) six times. A 1:200 dilution of serum diluted in 100 μl blocking buffer was added to the wells and incubated for 2 h at room temperature. After incubating with the primary Ab, the wells were washed six times with PBS with 0.05% Tween 80 (wash buffer), followed by the addition of 100 μl 1:5000 dilution of the secondary anti-human polyvalent Ab (Sigma-Aldrich) for 2 h. Following washing the wells with PBS six times, 100 μl p-nitrophenylphosphate substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added. The absorbance at 405 nm was read using a VersaMax Pro plate reader ( Molecular Devices, Sunnyvale, CA) at 15 min. The cutoff for positivity was considered to be 3 times the background OD average for the non-endemic control sera (n = 23) determined by binding BSA with a 1:200 serum dilution (cutoff 0.411).

**Multicytokine and multichemokine assay**

The concentrations of 19 analytes (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IFN-γ, IFN-γ-induced protein 10 [IP-10; CXCL10], G-CSF, GM-CSF, MCP-1 [CCL2], monokine induced by IFN-γ [CXCL9], MIP-1β [CCL4], and TNF) in supernatants from 24-h whole-blood cultures (WBC) were measured using the Bio-Plex suspension array system powered by Luminex xMap multiplex technology (Bio-Rad Laboratories, Veenendaal, The Netherlands) as described (17). After prewetting the filter with assay solution, the magnetic beads were washed twice with washing solution using 96-well multiscreen filter plates (Millipore), an Aurum vacuum manifold, and a vacuum pump (Bio-Rad Laboratories). Supernatant samples (50 μl) were added to the plates, and the plates were incubated for 45 min at room temperature in the dark at 300 rpm on a plate shaker. After three washing steps, 12.5 μl detection Ab mixture was added per well, and plates were incubated at room temperature in the dark for 30 min on a plate shaker. After three washes, 25 μl streptavidin-PE solution was added per well and incubated for 10 min. After three washes, 80 μl assay buffer 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 was 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 assigned the values of the limits of quantification of the cytokine or chemokine.

**Statistical analysis**

Differences in cytokine concentrations between test groups were analyzed with the two-tailed Mann-Whitney U test for nonparametric distribution using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Values of p were corrected for multiple comparisons. The statistical significance level used was p < 0.05.

**Results**

*IFN-γ responses to M. leprae* Ags in WBA in Bangladesh and South Korea

In a previous study, IFN-γ production by T cells from EC was observed in response to *M. leprae*-unique proteins (10). However, because these EC were derived from areas with high leprosy prevalence and also responded to *M. leprae* WBS in vitro, the observed cellular responses toward the *M. leprae*-unique proteins could still indicate *M. leprae* specificity. To investigate this, 17 *M. leprae* Ags were tested in an area highly endemic for leprosy (Dhaka, Bangladesh) and an area with low prevalence (South Korea) by analysis of IFN-γ production after 24-h incubation of whole-blood cultures stimulated with recombinant proteins in 10 TT/BT leprosy patients, 10 EC, and 10 HHC from Bangladesh, and the same numbers of EC and TB patients from South Korea. To ensure reproducibility, exactly the same batches of control Ags, recombinant *M. leprae* proteins, and ELISA kits were provided to both sites. ML0755, ML0091, ML0811, ML0953, ML2044, ML2055, ML2307, ML2313, and ML2666 were only tested in the Bangladeshi groups, in which they showed low responses, in tuberculosis patients, and/or in HHC (Supplemental Fig. 1A), and were therefore not investigated in other cohorts.

IFN-γ responses for the negative and positive controls (medium and PHA) were similar in individuals from both areas, indicating that the blood samples used for all five groups were equally able to produce IFN-γ (Fig. 1). *M. leprae* induced some variability in IFN-γ between the two EC groups. Nevertheless, median values were comparable for all groups, thereby excluding the use of IFN-γ responses to *M. leprae* WBS as a discriminatory readout. Importantly, significant differences in IFN-γ concentrations between exposed individuals versus individuals living in a population where they are less likely to be exposed were induced by ML0840 and ML2478 (both p < 0.0001): all Bangladeshi EC and none of the EC from South Korea recognized these proteins (Fig. 1). ML1601 was significantly better recognized in the EC group in Bangladesh (p = 0.0005), whereas 9 of 10 TB patients from South Korea also recognized this protein, which has an ortholog in *Mycobacterium avium paratuberculosis* (18). ML0009, ML0957, ML1976, and ML2531 did not show significant differences, although ML0009 (p = 0.0686) and ML2531 (p = 0.0342) showed...
a tendency toward higher responses in EC from Bangladesh (Supplemental Fig. 1B). Thus, IFN-γ responses in 24-h WBA using M. leprae-specific recombinant proteins ML2478 and ML0840, but not M. leprae WCS, correlate with differences in M. leprae exposure likelihood as estimated from EC living in high versus low leprosy prevalence areas.

Next, sera from these individuals were analyzed for the presence of Abs to the M. leprae homolog of Ag85B (ML2028), a synthetic analog of the M. leprae-specific PGL-I (ND-O-BSA), and M. leprae lipoarabinomannan (19). In contrast to the discriminatory IFN-γ patterns induced in 24-h WBA of EC (South Korea) versus EC (Bangladesh) (prevalence = 2.45/10,000), or healthy controls (EC; n = 10) and tuberculosis patients (TB; n = 10) from South Korea (prevalence <1/10,000). For each group, the number of IFN-γ responders (>100 pg/ml) versus the total number of individuals in the group is indicated below the x-axis. Background values were <50 pg/ml. Median values for each group are indicated by horizontal lines. Significant differences between test groups are indicated by p values.

**FIGURE 1.** IFN-γ responses in WBA from individuals in Bangladesh and South Korea. IFN-γ production in response to control stimuli (medium, PHA, and M. leprae WCS) or to recombinant proteins (ML0840, ML1601, and ML2478) in 24-h WBA of leprosy patients (TT/BT; n = 10), HHC (n = 10), and EC (n = 10) from Bangladesh (prevalence = 2.45/10,000), or healthy controls (EC; n = 10) and tuberculosis patients (TB; n = 10) from South Korea (prevalence <1/10,000). For each group, the number of IFN-γ responders (>100 pg/ml) versus the total number of individuals in the group is indicated below the x-axis. Background values were <50 pg/ml. Median values for each group are indicated by horizontal lines. Significant differences between test groups are indicated by p values.

To expand these findings using healthy controls from an area with low numbers of new leprosy cases and a group from an area with much higher leprosy endemicity (EC<sub>low</sub> versus EC<sub>high</sub>), we investigated reactivity to the above M. leprae Ags in EC in Fortaleza (Brazil), where pockets in the city have a prevalence of <0.2 per 10,000 (EC<sub>low</sub>) and another area with a leprosy prevalence of >4 per 10,000 (EC<sub>high</sub>). In addition, HHC and TT/BT patients from Fortaleza were included (Fig. 2). Because comparison of WBA and lymphocyte stimulation tests showed similar IFN-γ responses (Supplemental Fig. 3A), 6-d lymphocyte stimulation tests with PBMC were used as a test format in this part of the study to allow testing of more Ags.

Whereas PBMC of all groups were equally capable of producing IFN-γ after 6 d, as indicated by the response to PHA (Fig. 2A), ML2478 (p = 0.0029) again showed significantly higher induction of IFN-γ responses in PBMC from TT/BT patients, HHC, and, importantly, EC<sub>high</sub> as compared with PBMC from the EC<sub>low</sub> group from the same city. Thus, ML2478 (p = 0.0021), but not M. leprae WCS (p = 0.104), is useful to estimate differences in M. leprae exposure between EC defined by whether they reside in high versus low prevalence areas, even within the same city.
Based on the data obtained in Bangladesh, South Korea, and Brazil, we next included an African setting by studying the response induced by selected M. leprae Ags in EC from Ethiopia. Eighteen EC<sub>high</sub> were derived from a subcity of Addis Ababa (Kolfe Keranio) with a prevalence rate of 1.5 per 10,000, whereas 17 EC<sub>low</sub> were derived from areas in Addis Ababa with a prevalence rate of 0.36 per 10,000. All individuals responded equally well to the positive control stimulus PHA (Fig. 3A), but responses to M. leprae WCS differed between the two EC groups (Fig. 3B). Importantly, ML2478 again induced much higher concentrations (\(p = 0.0001\)) of IFN-\(\gamma\) in the WBA of Ethiopian EC<sub>high</sub> compared with Ethiopian EC<sub>low</sub> (Fig. 3C; \(p = 0.0001\)). In contrast to responses observed for EC from Bangladesh, ML0840 induced low responses in all Ethiopian EC (data not shown) and was not discriminatory with respect to M. leprae exposure. Thus, ML2478 combined with IFN-\(\gamma\) as a readout can also be used in 24-h WBA to estimate differences in M. leprae exposure be EC in areas with different leprosy prevalence even when located in one city.

**Multiplex analysis of cytokines and chemokines in response to M. leprae Ags in WBA in Bangladesh, South Korea, and Ethiopia**

In our previous study (10), only IFN-\(\gamma\) was determined after stimulation of whole blood or PBMC. Recent studies on TB show that other (combinations of) cytokines are likely to be suitable for application in diagnostic assays (13, 20, 21). Because IFN-\(\gamma\) production induced by recombinant proteins was found in the current study not to be significantly different between the three different groups in Bangladesh (TT/BT, HHC, and EC), IFN-\(\gamma\) cannot be used as a single biomarker to discriminate between leprosy patients (TT/BT) and those merely exposed to M. leprae (EC). Therefore, 18 additional cytokines and chemokines were tested using aliquots of WBA supernatants (described in Fig. 1). In striking contrast to IFN-\(\gamma\), the concentrations of IL-1\(\beta\), MIP-1\(\beta\) (or CCL4), and MCP-1 (or CCL2) were significantly enhanced in TT/BT patients after stimulation with M. leprae WCS compared with Bangladeshi EC (\(p = 0.0006\), \(p = 0.0007\), and \(p = 0.0021\) respectively; Fig. 4A–C).

When cumulative values were considered (Fig. 4D), even higher degrees of significance were observed between EC and TT/BT groups in Bangladesh (\(p < 0.0001\)), as well as between EC and TB groups in South Korea (\(p = 0.0032\)). Thus, in contrast to IFN-\(\gamma\), the levels of MCP-1, MIP-1\(\beta\), and IL-1\(\beta\) induced in leprosy patients as well as TB patients are increased compared with EC from the same areas, potentially reflecting immune responses associated with mycobacterial infection.

To further analyze the potential of MCP-1, MIP-1\(\beta\), and IL-1\(\beta\) as biomarker tools for leprosy diagnostics, receiver operating characteristics were analyzed (Table II), showing areas under the curve (AUC) ranging from 0.89 (IL-1\(\beta\)) to 0.94 (MIP-1\(\beta\)), thereby indicating good to excellent discrimination between the TT/BT and EC groups in Bangladesh. Combining the three biomarkers enhanced this diagnostic ability even more, as evident from the AUC value (0.99).

It is of interest that IL-1\(\beta\) concentrations in HHC were very heterogeneous, resulting in two subgroups. This could indicate that some individuals in this group may induce similar immune responses as TT/BT patients. Longitudinal cytokine analysis of these HHC may reveal whether such immune responses could correlate with progression to disease. Interestingly, TB patients from South Korea produced significantly higher concentrations of...
MCP-1 than EC ($p = 0.0001$), arguing for a specific role of MCP-1 in mycobacterial diseases.

Despite some interindividual differences, the data revealed that the overall concentrations for most cytokines (IL-10, IL-17, IL-2, IL-6, IL-8, G-CSF, GM-CSF, IP-10, monokine induced by IFN-$\gamma$, and TNF) showed no significant differences between TT/BT, HHC, and EC from Bangladesh (Fig. 4 and data not shown). In all test groups, the remaining cytokines, IL-4, IL-5, IL-7, IL-12p70, and IL-13, were hardly detected (median <50 pg/ml; data not shown). Thus, these multiplex analyses demonstrate that cytokines/chemokines other than IFN-$\gamma$, namely IL-1b, MIP-1b, and MCP-1, have the potential to distinguish pathogenic immune responses as present in patients of mycobacterial diseases from those induced during asymptomatic exposure to M. leprae.

The multiplex cytokine analysis of WBA of Ethiopian EC high and EC low (Fig. 5) implied a comparison between two test groups of healthy individuals, and thus does not necessarily reveal biomarkers related to pathogenic immune responses. IP-10 or CXCL10 has been shown to be a useful biomarker for diagnosis of M. tuberculosis infection (21). In Fig. 5, it is shown that, in line with the differences in IP-10 observed between EC from Bangladesh and South Korea (Fig. 4), IP-10 responses correlated with prevalence-estimated M. leprae exposure density, as EC high produced substantially higher concentrations of IP-10 than EC low ($p < 0.0001$).

Concentrations of MCP-1 were slightly increased in the EC high group, but not as significantly as IP-10. In contrast, IL-1b and MIP-1b that were increased in TT/BT patients in Bangladesh, compared with EC from that area, did not show significant differences between the two Ethiopian EC groups. This is similar to the finding that these cytokines did not differ significantly between EC from Bangladesh and from South Korea either, whereas IP-10 concentrations could distinguish between these groups (Fig. 4). None of the other cytokines tested displayed concentrations that differed sufficiently between patients and EC (data not shown).

Stimulation with the M. leprae-unique protein ML2478 instead induced a cytokine pattern similar to that of M. leprae WCS-stimulated whole-blood cultures for IP-10 and to a slightly lesser extent for MCP-1 (Fig. 5E, 5F), indicating that, in addition to IFN-$\gamma$, IP-10 can also be used as a biomarker tool to measure M. leprae exposure. No MCP-1, MIP-1b, and IL-1b were induced by ML2478 in nonendemic controls (Supplemental Fig. 3B).

Determination of IFN-$\gamma$/IL-10 ratios in WBA

Because both pro- and anti-inflammatory cytokines play a role in protection from and pathogenesis of mycobacterial diseases, their balance may control or predict an eventual clinical outcome. In this respect, the IFN-$\gamma$/IL-10 ratio has been described to significantly correlate with TB cure and severity (22–25). Determination of the IFN-$\gamma$/IL-10 ratio for individuals from Bangladesh showed a higher IFN-$\gamma$/IL-10 ratio for EC than for HHC and TT/BT, a difference that was not observed by separate analysis of these two cytokines (Fig. 6). Similarly, TB patients in South Korea also had a decreased IFN-$\gamma$/IL-10 ratio compared with EC from that area. This corroborates the value of this ratio as an indicator for pathogenic responses to mycobacteria.

Discussion

The stagnant decline in new leprosy cases demonstrates that transmission of M. leprae is persistent and not affected sufficiently by current control measures (1, 26, 27). In part, this is the consequence of the present practice of leprosy diagnosis, which is mainly based on recognition of clinical symptoms, requiring
special, frequently not available, expertise. Major obstacles in leprosy diagnostics are the lack of good surrogate markers for subclinical or latent *M. leprae* infection, as well as the long incubation time that hinders early detection of leprosy and its modes of transmission. Thus, to overcome inadequate leprosy diagnostics, the development of rapid tests that can be applied in nonexpert settings and allow identification of leprosy at early (subclinical) stages is high on the research agenda.

In the current study, we show that IFN-γ production induced by *M. leprae* WCS in 24-h WBA of leprosy patients (TT/BT; *n* = 10), HHC (*n* = 10), and EC (*n* = 10) from Bangladesh, or healthy controls (EC; *n* = 10) and tuberculosis patients (TB; *n* = 10) from South Korea. Median values per test group are indicated by horizontal lines. Background values varied from <50 pg/ml for IFN-γ to <2000 pg/ml for MIP-1β.

**FIGURE 4.** Multiplex cytokine analyses in WBA from individuals in Bangladesh and South Korea. Concentrations (all corrected for background values) of IL-1β (A); MIP-1β (B); MCP-1 (C); and IL-β, MIP-1β, and MCP-1 combined (D); or IP-10 (E) induced by stimulation with *M. leprae* WCS in 24-h WBA of leprosy patients (TT/BT; *n* = 10), HHC (*n* = 10), and EC (*n* = 10) from Bangladesh, or healthy controls (EC; *n* = 10) and tuberculosis patients (TB; *n* = 10) from South Korea. Median values per test group are indicated by horizontal lines. Background values varied from <50 pg/ml for IFN-γ to <2000 pg/ml for MIP-1β.

Because a *M. leprae*-resistant phenotype is generally believed to be associated with the emergence of a protective Th1-based response characterized by consistent secretion of IFN-γ in association with moderate amounts of proinflammatory cytokines, we and others have previously used IFN-γ release assays (IGRAs) as a readout of cell-mediated immune responses to investigate which *M. leprae* Ags can be useful for the diagnosis of leprosy (7, 9, 12). This was partly based on the initial promising reports on QuantiFERON-TB, an IGRA for diagnosis of TB (28). However, a recent meta-analysis showed that neither IGRA nor the tuberculin skin tests have high accuracy for the prediction of incident-active TB in endemic areas (29). Our study shows that this is also the case for leprosy because the positive IFN-γ responses measured in WBA after stimulation with *M. leprae*-unique Ags depended on the level of endemicity in the investigated area and were not specific for disease. Importantly, however, in this study,
we have identified *M. leprae*-unique proteins, in particular ML2478, which can be used with IFN-γ as a readout in the context of various genetic backgrounds (African, Asian, and South American) to point out distinct degrees of *M. leprae* exposure even if these occur in individuals residing in distinct areas of the same city. Therefore, such *M. leprae* proteins, combined with IGRAs, can be relevant as new tools for predicting the magnitude of *M. leprae* transmission in a given population and for identification of individuals who are at risk for acquiring *M. leprae* infection and possibly developing leprosy. Besides these data for ML2478, which is a hypothetical unknown protein lacking transmembrane regions and weakly similar to a probable metalloproteinase from *Streptomyces avermitilis* (33% identity), similar data were recently found by us using *M. leprae*-specific peptides instead of proteins, and further support our findings (M. Martins, M. da S. Guimarães, J. Spencer, M. Hacker, L. Costa, F. Carvalho, A. Geluk, J. van der Ploeg-van Schip, M. Pontes, H. Gonçalves, J. de Morais, T. Bandeira, M. Pessolani, P. Brennan, and G. Pereira, submitted for publication) (18). The *M. leprae*-specific IFN-γ response detected in this study in EC in areas hyperendemic for leprosy is consistent with earlier findings on the presence of *M. leprae* in nasal swaps of EC in Indonesia (30). Thus, this indicates that a vast proportion of leprosy patients probably acquires *M. leprae* infection from unidentified infected individuals or subclinical leprosy cases in the community and not necessarily from diagnosed leprosy patients.

The IP-10 production measured in WBA in this study displayed a pattern similar to that of IFN-γ, although the overall IP-10 concentrations were higher. Thus, our finding that IP-10 can differentiate between *M. leprae* exposure levels in two Ethiopian EC groups corroborates the potential of this cytokine as a biomarker for *M. tuberculosis* exposure/infection (31). In this respect, it is noteworthy that IP-10 has also been shown to be a promising biomarker for TB in HIV+ individuals, as the use of IP-10 as a readout, with or without IFN-γ, was reported to be much less influenced by CD4 cell count than the Quantiferon-TB Gold In-Tube (32). Although IFN-γ is directly involved in inducing IP-10 production, IP-10 is produced primarily by monocytes and might be induced by CD4 T cell- and IFN-γ–independent pathways. Alternatively, the higher concentrations of IP-10 produced may render this biomarker less sensitive to the effect of immune suppression.

The outcome of the immune response to *M. leprae* is determined by chemokines and cytokines that act as molecular signals for communication between cells of the immune system, which renders them useful biomarkers predicting either protection or progression to disease. In this study, we identified secreted chemokines/cytokines (IL-1β, MIP-1β, and MCP-1) that, in contrast to IFN-γ, could discriminate in 24-h WBA between patients (leprosy and TB) and healthy EC in the same endemic areas, thereby possibly reflecting differences between *M. leprae* exposure and pathogenic immunity against *M. leprae*.

The chemokine that was very significantly increased in TT/BT leprosy patients compared with healthy EC from Bangladesh was MCP-1 (or CCL2). This molecule recruits monocytes, memory T cells, and dendritic cells to sites of tissue injury and infection (33), and it has been suggested to play a role in maintaining the integrity of the granuloma in asymptomatic individuals with latent infection in high TB burden settings (34). For TB patients, MCP-1 production by *M. tuberculosis*-stimulated PBMC was associated with TB disease severity (35). In contrast, for lepromatous leprosy
patients, MCP-1 was found to be lower than for TB patients (36). Similar data for tuberculoid leprosy patients have not been reported, yet the data in this study indicate that TT/BT patients are more inclined toward a phenotype resembling that of TB patients with elevated MCP-1 production.

The second potential immunological biomarker we identified, MIP-1β (or CCL4), is a chemoattractant for monocytes and can inhibit T cell activation by interfering with TCR signaling (37). The exact role of MIP-1β in leprosy pathogenesis is still not clear.

Thirdly, our data showed increased IL-1β concentrations in WBA of TT/BT compared with EC in Bangladesh. IL-1β is produced by activated macrophages, plays a major role in host resistance to M. tuberculosis (38), and is involved in the TLR2/1-induced vitamin D antimicrobial pathway leading to induction of the antimicrobial peptide defensin β4A. Recently, reduced expression of the IL1B gene was reported for lesions of lepromatous leprosy patients who typically lack good cellular responses (39). In view of our finding that TT/BT patients produce more IL-1β in response to M. leprae, this cytokine could be useful to indicate leprosy subtypes as well. Thus, although we cannot absolutely explain the observed difference in IL-1β, MIP-1β, and MCP-1 secretion in the WBA in the various test groups, we cannot rule out any effect of M. leprae-specific recall responses that may affect these innate responses (40).

In leprosy, the quality and quantity of the innate and adaptive immune response determine the outcome of infection; whereas the proinflammatory cytokine IFN-γ provides protection against mycobacteria, the anti-inflammatory cytokine IL-10 has been shown to be associated with dampening Th1 cells’ responses toward mycobacteria (41, 42). Besides measuring single cytokines, the ratios of such cytokines can provide important information because both pro- and anti-inflammatory cytokines play a role in protection from and pathogenesis of mycobacterial diseases, and their balance may control or predict the eventual clinical outcome. The IFN-γ/IL-10 ratio has been described to significantly correlate with TB cure (22–25). Also, the IFN-γ/IL-10 ratio positively correlated with tuberculin skin test induration, suggesting that the ratio between PPD-induced IFN-γ and IL-10 in peripheral blood may be important in controlling tuberculin skin test reactivity (43). In this study, IFN-γ/IL-10 ratios were higher for EC compared with either leprosy or TB patients, despite the lack of significant differences if only IFN-γ was measured. Thus, changes in the IFN-γ/IL-10 ratio, especially when measured longitudinally in one individual, may provide information about potential disease development or response to treatment.

Because the HIV burden in most leprosy-endemic areas is quite severe, it should be analyzed whether IL-1β, MIP-1β, MCP-1, IFN-γ, and IP-10 as well as the ratios of Th1/Th2 cytokines can be applied as biomarkers in immunocompromised individuals. Therefore, we are currently investigating such potential biomarkers, in combination with M. leprae-specific Ags, in HIV+ individuals as well as HIV+ leprosy patients.

WBA using M. leprae Ags thus induce a fingerprint of (the ratio of) Th1 or Th2 cytokines that may, combined with detection of anti–PGL-I Abs, be used to specify disease type in the leprosy spectrum. Recently, we reported the development of a robust, user-friendly lateral flow assay based on upconverting phosphor technology that allows simultaneous detection of cellular and humoral immune responses in one sample (44, 45). Using ML2478-stimulated WBA, this upconverting phosphor technology assay can now be used in poorly equipped laboratories to estimate levels of M. leprae, by measuring both Th1 (IFN-γ/IP-10) and Th2 (IL-10) as well as anti–PGL-I IgM Abs. Currently, the development of this rapid lateral flow assay for detection of IL-1β, MIP-1β, and MCP-1 is in progress.

Because the majority of those exposed to M. leprae develop a protective immune response against the bacterium, large-scale, longitudinal follow-up studies, allowing intraindividual comparison of immune profiles in healthy controls from leprosy-endemic areas worldwide, will be essential to analyze whether the biomarkers identified in this study can be applied as tools for prediction of pathogenic immune responses to M. leprae.

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Disclosures
The authors have no financial conflicts of interest.

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Supplementary Figure S1A:

IFN-γ responses in WBA from individuals in Bangladesh. IFN-γ production in response to recombinant proteins (ML0091, ML0811, ML2044 and ML2055) in 24 hour WBA of leprosy patients (TT/BT; n = 10), healthy household contacts (HHC; n = 10) and endemic controls (EC; n=10) from Bangladesh. Background values were <50 pg/ml. Median values for each group are indicated by horizontal lines.

Supplementary Figure S1B:

IFN-γ responses in WBA from individuals in Bangladesh and South Korea. IFN-γ production in response to recombinant proteins (ML0009, ML0957, ML1976 and ML2531) in 24 hour WBA of leprosy patients (TT/BT; n = 10), healthy household contacts (HHC; n = 10) and endemic controls (EC; n=10) from Bangladesh (prevalence = 2.45/10,000) or healthy controls (EC; n=10) and tuberculosis patients (TB; n=10) from South Korea (prevalence <1/10,000). For each group the number of IFN-γ responders (>100 pg/ml) versus the total number of individuals in the group is indicated below the x-axis. Background values were <50 pg/ml. Median values for each group are indicated by horizontal lines.

Supplementary Figure S2:

Antibody responses in sera from healthy individuals in Bangladesh and South Korea. Reactivity of sera from endemic controls (EC; n=10) from Bangladesh and healthy controls (EC; n=10) from South Korea toward synthetic PGL-I antigen (ND-O-
BSA; A), native *M. leprae* LAM (LepLAM; B) and recombinant protein ML2028 (Ag85B; C) by ELISA. Optical density readings were performed using a 1:200 serum dilution. Median values for each group are indicated by horizontal lines.

**Supplementary Figure S3: Comparison of cytokine production in 24h WBA.**

**A**: IFN-γ production (corrected for background levels) using 24 hour WBA versus 6 days LST in response to ML2478 recombinant protein (10 μg/ml) for 4 Brazilian leprosy patients (●) and two Dutch non endemic controls (▼).

**B**: Cytokine/chemokine production (corrected for background levels) measured in ELISAs specific for IFN-γ, MCP-1, IL-1β and MIP-1β in response to ML2478 recombinant protein (10 μg/ml) in 24 hour WBA of one leprosy patient living in The Netherlands and Dutch non endemic controls (n= 3).
Geluk et al. Supplementary Figure S1A
Supplementary Figure S1B

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