Published in final edited form as:
J Immunol. 2012 May 15; 188(10): 4782–4791. doi:10.4049/jimmunol.1103452.

New Biomarkers with Relevance to Leprosy Diagnosis Applicable in Areas Hyperendemic for Leprosy[1]

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Abstract

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 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 read-out. 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 to EC, whereas IP-10, like IFN-γ, differed between EC from areas with dissimilar leprosy prevalence.

[1]This study was supported by the Netherlands Leprosy Relief Foundation (NLR; ILEP#: 7.01.02.48) together with the Turing Foundation as part of the IDEAL Consortium, and the Order of Malta-Grants-for-Leprosy-Research. AG received additional support for this study from the Q.M. Gastmann-Wichers Foundation. JSS and HJK received some support from the NIH/NIAID grant R01 AI-082575. The authors declare no conflict of interest.

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This study identifies *M. leprae*-unique antigens, particularly ML2478, as biomarker tools to measure *M. leprae* exposure using IFN-γ or IP-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*.

Keywords

Mycobacterium leprae (M. leprae); biomarkers; leprosy; multiplex analysis

INTRODUCTION

Leprosy is a treatable immuno-pathogenic infection caused by *Mycobacterium leprae* (*M. 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 amongst 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 antibodies against phenolic glycolipid-1 (PGL-I), an *M. leprae* specific cell-surface antigen. Although it is useful for detection of most multibacillary (MB) leprosy patients, it has limited value in identifying paucibacillary (PB) leprosy patients, since the latter typically develop cellular rather than humoral immunity (32). The Mitsuda skin test, on the other hand, 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 *M. tuberculosis* and thus does not represent an adequate tool to measure *M. leprae* exposure or latent infection (29,36).

Since the methods and knowledge available to date have obviously not been sufficient to eliminate leprosy, the WHO 2011–2015 global strategy highlighted the need for early diagnosis and treatment (5) which will block development 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 antigens since the availability of the *M. leprae* genome sequence about one decade ago (9). Subsequently, these (hypothetical) antigens were used as recombinant proteins or synthetic peptides in *in vitro* T cell stimulation assays, mostly assessing IFN-γ production (3,12,15,16,41,43). Although it is not an immunological correlate of protection, the number of IFN-γ-releasing antigen-specific T cells and the amount of total IFN-γ released remain widely used as surrogate markers for the pro-inflammatory immune response against *M. leprae* and *M. tuberculosis* (45). 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* antigens.

In a previous study we tested recombinant proteins that had been selected based on their unique sequence in *M. leprae* (16). Notwithstanding this selection, IFN-γ production by EC-derived PBMC or whole blood was observed in response to most of these *M. leprae* proteins. Since 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 towards 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 antigens.

Since host immunity and immuno-pathogenicity in response to *M. leprae* involves 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, here we have analyzed the concentrations of multiple cytokines, besides IFN-γ, after 24 hour whole blood stimulation with 17 *M. leprae* antigens in various cohorts from leprosy endemic areas in Bangladesh, Brazil and Ethiopia. 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 MDT.

MATERIALS AND METHODS

General procedure of the study

Patients and controls were recruited at: International Center for Diarrhoeal Disease Research Bangladesh (ICDDR,B), Dhaka, Bangladesh, Yonsei University (YU), Seoul, South Korea, Fiocruz Fortaleza, Brazil and the Armauer Hansen Research Institute (AHRI) in Addis Ababa, Ethiopia. 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 (SOP) 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 *E. 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 antibody (Invitrogen) to confirm size and purity. Endotoxin contents were below 50 EU (endotoxin unit) per mg of recombinant protein as tested using a Limulus Amebocyte Lysate (LAL) QCL-1000 assay (Lonza Inc., Basel, Switzerland). Recombinant proteins tested in this study (n = 17) included: ML0009, ML0091, ML0755, ML0811, ML0840, ML0953, ML0957, ML1601, ML1976, ML2044, ML2055, ML2307, ML2313, ML2478, ML2531, ML2532 and ML2666. ML0091, ML0811, ML2044 and ML2055 were kindly provided by Dr. M.S. Duthie (Seattle, USA).

Recombinant proteins were tested to exclude protein non-specific T cell stimulation and cellular toxicity in IFN-γ release assays using PBMC of *in vitro* 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 whole cell sonicate (WCS)

Irradiated armadillo-derived *M. leprae* whole cells were probe sonicated with a Sanyo sonicator to >95% breakage. This material was provided through the NIH/NIAID "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 TT/BT leprosy patients (Leprosy Control Institute & Hospital, Dhaka), 10 healthy household contacts of BL/LL patients (HHC), 10 healthy individuals from the same endemic area (EC); in South Korea (prevalence <1/10,000): 10 smear positive, pulmonary tuberculosis patients (TB) 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 sub city of Addis Ababa (Kolfe Keranio) with a prevalence rate of 1.5 per 10,000 (72 in 465,811), whereas17 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 (35) by qualified personnel. Patients were treated with chemotherapy for less than 3 months with no signs of leprosy reactions. HHC were defined as adults living in the same house as a BL/LL index case for at least the preceding six months. TB patients were diagnosed based on a positive culture of *M. tuberculosis* in sputum and were recruited at the outpatient clinic of the Pulmonary Division, Severans Hospital, Yonsei University Health System (YUHS) and had been on chemotherapy for at least 3 months to enable recovery of T cell function. EC were assessed for the absence of signs and symptoms of tuberculosis 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 ICDDR,B; in South Korea: Institutional Review Board for the Protection of Human Subjects at YUHS; in Brazil: Brazilian National Council of Ethics in Research (CONEP); in Ethiopia: National Health Research Ethical Review committee (NERC). Informed consent was obtained from all individuals before venepuncture.

Whole blood assays (WBA)

Within 3 hours of collection, venous heparinized blood (450 μl per well) was incubated in 48-well plates at 37°C at 5% CO_2, 90% relative humidity with 50 μl of antigen solution (100μg/ml). After 24 hour 150μl of supernatants were removed from each well and frozen in aliquots at −20°C until further analysis.

Lymphocyte stimulation tests (LST)

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 Corporation, Cambridge, Mass.) in 200 μl/well of serum free Adoptive Immunotherapy medium (AIM-V, Invitrogen, Carlsbad, CA). Recombinant protein, *M. leprae* WCS or PPD (purified protein derivative of *M. tuberculosis*, Statens Serum Institut, Copenhagen,

Denmark) were added at final concentrations of 10 μg/ml. As a positive control 1 μg/ml PHA (phytoheamagglutinin; Remel, Oxoid, Haarlem, The Netherlands) was used. After 6 days of culture at 37°C at 5% CO_2, 90% relative humidity, 75 μl of supernatant were removed from each well, triplicates were pooled and frozen in aliquots at −20°C until further analysis.

IFN-γ ELISA

IFN-γ concentrations were determined by ELISA (U-CyTech, Utrecht, The Netherlands) as described (17). The cut-off 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 Antibody 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 (LepLAM) were coated onto high-affinity polystyrene Immulon IV 96-well ELISA plates (Dynex Technologies, Chantilly, VA) using 50 ng per well in 100 μl of 0.1M sodium carbonate buffer, pH 9.0 at 4°C overnight. Unbound antigen 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 antibody, the wells were washed six times with PBS with 0.05% Tween 80 (wash buffer), followed by the addition of 100 μl of a 1:5,000 dilution of the secondary anti-human polyvalent antibody (Sigma) for 2 h. Following washing the wells with PBS six times, 100 μl of *p*-nitrophenylphosphate substrate (Kirkegaard and Perry Labs, Gaithersburg, MD) was added. The absorbance at 405 nm was read using a VersaMax Pro plate reader (Molecular Devices, Sunnyvale, CA) at 15 minutes. The cutoff for positivity was considered to be three times the background O.D. average for the non-endemic control sera (n = 23) determined by binding BSA with a 1:200 serum dilution (cutoff 0.411).

Multi-cytokine and -chemokine 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, IP-10 (CXCL10), G-CSF, GM-CSF, MCP-1 (CCL2), MIG (CXCL9), MIP-1β (CCL4) and TNF] in supernatants from 24 hour 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) (18). After pre-wetting 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, Veenendaal, The Netherlands). Supernatant samples (50 μl) were added to the plates and the plates were incubated for 45 minutes at room temperature in the dark at 300 rpm on a plate shaker. After three washing steps, 12.5 μl detection antibody cocktail was added per well and plates were incubated at room temperature in the dark for 30 minutes on a plate shaker. After three washes, 25 μl strepavidin-PE solution was added per well and incubated for 10 minutes. After three washes, 80 μl of 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 were analyzed for fluorescence. A curve fit was applied to each standard curve according to the manufacturer's manual. Sample concentrations were interpolated from these standard curves. Analyte

concentrations outside the upper- or lower limits of quantification were assigned the values of the limits of quantification of the cytokine or chemokine.

Statistical analysis

Differences in cytokine concentrations between test groups were analysed with the two-tailed Mann-Whitney U test for non-parametric distribution using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego California USA; www.graphpad.com). P-values were corrected for multiple comparisons. The statistical significance level used was $p<0.05$.

RESULTS

IFN-γ responses to *M. leprae* antigens 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 (16). However, since these EC were derived from areas with high leprosy prevalence and also responded to *M. leprae* WCS *in vitro*, the observed cellular responses towards the *M. leprae*-unique proteins could still indicate *M. leprae*-specificity. To investigate this, 17 *M. leprae* antigens 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 hour 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 antigens, 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 (Supplementary Figure S1A), 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 (Figure 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 read-out. 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 (Figure 1). ML1601 was significantly better recognized in the EC group in Bangladesh ($p=0.0005$), whereas 9 out of 10 TB patients from South Korea also recognized this protein which has an orthologue in *M. avium paratuberculosis* (4). ML0009, ML0957, ML1976 and ML2531 did not show significant differences, although ML0009 ($p=0.0686$) and ML2531 ($p=0.0342$) showed a tendency towards higher responses in EC from Bangladesh (Supplementary Figure S1B). Thus, IFN-γ responses in 24 hour 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 antibodies (Ab) 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 (LepLAM) (44). In contrast to the discriminatory IFN-γ patterns induced in 24 hour WBA of EC (South Korea) vs. EC (Bangladesh) with ML2478 and ML0840, the Ab concentrations to the three *M. leprae* antigens tested could not differentiate between these two EC groups (Supplemental Figure S2).

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

In order 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} vs. EC_{high}), we investigated reactivity to the above *M. leprae* antigens in EC in Fortaleza (Brazil), where pockets in the city have a prevalence of less than 0.2 per 10,000 (EC_{low}) and another area with a leprosy prevalence of more than 4 per 10,000 (EC_{high}). In addition, HHC and TT/BT patients from Fortaleza were included (Figure 2). Since comparison of WBA and lymphocyte stimulation tests (LST) showed similar IFN-γ responses (Supplementary Figure S3), 6 day LST with PBMC were used as a test format in this part of the study to allow testing of more antigens.

Whereas PBMC of all groups were equally capable of producing IFN-γ after 6 days as indicated by the response to PHA (Figure 2A), ML2478 (p=0.0029) again showed significantly higher induction of IFN-γ responses in PBMC from TT/BT patients, HHC, and importantly, from EC_{high} as compared to 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.

IFN-γ responses to *M. leprae* antigens 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* antigens in EC from Ethiopia. Eighteen EC_{high} were derived from a sub city 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 (Figure 3A) but responses to *M. leprae* WCS differed between the two EC groups (Figure 3B). Importantly, ML2478 again induced much higher concentrations (p=0.0001) of IFN-γ in the WBA of Ethiopian EC_{high} compared to Ethiopian EC_{low} (Figure 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 read-out, can also be used in 24h WBA to estimate differences in *M. leprae* exposure between EC in areas with different leprosy prevalence even when located in one city.

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

In our previous study (16) 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 (8,39,45). Since 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 Figure 1). In striking contrast to IFN-γ, the concentrations of IL-1β, macrophage inflammatory protein-1β (MIP-1β or CCL4) and monocyte chemotactic protein-1 (MCP-1 or CCL2) were significantly enhanced in TT/BT patients after stimulation with *M. leprae* WCS compared to Bangladeshi EC (p=0.0006, p= 0.0007 and p= 0.0021 respectively; Figure 4A–C).

When cumulative values were considered (Figure 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 to 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, ROC (receiver operating characteristics) were analyzed (Table II), showing AUC (areas under the curve) 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 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, MIG and TNF) showed no significant differences between TT/BT, HHC and EC from Bangladesh (Figure 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} (Figure 5) implied a comparison between two test groups of healthy individuals and thus does not necessarily reveal biomarkers related to pathogenic immune responses. IFN-γ induced protein 10 (IP-10 or CXCL10) has been shown to be a useful biomarker for diagnosis of *M. tuberculosis* infection (39). In Figure 5 it is shown that, in line with the differences in IP-10 observed between EC from Bangladesh and South Korea (Figure 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 to 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 (Figure 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 (Figure 5E and 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 was induced by ML2478 in NEC (Supplementary Figure S3B).

Determination of IFN-γ/IL-10 ratios in WBA

Since 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 (13,22,24,40). 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 (Figure 6). Similarly, TB patients in South Korea also had a decreased IFN-γ/IL-10 ratio compared to 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 (1,37,42). 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 hinder 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 non-expert settings and allow identification of leprosy at early (subclinical) stages is high on the research agenda.

In the present study we show that IFN-γ production induced by *M. leprae*-unique proteins can identify individuals highly exposed to *M. leprae* and therefore more at risk of developing disease and/or transmitting the bacterium.

Since an *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 pro-inflammatory cytokines, we and others have previously used IFN-γ release assays (IGRAs) as a readout of cell-mediated immune responses (CMI) to investigate which *M. leprae* antigens can be useful for the diagnosis of leprosy (3,15,43). This was partly based on the initial promising reports on QuantiFERON®-TB, an IGRAs for diagnosis of TB (33). 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 (34). Our study shows that this is also the case for leprosy since the positive IFN-γ responses measured in WBA after stimulation with *M. leprae*-unique antigens depended on the level of endemicity in the investigated area and was not specific for disease. Importantly, however, here we have identified *M. leprae*-unique proteins, in particular ML2478, which can be used with IFN-γ as a read-out 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 of 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 metallopeptidase from *Streptomyces avermitilis* (33% identity), similar data, were recently found by us using *M. leprae*-specific peptides instead of proteins, further support our findings (Martins *et al.* submitted; (4). The *M. leprae*-specific IFN-γ response detected in this study in EC in areas hyperendemic for leprosy are consistent with earlier findings on the presence of *M. leprae* in nasal swaps of EC in Indonesia (21). Thus, this indicates that a vast proportion of leprosy patients probably acquire *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 (38). 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 read-out, with or without IFN-γ, was reported to be much less influenced by CD4 cell count than the QuantiFERON®-TB Gold In-Tube (2). 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 24h 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 to 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 (7) 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 has been suggested (23). For TB patients MCP-1 production by *M. tuberculosis*-stimulated PBMC was associated with TB disease severity (19). On the other hand, for lepromatous leprosy (LL) patients MCP-1 was found to be lower than for TB patients (20). Similar data for tuberculoid leprosy patients have not been reported, yet the data in this study indicate that TT/BT patients are more inclined towards 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 chemo-attractant for monocytes and can inhibit T cell activation by interfering with TCR signaling (25). 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 to EC in Bangladesh. IL-1β is produced by activated macrophages, plays a major role in host resistance to *M. tuberculosis* (30) 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 LL patients who typically lack good cellular responses (28). 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 can not absolutely explain the observed difference in IL-1β, MIP-1β and MCP-1 secretion in the WBA in the various test groups we can not rule out any effect of *M. leprae*-specific recall responses that may affect these innate responses (26).

In leprosy the quality and quantity of the innate and adaptive immune response, determine the outcome of infection: whereas the pro-inflammatory cytokine IFN-γ provides protection against mycobacteria, the anti-inflammatory cytokine IL-10 has been shown to be associated

with dampening Th1 cells' responses towards mycobacteria (27,31). Besides measuring single cytokines, the ratios of such cytokines can provide important information since 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 (13,22,24,40). Also, the IFN-γ/IL-10 ratio positively correlated with TST induration suggesting that the ratio between PPD induced IFN-γ and IL-10 in peripheral blood may be important in controlling TST reactivity (6). In this study IFN-γ/IL-10 ratios were higher for EC compared to 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.

Since 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 immuno-compromised individuals. Therefore, we are currently investigating such potential biomarkers, in combination with *M. leprae* specific antigens, in HIV^+ individuals as well as HIV^+ leprosy patients.

WBA using *M. leprae* antigens thus induce a 'fingerprint' of (the ratio of) Th1 or Th2 cytokines that may, combined with detection of anti-PGL-I antibodies, 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 up-converting phosphor technology (UCP-LF) that allows simultaneous detection of cellular and humoral immune responses in one sample (10,11). Using ML2478-stimulated WBA, this UCP-LF assay can now be used in poorly equipped laboratories to estimate levels of *M. leprae* exposure, by measuring both Th1 (IFN-γ/IP-10) and Th2 (IL-10) as well as anti-PGL-I IgM antibodies. Currently, the development of this rapid lateral flow assay for detection of IL-1β, MIP-1β and MCP-1 is in progress.

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

AHRI, CSU, Fiocruz, ICDDR, B, LSHTM and LUMC are part of the IDEAL (***I***nitiative for ***D***iagnostic and ***E***pidemiological ***A***ssays for ***L***eprosy) Consortium. We thank Yonas Fantahun (AHRI) for help with recruitment of blood donors, Dr. Young Ae Kang (Severance Hospital, YUHS) for recruitment of tuberculosis patients and Dr. William Wheat for critically reading the manuscript.

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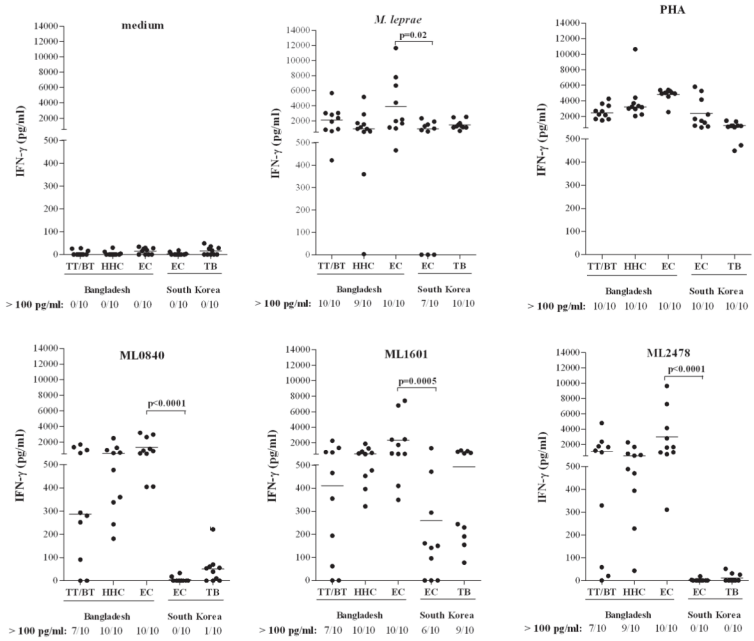

Figure 1. IFN-γ responses in WBA from individuals in Bangladesh and South Korea

IFN-γ production in response to control stimuli (medium, PHA and *M. leprae* WCS) or to recombinant proteins (ML0840, ML1601 and ML2478) in 24 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. Significant differences between test groups are indicated by p-values.

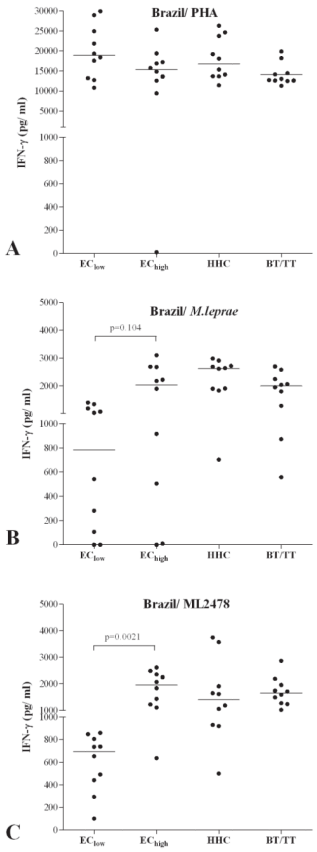

Figure 2. IFN-γ responses to *M. leprae* antigens in PBMC from EC_{high} and EC_{low} in Brazil

IFN-γ production (corrected for background values) induced using PHA (**A**), *M. leprae* (**B**) or ML2478 recombinant protein (**C**) in 6 day 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, healthy household contacts (HHC) of MB leprosy patients and TT/BT patients. Median values for each group are indicated by horizontal lines. Background values were <20 pg/ml.

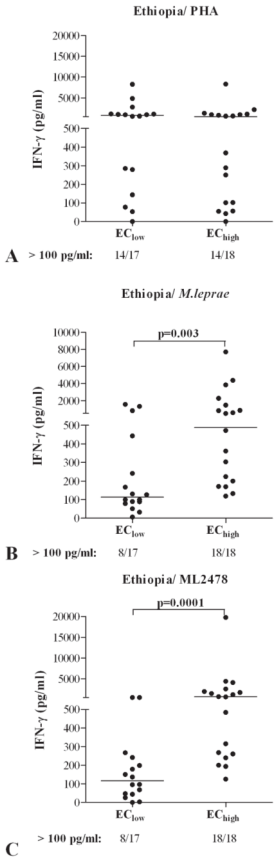

Figure 3. IFN-γ responses to *M. leprae* proteins in WBA from EC_{high} and EC_{low} in Ethiopia

IFN-γ production (corrected for medium values) in response to PHA (**A**), *M. leprae* WCS (**B**) or recombinant protein ML2478 (**C**) in 24 hour 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.

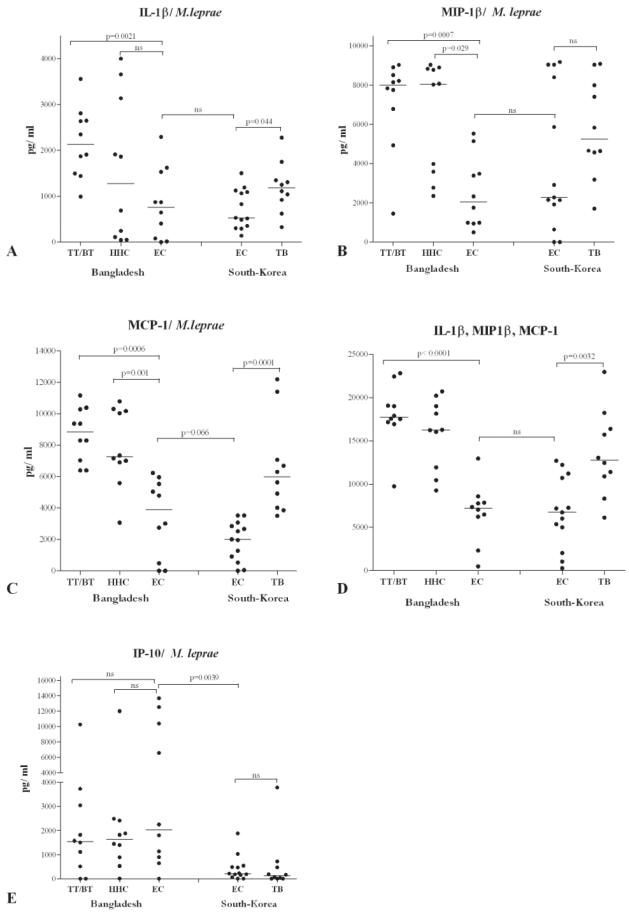

Figure 4. Multiplex cytokine analyses in WBA from individuals in Bangladesh and South Korea

Concentrations (all corrected for background values) of IL-β (**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 hour WBA of leprosy patients (TT/BT; n = 10), healthy household contacts (HHC; n =10) and endemic controls (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. ns = not significant.

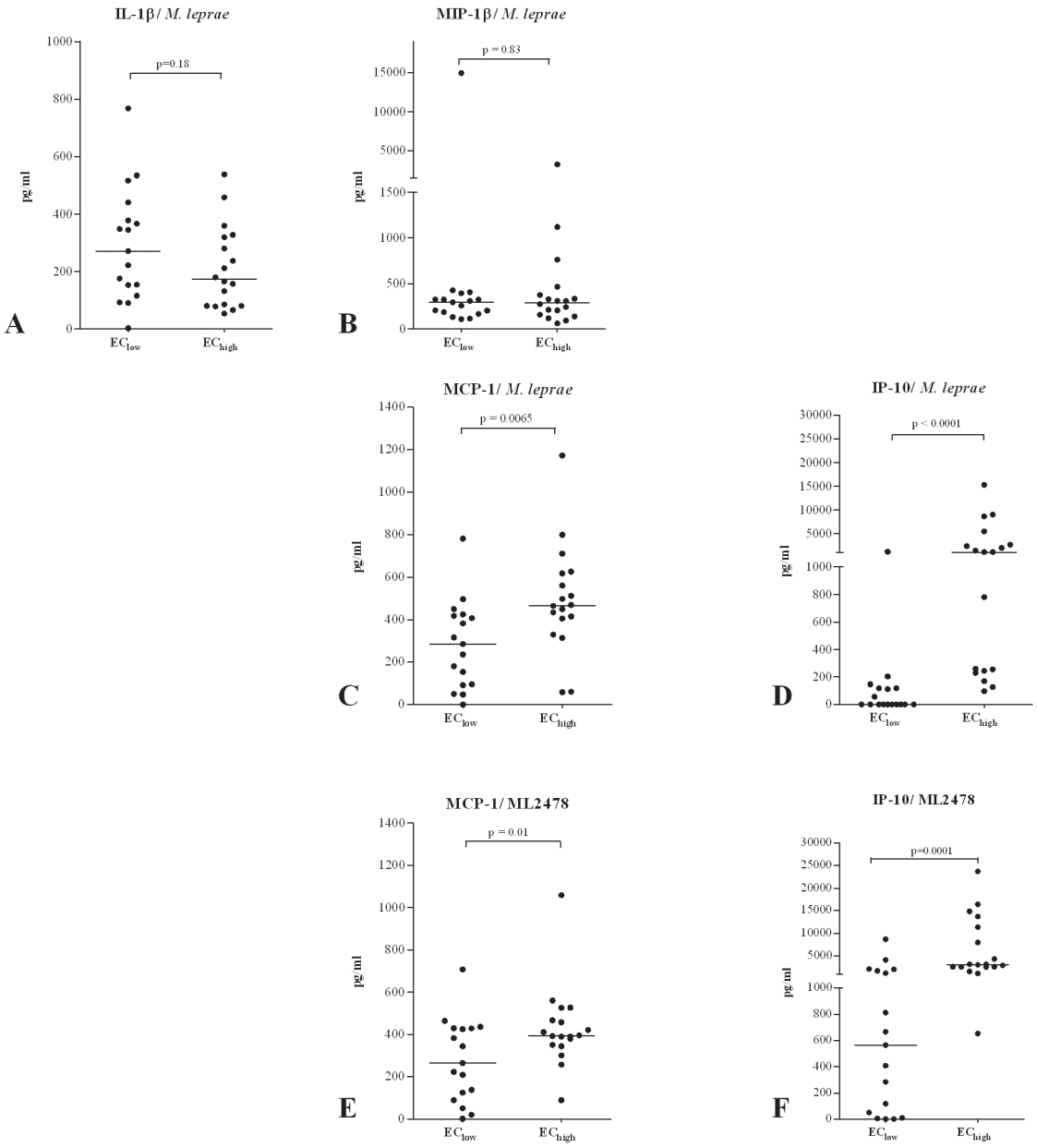

Figure 5. Multiplex cytokine analyses in whole blood cultures from EC in Ethiopia

Concentrations (all corrected for background values) of IL-β (**A**), MIP-1β (**B**), MCP-1 (**C, E**), IP-10 (**D, F**) induced by stimulation with *M. leprae* WCS (**A–D**) or ML2478 (**E**, **F**) 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 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.

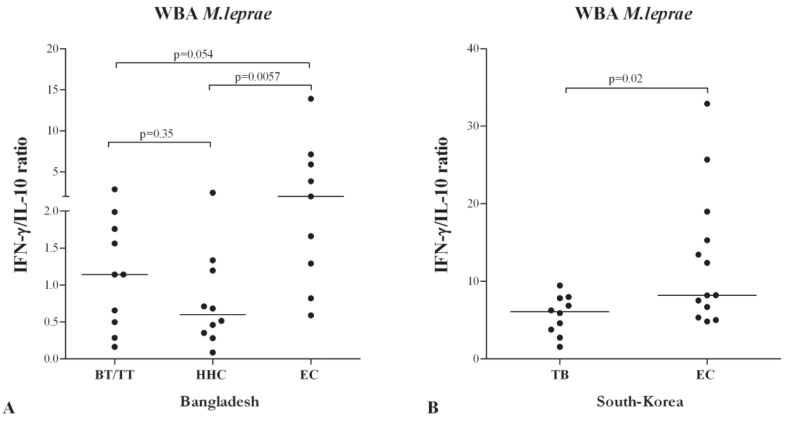

Figure 6. IFN-γ/IL-10 ratio in *M. leprae* stimulated WBA

Ratios of IFN-γ concentrations (corrected for background values) with respect to IL-10 concentrations (corrected for background values) induced by stimulation with *M. leprae* WCS in 24 hour WBA in individuals from Bangladesh (**A**) and South Korea (**B**).

Table I

Participating study sites and study groups

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

[a] Prevalence per 10,000 individuals at the end of 2010.

[b] TT/BT: tuberculoid leprosy/borderline tuberculoid leprosy; HHC: healthy household contact; EC: endemic control.

[c] BI: bacterial index (mean).

[d] not applicable.

Table II

Receiver operating characteristics (ROC) for cytokines induced in WBA

Cytokine	median TT/BT	median EC	p-value	AUC[a]
MCP-1	8829	4794	0.0006	0.94
MIP-1β	7995	2045	0.0007	0.92
IL-1β	2129	760	0.0021	0.89
MCP-1/MIP-1β/IL-1β	17735	7109	<0.0001	0.99
IP-10	1578	2029	0.3258	0.63
IFN-γ	1268	1548	0.1417	0.70

[a]AUC: area under the curve;

[b]Data from 24h WBA including leprosy patient, HHC and EC from one area hyperendemic for leprosy (Bangladesh) were considered for the calculations of ROC-values.