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

Annemieke Geluk,* Kidist Bobosha,*,† Jolien J. van der Ploeg-van Schip,* John S. Spencer,‡ Sayera Banu,§ Marcia V. S. B. Martins,¶ Sang-Nae Cho,‖ Kees L. M. C. Franken,* Hee Jin Kim,# Yonas Bekele,† Mohammad K. M. Uddin,§ Sheikh Abdul Hadi,§ Abraham Aseffa,† Maria C. V. Pessolani,¶ Geraldo M. B. Pereira,# Hazel M. Dockrell, and Tom H. M. Ottenhoff***

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*. *The Journal of Immunology*, 2012, 188: 4782–4791.

Leprosy is a treatable immunopathogenic infection caused by *Mycobacterium leprae*. It mainly affects skin and peripheral nerves and ranks as the second most pathogenic mycobacterial infectious disease after tuberculosis (TB). Despite a spectacular decrease in global prevalence since 1982, leprosy is still considered a public health problem in 32 countries, mostly from the African, Asian, and South American continents that cover 92% of all registered patients (1). Transmission of leprosy is sustained, as evidenced by the hundreds of thousands of new cases of leprosy that keep being detected globally every year: 228,474 new cases were detected in 2010, among whom 20,472 were children (1). However, our understanding of the mode of *M. leprae* transmission has been complicated due to the long incubation time of leprosy and the lack of tests that detect asymptomatic *M. leprae* infection, a presumed major source of transmission, or predict possible progression of infection to clinical disease. Tests used in leprosy diagnostics include a serological test detecting IgM Abs against phenolic glycolipid-I (PGL-I), a *M. leprae*–specific cell surface Ag. Although it is useful for detection of most multibacillary leprosy patients, it has limited value in identifying paucibacillary leprosy patients, because the latter typically develop cellular rather than humoral immunity (2). The Mitsuda skin test, in contrast, evaluates the in vivo immune response against *M. leprae* bacilli (lepromin) and is used for classification of leprosy. However, this test is not specific for *M. leprae*, as it can also be mediated by lymphocytes responsive to *Mycobacterium tuberculosis*, and thus does not represent an adequate tool to measure *M. leprae* exposure or latent infection (3, 4).

Because the methods and knowledge available to date have obviously not been sufficient to eliminate leprosy, the World Health Organization 2011–2015 global strategy highlighted the need for early diagnosis and treatment (5), which will block development

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Abbreviations used in this article: AUC, area under the curve; EC, endemic control; HHC, healthy household contact; IGRA, IFN-γ release assay; IP-10, IFN-γ–induced protein 10; PGL-I, phenolic glycolipid-I; PPD, purified protein derivative of *M. tuberculosis*; TB, tuberculosis; TT/BT, tuberculoid leprosy/borderline tuberculoid leprosy; WBA, whole-blood assay; WCS, whole-cell sonicate.

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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 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 endemicity 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 (Mereiles; prevalence <0.2/10,000; EC_{low}), and 10 healthy controls living in an area of Fortaleza with high prevalence (Bom Jardin; prevalence >4/10,000; EC_{high}); in Ethiopia, 35 healthy controls were tested, 18 EC_{high} who 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 endemicity 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% CO_2, 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 by Ficoll density centrifugation from venous, heparinized blood and plated in triplicate cultures (2×10^5 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, Oxoid, Haarlem, The Netherlands) was used. After 6 d of culture at 37°C at 5% CO_2, 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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Table I. Participating study sites and study groups

Site	Prevalence[a]	Category	BI[b]	Sex Ratio (M/F)	Mean Age (y)	Age Range (y)
Bangladesh (Dhaka)	0.28	TT/BT	0	7/3	38.5	22–65
		HHC	NA	6/4	35.7	20–70
		EC	NA	7/3	28.1	24–35
South Korea (Seoul)	<0.1	EC	NA	9/1	23	21–25
		TB	NA	4/6	51.2	24–77
Ethiopia (Addis Ababa)	0.36	EC_{low}	NA	5/13	27.6	18–40
	1.5	EC_{high}	NA	8/9	23.1	18–38
Brazil (Fortaleza)	<0.2	EC_{low}	NA	5/5	34.7	22–60
	>4	EC_{high}	NA	5/5	36.6	18–58

[a]Prevalence per 10,000 individuals at the end of 2010.
[b]BI, bacterial index (mean).
NA, Not applicable.

IFN-γ ELISA

IFN-γ concentrations were determined by ELISA (U-CyTech, Utrecht, 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 buffycoat (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* lipoarabinomannan were coated onto high-affinity polystyrene Immulon IV 96-well ELISA plates (Dynex Technologies, Chantilly, VA) using 50 ng/well in 100 μl 0.1 M sodium 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 assays (WBA) were measured using the Bio-Plex suspension array system powered by Luminex xMap multiplex technology (Bio-Rad Laboratories, Veenendaal, The Netherlands) and analyzed using the Bio-Plex Manager software 6.0 (Bio-Rad Laboratories, Veenendaal, The Netherlands) (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* WCS 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 tuberculoid 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* WCS 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

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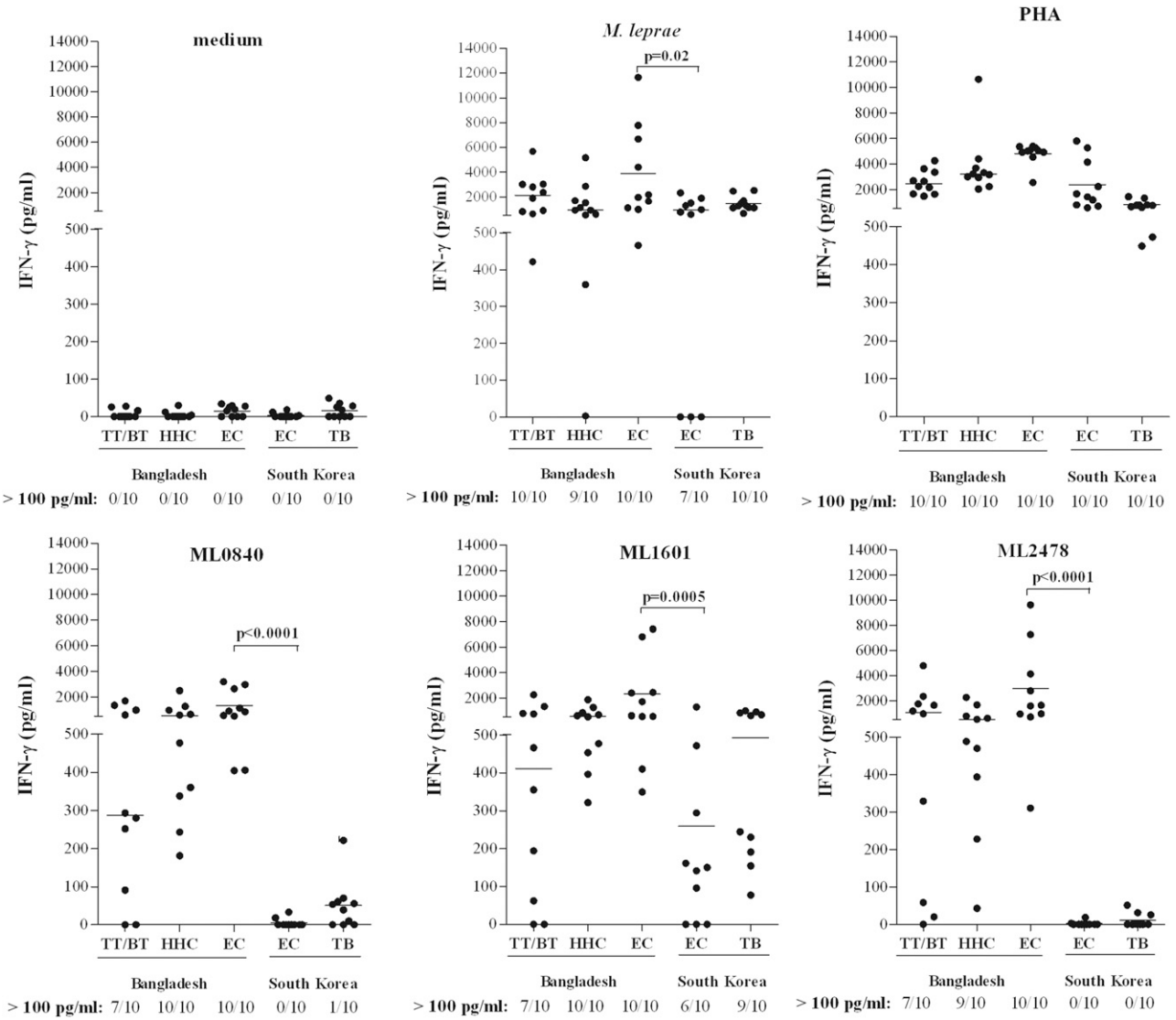

FIGURE 1. IFN-γ responses in WBA from individuals in Bangladesh and South Korea. IFN-γ production in response to control stimuli (medium, PHA, and *M. leprae* WCS) or to recombinant proteins (ML0840, ML1601, and ML2478) in 24-h WBA of leprosy patients (TT/BT; $n = 10$), HHC ($n = 10$), and EC ($n = 10$) 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.

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) with ML2478 and ML0840, the Ab concentrations to the three *M. leprae* Ags tested could not differentiate between these two EC groups (Supplemental Fig. 2).

IFN-γ responses to M. leprae *Ags in EC_{high} and EC_{low} from the same city*

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_{low} versus EC_{high}), 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_{low}) and another area with a leprosy prevalence of >4 per 10,000 (EC_{high}). 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_{high} as compared with PBMC from the EC_{low} 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.

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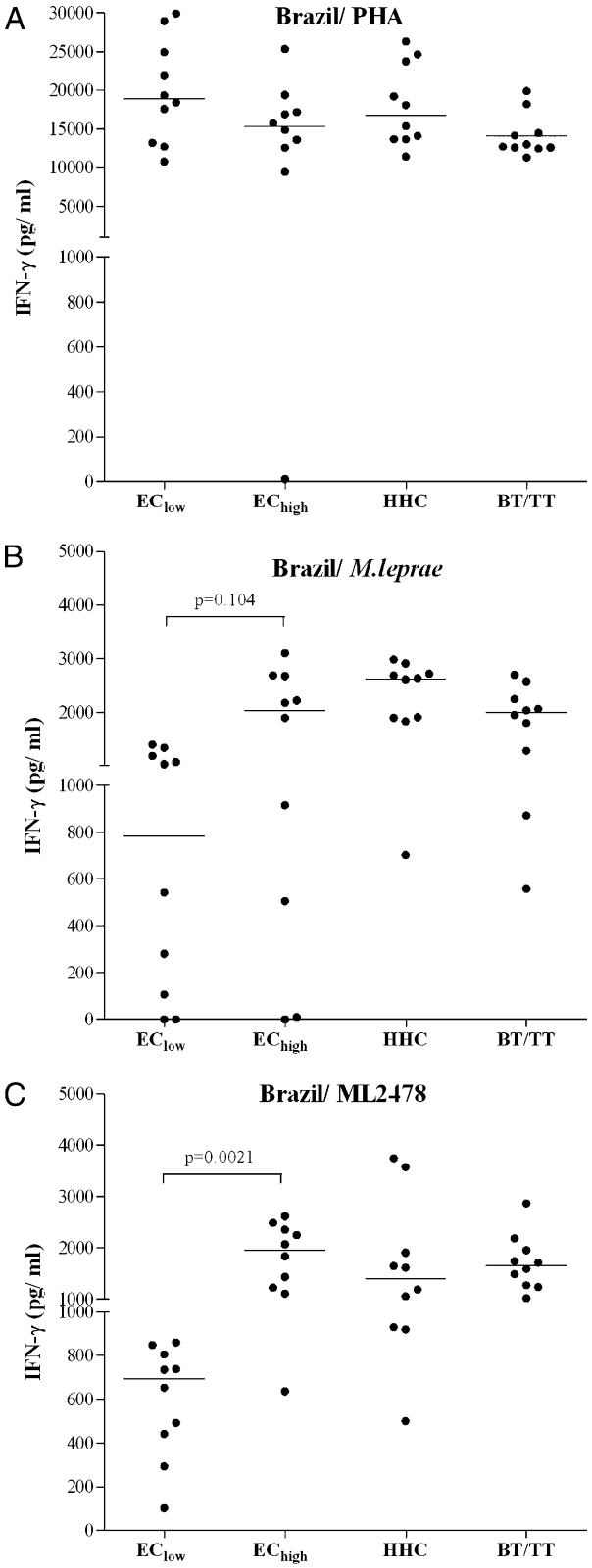

FIGURE 2. IFN-γ responses to *M. leprae* Ags in PBMC from EC_{high} and EC_{low} in Brazil. IFN-γ production (corrected for background values) induced using PHA (**A**), *M. leprae* (**B**), or ML2478 recombinant protein (**C**) in 6-d cultures of PBMC from healthy individuals from an area of Fortaleza, Brazil, with low (EC_{low}; prevalence <0.2/10,000; n = 10) or high (EC_{high}; prevalence >4/10,000; n = 10) leprosy prevalence, HHC of multibacillary leprosy patients, and TT/BT patients. Median values for each group are indicated by horizontal lines. Background values were <20 pg/ml.

IFN-γ responses to M. leprae *Ags in WBA in EC_{high} and EC_{low} in Ethiopia*

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_{high} were derived from a subcity of Addis Ababa (Kolfe Keranio) with a prevalence rate of 1.5 per 10,000, whereas 17 EC_{low} 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-γ in the WBA of Ethiopian EC_{high} compared with Ethiopian EC_{low} (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-γ 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-γ 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-γ 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-γ 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-γ, the concentrations of IL-1β, MIP-1β (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-γ, the levels of MCP-1, MIP-1β, and IL-1β 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-1β, and IL-1β as biomarker tools for leprosy diagnostics, receiver operating characteristics were analyzed (Table II), showing areas under the curve (AUC) ranging from 0.89 (IL-1β) to 0.94 (MIP-1β), 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-1β 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

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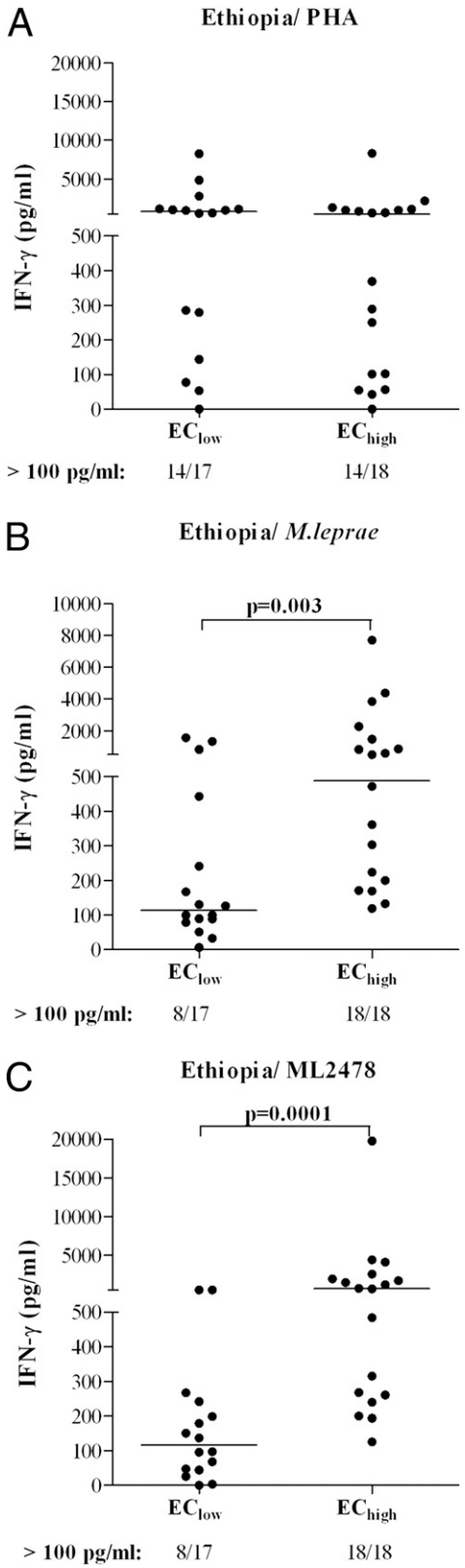

FIGURE 3. IFN-γ responses to *M. leprae* proteins in WBA from EC_{high} and EC_{low} in Ethiopia. IFN-γ production (corrected for medium values) in response to PHA (**A**), *M. leprae* WCS (**B**), or recombinant protein ML2478 (**C**) in 24-h WBA of healthy individuals from areas in Addis Ababa, Ethiopia, with low (EC_{low}; prevalence = 0.36/10,000; n = 17) and high (EC_{high}; prevalence = 1.5/10,000; n = 18) leprosy endemicity. Median values per test group are indicated by horizontal lines. For each group, the number of IFN-γ responders versus the total number of individuals in the group is indicated below the x-axis.

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-γ, 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-γ, namely IL-1β, MIP-1β, 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-1β and MIP-1β 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-γ, IP-10 can also be used as a biomarker tool to measure *M. leprae* exposure. No MCP-1, MIP-1β, and IL-1β were induced by ML2478 in nonendemic controls (Supplemental Fig. 3B).

Determination of IFN-γ/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-γ/IL-10 ratio has been described to significantly correlate with TB cure and severity (22–25). Determination of the IFN-γ/IL-10 ratio for individuals from Bangladesh showed a higher IFN-γ/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-γ/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

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