In search of biomarkers for leprosy diagnosis : in silico identification, screening & field application

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Chapter 3

Potential biomarkers for leprosy

3.1 Peptides Derived from *Mycobacterium leprae* ML1601c Discriminate Between Leprosy Patients and Healthy Endemic Controls

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Abstract

The stable incidence of new leprosy cases suggests that transmission of infection continues despite worldwide implementation of MDT. Thus, specific tools are needed to diagnose early stage *Mycobacterium leprae* infection, the likely sources of transmission. *M. leprae* antigens that induce T-cell responses in *M. leprae* exposed and/ or infected individuals thus are major targets for new diagnostic tools.

Previously, we showed that ML1601c was immunogenic in patients and healthy household contacts (HHC). However, some endemic controls (EC) also recognized this protein. To improve the diagnostic potential, IFN-γ responses to ML1601c peptides were assessed using PBMC from Brazilian leprosy patients and EC. Five ML1601c peptides only induced IFN-γ in patients and HHC. Moreover, in 24-hour whole blood assays (WBA), two ML1601c peptides could assess the level of *M. leprae* exposure in Ethiopian EC. Besides IFN-γ, also IP-10, IL-6, IL-1β, TNF-α and MCP-1 were increased in EC from areas with high leprosy prevalence in response to these ML1601c peptides.

Thus, ML1601c peptides may be useful for differentiating *M. leprae* exposed or infected individuals and can be used to indicate the magnitude of *M. leprae* transmission even in the context of various HLA alleles as present in different genetic backgrounds.

Introduction

Leprosy is a treatable infection caused by *Mycobacterium leprae* (*M. leprae*) involving skin and peripheral nerves and is influenced by genetic and environmental factors [1-3]. The infection can result in skin lesions, nerve degeneration and deformities. Despite a spectacular decrease in global prevalence since 1982, transmission of leprosy is sustained as evidenced by the hundreds of thousands new cases of leprosy that keep being detected globally every year: 244,796 new cases of leprosy were detected during 2009 amongst whom 22,485 were children and the registered prevalence at the beginning of 2010 was 211,903 cases [4].

In Brazil, for example, the number of new cases detected during 2009 was 37,610 resulting in a registered prevalence of 38,179 at the end of first quarter of 2010 [4]. These figures demonstrate that *M. leprae* infected contacts and persons with subclinical, undiagnosed leprosy, likely the major sources of unidentified transmission, are an incessant source of active transmission. Despite many efforts, prediction of disease development in affected individuals is still not possible nor can we detect asymptomatic *M. leprae* infection. Diagnosis of leprosy is usually based on clinical features and skin smear results including the number of skin lesions. *M. leprae* is not cultivable and bacterial enumeration by microscopic examination is required for leprosy classification, choice in choosing and monitoring chemotherapy regimens, and diagnosis of relapse. However, detection and quantification using standard microscopy yields data of limited specificity and sensitivity. Thus, in order to complement current clinical methods, especially for PB patients, and to allow informed decision making on who needs treatment at a preclinical stage, several groups are investigating design of improved diagnostic tools. These tools will reduce transmission, prevent functional disabilities and stigmatizing deformities and facilitate leprosy eradication, especially in individuals at risk for developing leprosy such as close contacts of leprosy patients.

Assays have been developed that detect *M. leprae* specific IgM antibodies against PGL-I [5;6], which are able to identify multibacillary (MB) leprosy patients (with strong humoral immunity to *M. leprae*), but these fail to detect most paucibacillary (PB) leprosy patients and leprosy patients' contacts as these typically develop strong cellular but not humoral immunity. One of the hurdles hampering T-cells based diagnostic tests is that *M. leprae* antigens can cross-react at the T-cells level with antigens present in other mycobacteria, like *M. tuberculosis* or BCG even if the homology is relatively low as is the case for ESAT-6 and CFP-10 [7;8]. Using comparative genomics, we previously identified candidate proteins highly restricted to *M. leprae* which showed promising features with respect to application in leprosy diagnostics [9;10].

For specific detection of *M. tuberculosis* infection, commercially available IFN-γ release assays (IGRAs) like QuantiFERON®-TB Gold have been developed [11]: these tests are based on cellular immune responses induced by a cocktail of peptides derived from ESAT-6 (Rv3875), CFP-10 (Rv3874) and TB7.7 (Rv2654) that are selectively expressed by *M. tuberculosis* and deleted from all (non-virulent) BCG strains and most other NTM [11]. This has inspired research into the feasibility of developing similar peptide-based assays for the identification of asymptomatic leprosy: encouraging results have been generated indicating that some synthetic peptides induce specific responses in individuals exposed to *M. leprae* and could potentially be developed into a rapid test for the detection of *M. leprae* infection [10;12;13]. In contrast to TB, however, ESAT-6 or CFP-10-derived peptides will not be useful due to the crossreactive T-cells responses they induce in TB patients [7;8].

Since T-cell reactivity to peptides are HLA-restricted [14-16], the use of a pool composed of several different *M. leprae* peptides, in analogy to the pool of peptides applied in the QuantiFERON®-TB Gold tests, will increase sensitivity [17;18], while avoiding T-cells cross-reactivity. In order to improve sensitivity of a specific diagnostic peptide mixture, we have in this study extended the number of peptides with potential to distinguish exposure to *M. leprae* from BCG vaccination and exposure to other mycobacteria in a future diagnostic tool.

The protein ML1601c was previously identified by us as highly immunogenic in *M. leprae* exposed Brazilian individuals [9], and aalthough it does not contain a homologous sequence in *M. tuberculosis*, it does have an orthologue in *M. avium paratuberculosis*, MAP3249 which is 33 % identical to ML1601c.

Table I
ML1601c synthetic peptides

Peptide#	Amino acid sequence*	Amino acid identity**
11	A**HH**N**AHAA**PAF**L**WSGLVSA	42 % (8/19)
12	F**L**W**S**GLVSA**AVL**IA**DGRGE**	52 % (10/19)
13	**AVL**IA**DGRGE**DTYLPIISIY	40 % (8/20)
14	DTYLPIISIYLA**R**GNE**L**KPN	10 % (2/20)
15	LA**R**GNE**L**KPNPL**L**S**V**IYVE**H**	25 % (5 /20)
16	PL**L**S**V**IYVE**H**L**L**V**L**F**Y**Q**S**VG	35 % (7/20)
17	L**L**V**L**F**Y**Q**S**VGD**H**C**GF**G**R**Y**D**F	45 % (9/20)
18	D**H**C**GF**G**R**Y**D**FG**K**T**M**VL**A**C**YG**	50 % (10/20)
19	G**K**T**M**VL**A**C**YG**CVGTRSL**L**SG	30 % (6/20)
20	CVGTRSL**L**S**GR**DDDLVTSVP	15 % (3/20)
3	RDDDLVTSVPPCGRASVVHRS	0 % (0/21)

** Synthetic peptides overlapping ML1601c are shown in single letter amino acid code.*
***Amino acids sequences of ML1601c* (*M. leprae* TN and BR4923) *peptides were analyzed using BLAST® (http://blast.ncbi.nlm.nih.gov); amino acids that are identical to the MAP3249 are depicted in bold.*

To identify single peptides that are only recognized by *M. leprae* exposed and/or infected individuals, we here analyzed IFN-γ production in Brazilian leprosy patients and controls in response to overlapping ML1601c peptides covering the whole protein.

Materials and Methods

Synthetic peptides. ML1601c overlapping peptides (Table I: two 19-mers with 9 amino acid overlap; eight 20-mers with 10 amino acid overlap; one 21-mer with 10 amino acids overlap) were purchased from Peptide 2.0 Inc. (Chantilly, VA, USA). Homogeneity and purity were confirmed by analytical HPLC and by mass spectrometry. Purity of all peptides was ≥ 80%. All impurities consist of shorter versions of the peptides caused by < 100% coupling

efficiency in each round of synthesis. Aliquots of identical batches of the synthetic peptides were tested in Brazil, Ethiopia and The Netherlands.

Recombinant ML1601c protein. The ML1601c gene was 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) [19]. 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. 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 IU per mg recombinant protein as tested using a Limulus Amebocyte Lysate (LAL) assay (Cambrex, East Rutherford, NJ). Recombinant ML1601c protein was tested to exclude protein non-specific T-cells 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. 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 (these reagents are now available through the Biodefense and Emerging Infections Research Resources Repository listed at
http://www.beiresources.org /TBVTRMResearchMaterials/tabid/1431/Default.aspx).

Study subjects. Twenty two Brazilian leprosy patients (11 paucibacillary (PB) leprosy patients and 11 multibacillary (MB)) were recruited from the Leprosy Out-Patient Unit, Leprosy Laboratory (Oswaldo Cruz Institute, city of Rio de Janeiro) and from the Duque de Caxias Outpatient Units (Health Department, city of Duque de Caxias, Rio de Janeiro State). Leprosy patients were diagnosed and classified based on clinical, bacteriological, and if possible histopathological findings. MB patients were treated with rifampicin, dapsone and clofazimine. PB patients were treated with rifampicin and dapsone. All MB patients were skin slit smear-positive whereas PB patients were all skin slit smear negative. All patients were tested before MDT was initiated. As controls, 19 healthy household contacts of MB leprosy patients (HHC), 8 tuberculosis patients (TB) and 17 healthy endemic controls (EC) were recruited from Duque de Caxias (n = 7) and the city of Rio de Janeiro (n = 10). Leprosy detection rates at the time of recruitment were 1.26 per 10,000 in Rio de Janeiro and 3.40 per 10,000 in Duque de Caxias (Ministry of Health of Brazil; www.datasus.gov.br). From Ethiopia 34 healthy controls were tested: 18 EC_{high} who were derived from a subcity of Addis Ababa (Kolfe Keranio Clinic) with a prevalence rate of 1.5 per 10,000 (72 in 465,811), whereas16 EC_{low} were derived from areas with a prevalence rate of 0.36 per 10,000 (10 in 273,310). Prevalence rates in Ethiopia were calculated based on the number of patients in the health centers provided by the personnel of each health center. TB patients were recruited from the Ambulatory Service, District Hospital Raphael de Paula e Souza, Rio de Janeiro. As non-endemic controls, 21 Dutch healthy individuals (NEC) were recruited at the Blood bank Sanquin, Leiden, The Netherlands. None of the NEC had experienced any known prior contact with leprosy patients. Informed consent was obtained from all individuals before venepuncture. Ethical approval of the study protocol was obtained through the appropriate local ethics committees.

Lymphocyte stimulation tests (LST). Venous blood was obtained from study participants in heparinized tubes and PBMC isolated by Ficoll density centrifugation. PBMC (1.5×10^6 cells/ ml) were plated in triplicate cultures in 96-well round bottom plates (Costar Corporation, Cambridge, Mass.) in 200 μl/well of Adoptive Immunotherapy medium

(AIM-V, Invitrogen, Carlsbad, CA). Synthetic peptides, recombinant protein, *M. leprae* whole cell sonicate or PPD (purified protein derivative of *M. tuberculosis*, Mycos, Loveland, Colorado) were added at final concentrations of 10 μg/ ml. As positive control stimuli SEB (staphylococcus enterotoxin B; 1 μg/ ml; Toxin Technology, Inc., Sarasota, FL, USA) or PHA (phytoheamagglutinin; 2 μg/ ml; Sigma, St. Louis, MO) were used. After 6 days of culture at 37°C at 5% CO_2, 90% relative humidity, 110 μl supernatants were removed from each well, triplicates were pooled and frozen in aliquots at –20°C until further analysis.

Whole blood assays (WBAs). Venous undiluted heparinized blood (450 μl per well) was incubated in 48-well plates at 37°C at 5% CO_2, 70% relative humidity with 50 μl of ML1601c peptides (p11 and p16) solution (10 μg/ ml final concentration). Blood was added to each well within 2 hours of collection. After 24 h of culture 180 μl of supernatants were removed from each well and frozen in aliquots at –20°C until further analysis.

IFN-γ ELISA. Detection of IFN-γ in culture supernatants of *in vitro* cultured cells was performed by ELISA (BD Bioscience) according to the manufacturer's instructions. OD values were converted into concentrations using Microplate Manager Software, version 5.2.1 (Bio-Rad Laboratories, Veenendaal, The Netherlands). The cut-off value to define positive responses was set beforehand at100 pg/ml. The assay sensitivity level was 20 pg/ml. Values for unstimulated whole blood cultures were typically < 30 pg/ml.

Multiplex determination of cytokines and chemokines. According to the manufacturer's guidelines, 18 inflammatory and immunomodulatory cytokines or chemokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1β (CCL4) and TNF) were measured in unstimulated, antigen-stimulated or mitogen-stimulated samples by Bio-Plex Suspension Array System powered by Luminex xMAP multiplex technology (Bio-Rad Laboratories, Veenendaal, The Netherlands) and analyzed with the Bio-Plex Manager™ Software 4.0 (Bio-Rad Laboratories, Veenendaal, The Netherlands). After pre-wetting the filter with assay-solution, the 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 45 minutes at room temperature in the dark at 300 rpm on a plate shaker. After three washes, 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, 100 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 100 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 levels between groups were analyzed with the two-tailed Mann-Whitney U test for non-parametric distribution using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA; www.graphpad.com). P-values were corrected for multiple comparisons. The statistical significance level used was $p<0.05$.

Factor Analysis. The factor analysis technique was applied to evaluate the IFN-γ production levels induced by ML1601c and the ML1601c-derived peptides in order to identify the different patterns of response associated with these stimuli, and to group together peptides inducing similar patterns of IFN-γ production. Three independent factors representing combinations of the original variables were determined. The factor loadings are the

correlation coefficients between the original variables or IFN-γ responses to a given peptide and the factors (StatSoft, Inc. 2010. STATISTICA, data analysis software system, version 9 www.statsoft.com).

Results

3.1. Identification of *M. leprae* specific T-cells epitopes of ML1601c in Brazilian population highly endemic for leprosy.

In view of its high immunogenicity in *M. leprae* exposed individuals [9], the recombinant protein ML1601c was tested for induction of IFN-γ in PBMC derived from multibacillary (MB) and paucibacillary (PB) leprosy patients, healthy house contacts (HHC), tuberculosis (TB) patients, healthy controls (EC) from Brazil and from 21 Dutch (non endemic) control (NEC) individuals (Figure 1). As controls, stimulation by *M. leprae* whole cell sonicate, purified protein derivative (PPD) of *M. tuberculosis* and staphylococcus enterotoxin B (SEB) were also analyzed. As can be seen from Figure 1 all groups responded well to the SEB control with median production > 2400 pg/ml. For the Dutch NEC the positive control PHA was used instead of SEB, inducing overall higher IFN-γ responses in this group (Figure 1A). PPD was highly immunogenic in all groups as well, be it that IFN-γ responses to *M. leprae* showed more variability as only three individuals responded well in the NEC group, two intermediately and 16 were non-responders for *M. leprae*. Additionally, the MB patients did not or only barely respond to *M. leprae*, which is a general phenomenon for this type of leprosy patients. Similar to PPD, ML1601c protein did not induce significantly different IFN-γ production in EC compared to NEC, nor compared to the HHC group, whereas MB patients again responded less well than the five other test groups. In summary, these data indicate that IFN-γ responses induced by ML1601c protein cannot be used to discriminate between *M. leprae* exposed and non-exposed individuals.

Table II
Use of factor analysis for grouping together peptides and protein inducing similar patterns of IFN-γ responses*

	Factor 1	Factor 2	Factor 3
p11	**0.9630**	0.0767	0.1875
p12	**0.9401**	0.0452	0.1643
p13	**0.9219**	0.0913	0.2498
p14	0.7010	0.0961	0.3563
p15	0.1877	-0.0058	**0.9473**
p16	**0.9243**	0.0214	0.1205
p17	0.1487	**0.9606**	0.0324
p18	0.5554	-0.0218	0.3223
p19	0.7157	-0.0095	0.3751
p20	0.3068	0.0327	**0.8787**
p3	**0.9524**	0.0701	0.1830
ML1601c	-0.0081	**0.9788**	-0.0103

** Values indicative of high correlation of a peptide IFN-γ response with one factor are depicted in bold. Factor analysis of the IFN-γ responses induced by the ML1601c protein and ML1601c peptides was performed with IFN-γ values observed in MB, PB, HHC, TB and EC (STATISTICA, data analysis software system, version 9). The 3 factors are new and independent variables that capture the characteristics of the original variables (IFN-γ responses to the peptides and ML1601c protein in the different groups). The factor loadings indicative of the correlation of the IFN-γ responses induced by a peptide with each factor are shown. The 0.80000 value was arbitrarily selected for indicating a high correlation of one peptide with a given factor.*

In view of this nondiscriminatory nature of the IFN-γ responses induced by the ML1601c protein in Brazilian individuals and due to the fact that ML1601c contains sequences similar or identical to *M. avium paratuberculosis* (MAP3249), peptides overlapping the entire sequence of ML1601c (Table I) were synthesized. Analysis of IFN-γ responses induced by these ML1601c synthetic peptides in PBMC is shown in Figure 2: again, similar to stimulation with ML1601c protein, MB patients responded less well than PB patients and HHC. In these latter two groups each ML1601c peptide was recognized by ≥ 1 (HHC) or ≥ 3 (PB) individuals with a maximum of 11 HHC recognizing p17. In contrast to the responses to the whole ML1601c protein, the synthetic peptides induced lower IFN-γ responses especially in NEC and TB, as none of the peptides was recognized by NEC and only p17 induced significant responses in three TB patients. Importantly, for the Brazilian EC only some peptides (p15, p17, p20 and to a lesser extent p18) induced significant responses in multiple donors. Thus, these data clearly indicate that peptide-induced IFN-γ production in response to ML1601c are more specific for and correspond with *M. leprae* exposure and/or infection.

3.2. Identification of ML1601c peptides with discriminatory capacity.

Since peptide responses are HLA-restricted, a combination of multiple *M. leprae* peptides will be required to render a diagnostic test for leprosy broadly applicable. Thus, for the selection of peptides with the best performance in discriminating individuals with *M. leprae* infection and/ or -exposure based on their capacity to induce IFN-γ production in PBMC, a factor analysis was performed using the IFN-γ data (Figure 2) induced by all ML1601c peptides and the ML1601c protein (Table II).

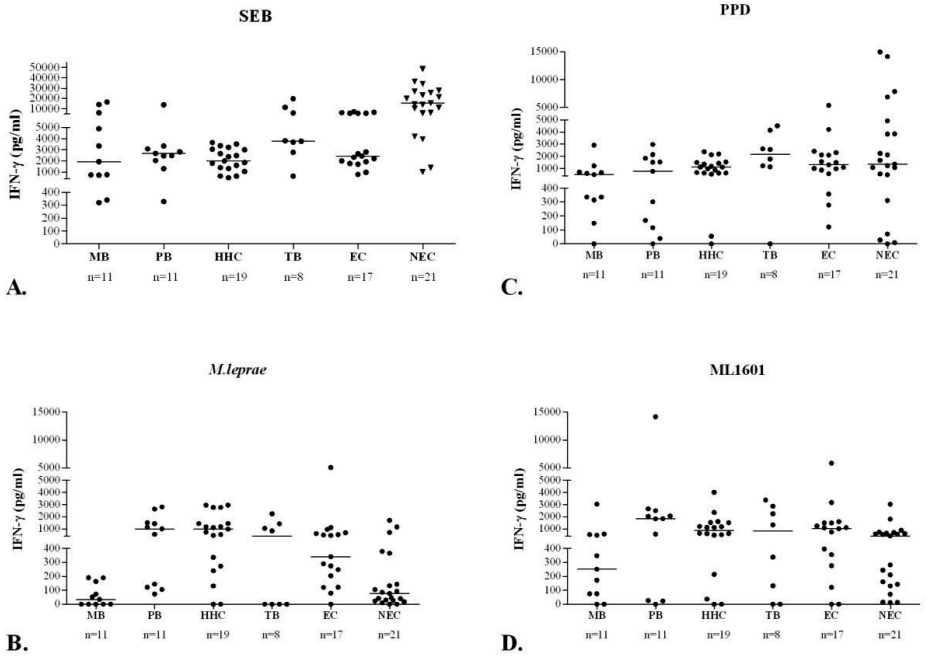

Figure 1 IFN-γ production by PBMC induced by SEB (**A.**), PPD (**B.**), *M.leprae* (**C.**) and ML1601c recombinant protein (**D.**) in MB (n = 11), PB (n = 11), HHC (n=19), TB (n = 8) and EC (n = 17) from Brazil as well as in Dutch NEC (n = 21). For NEC PHA was used instead of SEB. Values were corrected for background values. All background values were typically < 20 pg/ml. Horizontal bars indicate median responses.

This type of analysis has the potential to group together peptides inducing comparable patterns of IFN–γ responses, and as a consequence presenting high correlations (factor loadings) with the same factor. The 3 factors obtained from the IFN-γ responses to the ML1601c protein and the ML1601c peptides can in fact be linked to features relevant in the selection of peptides for use in diagnostic tests. Peptides presenting high correlations with factor 1 (factor loading > 0.8; p3, p11, p12, p13 and p16) induce high-level responses only in a subset of the exposed and/ or infected individuals (MB, PB, HHC) but not on those for which exposure is less likely (EC), absent (NEC) or TB patients. Therefore, IFN-γ production induced by these peptides was depicted for each peptide separately as well as for the sum of the IFN-γ values for all five of these ML1601c peptides combined (Figure 3). This figure shows that IFN-γ levels in response to p13 were most frequent but were also observed in three EC and in one NEC, whereas p3, p11 and p16 showed very specific responses only in leprosy patients and in *M. leprae* exposed HHC. This analysis clearly shows that *M. leprae*-specific IFN-γ responses can be induced selectively in PBMC derived from *M. leprae* exposed and/ or infected individuals by peptides derived from a protein that is not uniquely present in the *M. leprae* genome. The ML1601c protein and p17 were highly correlated with factor 2. Responsiveness to these two stimuli was present in the exposed and/ or infected groups, in the EC and in TB patients. So, the ML1601c protein and p17 (correlated to factor 2) are not useful antigens in terms of potentially discriminating *M. leprae* infection or disease. ML1601c p15 and p20 (correlated to factor 3) share with the factor 1 subset specificity for exposed and/ or infected individuals. However, p15 and p20 also stimulate EC rendering these peptides not useful for leprosy diagnostics either.

3.3.Whole blood assays using ML1601c peptides in Ethiopian healthy controls.

ML1601c p11 and p16 induced significant IFN-γ responses (> 200 pg/ml; Figure 3) in 10 and 11 *M. leprae* exposed or infected Brazilians, respectively, indicating recognition of these peptides in the context of various HLA-alleles. Since one of the aims of this study was to develop field-friendly test that is world-wide applicable, IFN-γ production in response to a mix of these peptides was analyzed in a 24-hour WBA [20] using 34 healthy controls from areas in Ethiopia with different leprosy prevalence (Figure 4; EC_{low}: n=16; EC_{high}: n=18). Although both groups responded equally well to the positive control stimulus PHA (Figure 4A), there was a significant difference ($p = 0.0067$) between IFN-γ responses induced by the ML1601c peptide mix in individuals from an area with low endemicity (EC_{low}) compared to those from an area with high endemicity (EC_{high}).

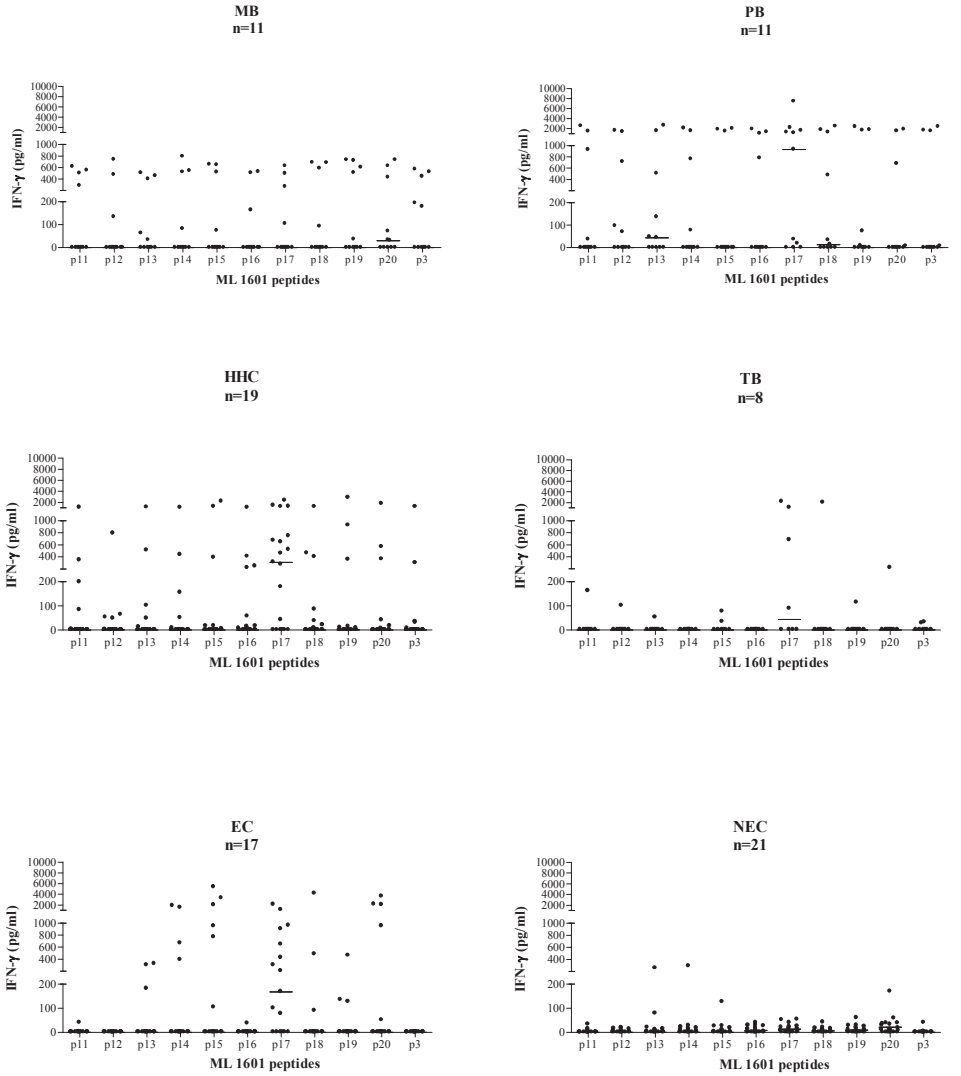

Figure 2 IFN-γ production by PBMC induced by ML1601c peptides (see Table I) in MB (n = 11), PB (n = 11), HHC (n=19), TB (n = 8) and EC (n = 17) from Brazil as well as Dutch NEC (n = 21). Values were corrected for background values. All background values were typically < 20 pg/ml. Horizontal bars indicate median responses.

Thus, WBA show that IFN-γ levels induced by ML1601c peptides selected on the basis of IFN-γ responses induced in *M. leprae* exposed or infected individuals in Brazil, can be detected as well in Ethiopian individuals exposed to *M. leprae*. IFN-γ responses to these peptides in a field-friendly 24-hour WBA can therefore be used as indication of the magnitude of the *M. leprae* transmission level in a given population.

3.4. Sequence homology of ML1601c peptides.

Since *M. avium paratuberculosis* contains a homologue of ML1601c (MAP3249), the sequence of ML1601c was aligned with that of MAP3249 and the amino acid identity was determined for ML1601 peptides (Table I). This alignment showed that the percentage identity in general was not very high, with 42 % and 35 % identical to MAP3249 for p11 and p16 respectively. Immunogenicity of the peptides did not correlate with the percentage identity, as p17 and p12 both had high percentage identical sequence (52 % and 45 %, respectively) but only p17 was recognized by many individuals. Thus, although a homologue of ML1601c protein is present in *M. avium paratuberculosis*, exact sequence identity is relatively low and allows induction of specific T-cells responses in *M. leprae* exposed individuals by ML1601c peptides.

3.5.Multiplex determination of cytokines and chemokines in response to ML1601c peptides.

Immunological correlates of protection in leprosy are still lacking: although antigen-specific IFN-γ production is often used as a biomarker for *M. leprae* infection [9], it is possible that additional cytokines might allow more specific or qualitatively different detection of immune responses against *M. leprae* peptides.

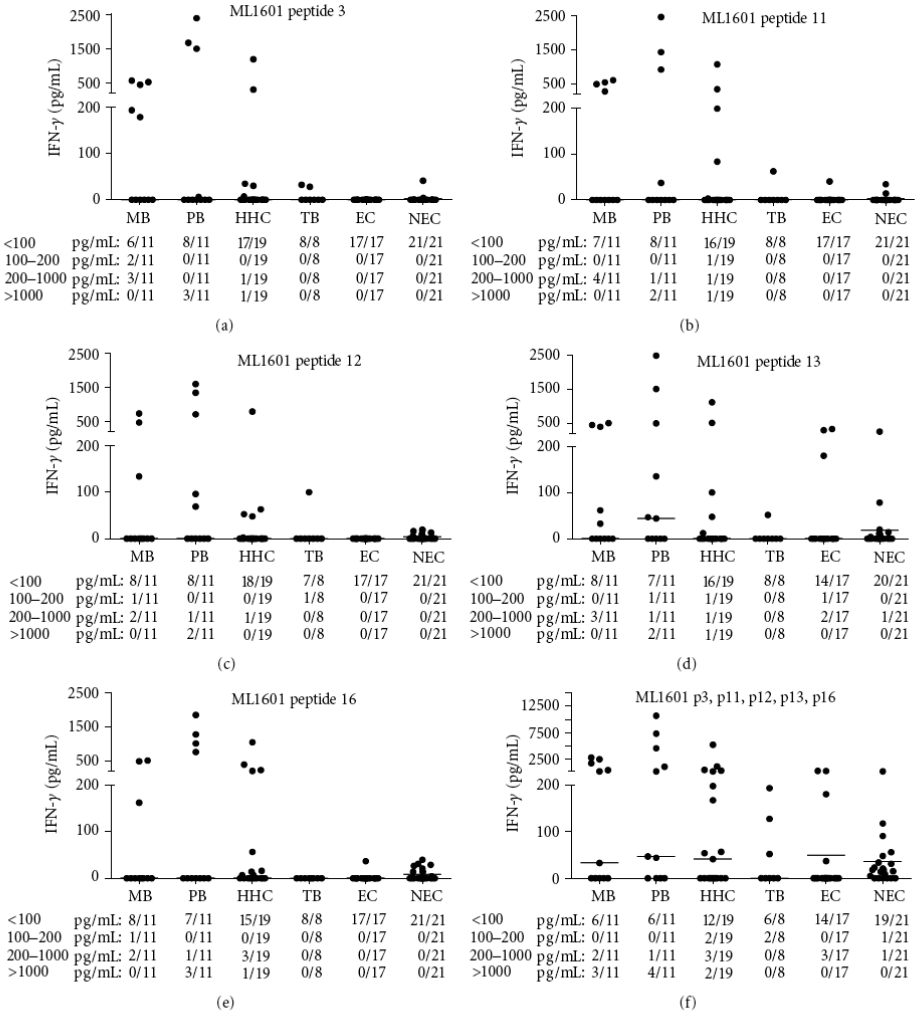

Figure 3: IFN-γ production by PBMC induced in all test groups by ML1601c peptides p3, p11, p12, p13, and p16 and the sum of the IFN-γ values for p3, p11, p12, p13, and p16 combined. The proportions of responders in each test group are indicated below the x-axis.

In order to further characterize the cellular immune response directed against ML1601c peptides, 15 additional cytokines and chemokines were tested in multiplex assays on identical supernatants as those used for IFN-γ (described above, Figure 4) obtained from the 24-hour WBA stimulated with a mix of ML1601c p11 and p16 using 34 healthy Ethiopian individuals. Although hardly any responses were detected for IL-17, G-CSF, VEGF, IL-1α, IL-10 and GM-CSF, nor any significant differences observed between EC_{low} and EC_{high} for the levels of IL-12, MIP-1β, MIG and IL-8 (data not shown), significantly different levels were observed between these two groups when IL-1β ($p = 0.0042$), IL-6 ($p = 0.0006$), IP-10 ($p = 0.0001$), TNF-α ($p < 0.0001$) or MCP-1 ($p = 0.0347$) were measured (Figure 5). Thus, in addition to IFN-γ, detection of these cytokines can also be used to indicate the magnitude of the *M. leprae* transmission level in a given population. Whether or not such cytokine responses also

indicate disease development or, alternatively, protection from disease will as yet have to be determined in longitudinal follow-up studies in HHC. Such studies are currently underway in highly leprosy endemic areas.

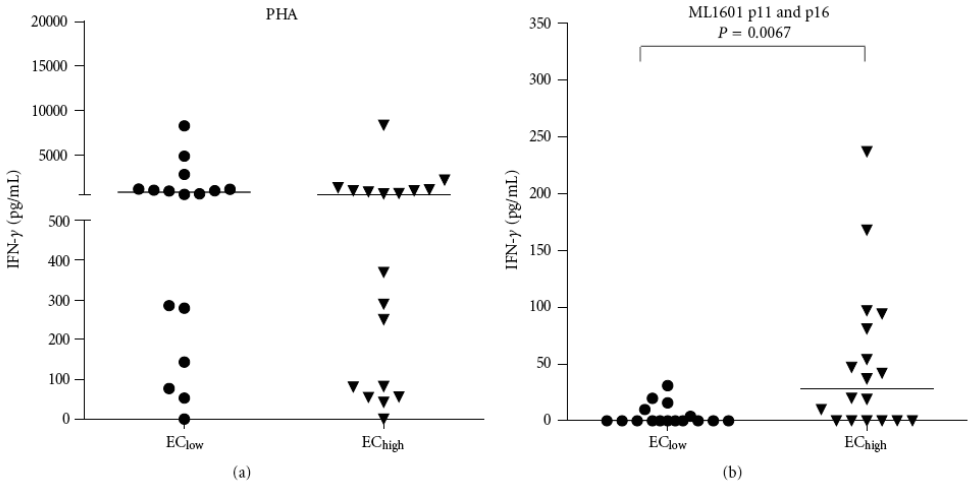

Figure 4: IFN-γ production in response to the positive control PHA (a) or a mix of ML1601c peptides no. 11 and no. 16 (b) measured after 24h culture of undiluted whole blood derived from 34 Ethiopian healthy controls (ECs) derived from areas with low endemicity for leprosy (EC_{low}; *n=16*) or from areas highly endemic for leprosy (EC_{high}; *n=18*).

Discussion

It is quite clear that elimination of leprosy requires, in addition to multidrug therapy (MDT), novel diagnostic tools that allow early detection of preclinical *M. leprae* infection, likely the major source of unidentified transmission. Also, the fact that children are still developing leprosy suggests that MDT has not substantially reduced transmission [2;3]. Therefore, identifying antigens that can be used as tools in diagnostic tests has been an important topic in leprosy research the last two decades.

In classical, PBMC-based IFN-γ release assays, *M. leprae* peptides have been shown to discriminate in a more specific fashion than proteins between *M. leprae* exposed contacts and patients as opposed to healthy controls from the same endemic area [10;12]. Our previous studies identified *M. leprae* peptides derived from proteins such as ML1989, ML1990, and ML2567 that induced IFN-γ in a 6 day proliferation assay using PBMC. The slight disadvantage of peptides though is that they usually induce significantly lower levels of IFN-γ than proteins, particularly when whole blood is used [13;20]. This could, however, be inherent to the selected peptides as for TB diagnosis; the combination of > 20 peptides is used successfully in WBA-based IGRA such as QuantiFERON®-TB Gold assay. Therefore, more peptides, shared in different *M. leprae* strains that can be applied in diagnostic tools for leprosy should be identified and tested in the context of various genetic backgrounds in South-America, Asia and Africa to enable development of a peptide-based WBA.

The Brazilian population can roughly be divided in three ethnic groups, namely from Caucasian, indigenous and African descent. Given this genetical diversity and the extraordinarily high leprosy endemicity compounded by poverty in several of its areas, Brazil is a suitable region for developing globally applicable T-cells based diagnostic tools. Indeed,

this study shows that even HLA-restricted, *M. leprae* peptides can be identified in a Brazilian population and applied to measure *M. leprae* exposure in an African population in Ethiopia. Two ML1601c peptides, p11 and p16, only induced IFN-γ production in PBMC from leprosy patients and HHC in Brazil and not, unlike ML1601c protein, in TB patients, EC or NEC. The combination of these peptides could be applied in a field-friendly, 24-h WBA in Ethiopia to estimate exposure to *M. leprae*. This is consistent with the findings of other *M. leprae* peptides (Martins *et al.,* submitted) thereby indicating that combinations of peptides can be designed and used efficiently to indicate substantial exposure to *M. leprae*.

The observation in this study that ML1601c protein induced significant IFN-γ responses in EC, TB as well as some NEC, is in agreement with the finding that the use of recombinant proteins coincides with an increased risk of detecting cross-reactive T-cells responses irrespective of overall sequence homology. In addition, purification and quality control assays for recombinant proteins are more labor-intensive than is the case for synthetic peptides. Therefore, despite the fact that T-cells responses to peptides are HLA-restricted, which may limit the applicability of single peptides with respect to diagnostic T-cells-based assays in genetically diverse populations [21], a cocktail of *M. leprae* peptides can be used to identify *M. leprae* exposure in genetically different populations.

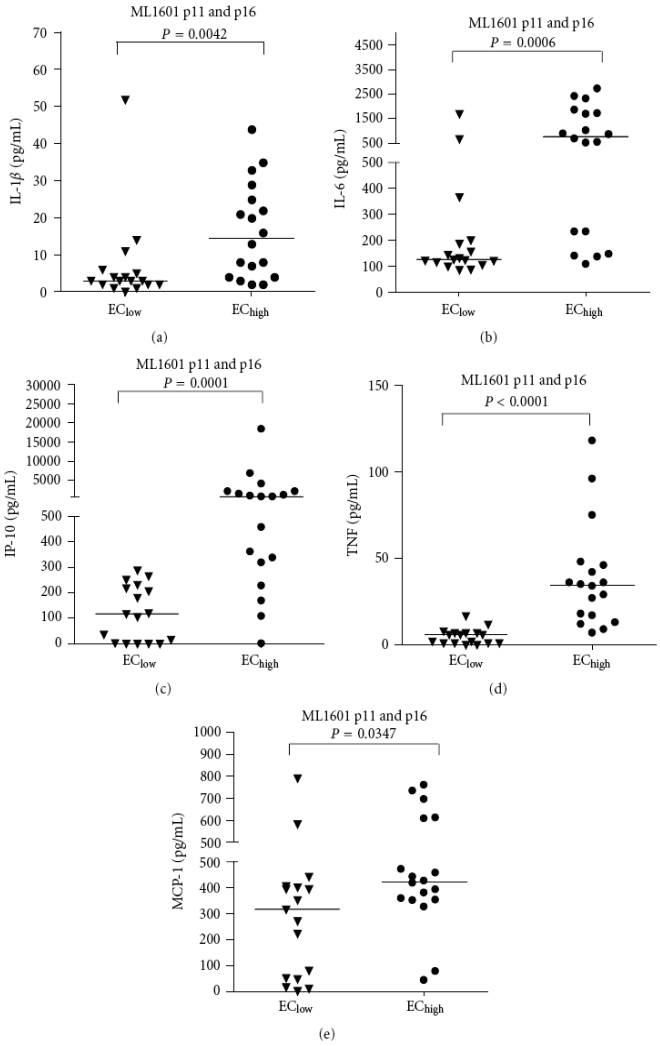

Figure 5: Production of IL-1β (a), IL-6 (b), IP-10 (c), TNF-α (d), and MCP-1 (e) in response to selected ML1601c peptides measured after 24h culture of undiluted whole blood derived from 34 Ethiopian healthy controls (ECs) derived from areas with low endemicity for leprosy (EC_{low}*: n=16*) or from areas highly endemic for leprosy (EC_{high}*: n=18*).

An alternative approach that we addressed here is that alternate cytokines or chemokines may be able to provide a distinction between progression to disease and containment of *M. leprae* infection. Therefore, we also tested supernatants of whole blood cultures stimulated with ML1601c p11 & p16 for 15 additional cytokines: significantly different levels were observed between EC_{low} and EC_{high} when IL-1β ($p = 0.0042$), IL-6 ($p = 0.0006$), IP-10 ($p = 0.0001$), TNF-α ($p < 0.0001$) or MCP-1 ($p = 0.0347$) were used as read-outs. Thus, in addition to IFN-γ, detection of these cytokines can also be used to estimate the magnitude of the *M. leprae* transmission level in a given population. The significant differences observed for both IL-1β and IL-6 suggest differences in the innate responses between the test groups [22]. For TB susceptibility it has been described that the polymorphism at the IL-1 locus influences the

cytokine response and may be a determinant of delayed-type hypersensitivity and disease expression in human tuberculosis [23]. For leprosy, however, no association with IL-1β polymorphism has been described [24].

In combination with classical detection of anti-PGL-I IgM antibodies, *M. leprae* peptide-based WBA measuring cytokines will not only allow detection of most forms of leprosy (PB and MB) but may also identify those at risk of developing disease by detecting preclinical forms of leprosy, thereby enabling installment of MDT at an early stage. Additional *M. leprae* peptides will presumably be identified in the future, but to ensure the success of developing an affordable, field-friendly test for the early diagnosis of leprosy, continued funding for these efforts will be critical.

Acknowledgements

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3.2 *Mycobacterium leprae* virulence-associated peptides are indicators of exposure to *M. leprae* in Brazil, Ethiopia and Nepal

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Abstract

Silent transmission of *Mycobacterium leprae*, as witnessed by the stable leprosy incidence in various countries, remains challenging despite worldwide implementation of multidrug therapy (MDT). The development of tools for early diagnosis of *M. leprae* infection should therefore be emphasized in leprosy research. As part of the continuous effort to identify antigens with diagnostic potential, *M. leprae* unique peptides derived from predicted virulence-associated proteins (groupIV.A; http://www.sanger.ac.uk/Projects/M_leprae/Ml_gene_list_hierarchical.shtml) were identified using advanced genome pattern programs and bioinformatics. Based on the presence of HLA-binding motifs, we selected 21 predicted promiscuous HLA-class I and 8 HLA-class II restricted T-cell epitopes as 9mers for further field-testing in Brazil, Ethiopia and Nepal. High levels of IFN-γ were induced by peptide ML2055 p35 in PBMC of TT/BT leprosy patients from Brazil and Ethiopia as well as controls from areas with high leprosy prevalence (EC_{high}) in Ethiopia, whereas Brazilian EC_{high} recognized ML1358 p20 and ML1358 p24. None of the peptides was recognized by non endemic controls. In addition, in Nepal peptide pools composed of these peptides induced IFN-γ by PBMC of leprosy patients as well as EC_{high}. Thus, these *M. leprae* virulence-associated peptides may be useful to differentiate *M. leprae* exposure in the context of different HLA polymorphisms.

Introduction

Despite the extensive decrease of the annual prevalence of leprosy since the introduction of multidrug therapy (MDT) over 30 years ago, a consistent number of new cases, including children, is still reported annually in a number of countries. This indicates continued and significant transmission at the population level, which challenges disease control efforts (WHO, 2011). The incubation period of *Mycobacterium leprae* (*M. leprae*) and the time elapsed before manifestation of symptoms in an infected individual ranges between 4 and 10 years but can even reach 30 years [7]. It is hypothesized that, most patients have been infected subclinically for a considerable amount of time before leprosy becomes apparent, thereby presumably representing a major source of *M. leprae* transmission.

Early detection of leprosy and prompt treatment with MDT will help to reduce transmission and infection, and thus have a major impact on preventing nerve damage, disabilities and deformities. However, there are currently no diagnostic tests available that can detect asymptomatic *M. leprae* infection. Development of a specific immunodiagnostic tool for leprosy requires adequate information about the specific antigens of the pathogen. The failure to grow *M. leprae* on artificial media [31] has greatly hampered leprosy research for many decades, including the development of specific diagnostic tools for leprosy. The recent availability of improved bioinformatics tools as well as the *M. leprae* genome sequence has opened up new possibilities in leprosy research, enabling the prediction of relevant proteins and potential HLA class I and class II epitopes that can activate T-cells [12]. The use in multiple studies of *M. leprae* unique antigens defined through such post-genomic approaches have led to the detection of *M. leprae*-protein or peptide specific T-cell responses that may help identify *M. leprae* exposed or -infected subjects [13;29;3;15;6;5].

Antigenic proteins usually contain multiple peptide epitopes and thus may be preferred as diagnostic tools in various populations containing different genetic backgrounds. However, an advantage of using peptides as diagnostic tools is the observed reduction in or lack of T-cell cross-reactivity compared to proteins [16;15]. Hence, analysing single *M. leprae* unique peptides, or pools thereof, in different leprosy endemic settings can be useful to identify promiscuous peptides with diagnostic potential across different genetic backgrounds. The immunogenic and diagnostic significance of *Mycobacterium tuberculosis* (*Mtb*) proteins ESAT-6 and CFP-10 and their peptides from the RD-1 region (involved in enhancing virulence) [4] have led us to search for similar possibilities in leprosy by using *M. leprae*-unique virulence-associated peptides. Hence, in the current study, the *M. leprae* whole proteome was *in silico* dissected into 20-mer amino acid peptides. Next, *M. leprae* unique peptides derived from group IV.A (virulence; http://www.sanger.ac.uk/Projects/M_leprae/Ml_gene_list_hierarchical. shtml) and predicted to bind promiscuously to HLA class I or II alleles were selected and synthesized. *In vitro* analysis of these synthetic peptides was performed using PBMC or whole blood derived from TT/BT and BL/LL leprosy patients and healthy endemic controls (EC) from areas with high or low leprosy prevalence in Brazil, Ethiopia and Nepal.

Materials and Methods

Peptide search strategy. The peptide identification procedure is depicted in Figure 1: all 20-mers in the *M. leprae* genome [9] were identified. A length of 20 amino acids was chosen since this may accommodate for both HLA class I and class II T-cell epitopes. 20-mers with an overlap of eight or more amino acids in a continuous stretch with sequences from other

mycobacteria, from completed or nearly complete genomes and from sequences available in the entire database of GenBank (www.ncbi.nlm.nih.gov/GenBank) were excluded from this study. This selection procedure is described in more detail below.

Bacterial genomes. All genomes used in this study were retrieved from GenBank and consist of a total of six completely sequenced mycobacterial genomes: *M. leprae* (GenBank Protein accession number: NC_002677), *M. tuberculosis* strains H37Rv (AL123456 and NC_000962) and CDC1551 (NC_002755), *M. bovis* (NC_002945) and *M. avium paratuberculosis* (NC_002944). Other (nearly) completely sequenced mycobacterial genomes (unpublished at the time of peptide selection) such as *M. avium*, *M. smegmatis*, *M. tuberculosis* strain 210 (www.tigr.org), *M. marinum*, *M. microti* (www.sanger.org), *M. paratuberculosis* and *M. ulcerans* (http://www.pasteur.fr/) were further included indirectly by using BLAST (described below) to give a broader picture of the conservation of 20-mers.

Bioinformatics tools. The complete *M. leprae* genome was divided into 20-mers with 19 amino acids overlap by using the GenomePatterns program resulting in the *M. leprae*-list. In order to check homology of *M. leprae* with closely related mycobacterial genomes that were completely sequenced, the GenomePatterns program was also used to generate a list of 20-mers with 19 amino acids overlap for *M. tuberculosis* strains H37Rv and CDC1551, *M. bovis* and *M. paratuberculosis* (the *MTB*-list). The *M. leprae*-list was then compared to the *MTB*-list and all 20-mers within the *M. leprae*-list which had a continuous stretch of eight or more identical amino acids to the *MTB*-list were excluded from further analysis. This resulted in 141,300 20-mers which are unique to the *M. leprae* genome. The 20-mers coded by any of the 1,116 pseudogenes of *M. leprae* were excluded, reducing the *M. leprae*-list to 138,938 20-mers derived from 1,546 different *M. leprae* candidate proteins. To narrow down the number of peptide candidates that needed to be blasted, we selected peptides derived from genes in functional classification group IV.A (virulence; including the following 13 genes: ML0360, ML0361, ML0362, ML0885, ML1214, ML1358, ML1811, ML1812, ML2055, ML2208, ML2466, ML2589, ML2711; http://www.sanger.ac.uk/Projects/M_leprae/Ml_gene_list_hierarchical.shtml, currently designated as *genes involved in virulence, detoxification and adaptation* or *genes involved in cell wall and cell processes* on http://mycobrowser.epfl.ch/leprosy.html, resulting in 886 candidate 20-mers. Next, we used a perl script "genediff.pl" that compares genomes using BLAST (CBS, script used internally) and excluded proteins homologous with the human genome and three homologs with *M. tuberculosis*. The overlapping *M. leprae*-derived 20-mers were assembled (if they occurred in sequential order) resulting in 40 protein fragments. BLAST was used to exclude fragments that were found in unfinished mycobacterial genome sequences of *M. smegmatis*, *M. tuberculosis* strain 210 and *M. microti* OV254 (www.sanger.org and www.tigr.org). In addition, the assembled *M. leprae* fragments were BLASTed against all sequences (not only mycobacterial) available in GenBank (http://www.ncbi.nlm.nih.gov/blast/). All hits that had an identity of more than eight amino acids with *M. leprae* peptides were again excluded. Out of the 40 fragments, 14 from 6 proteins remained unique for *M. leprae.*

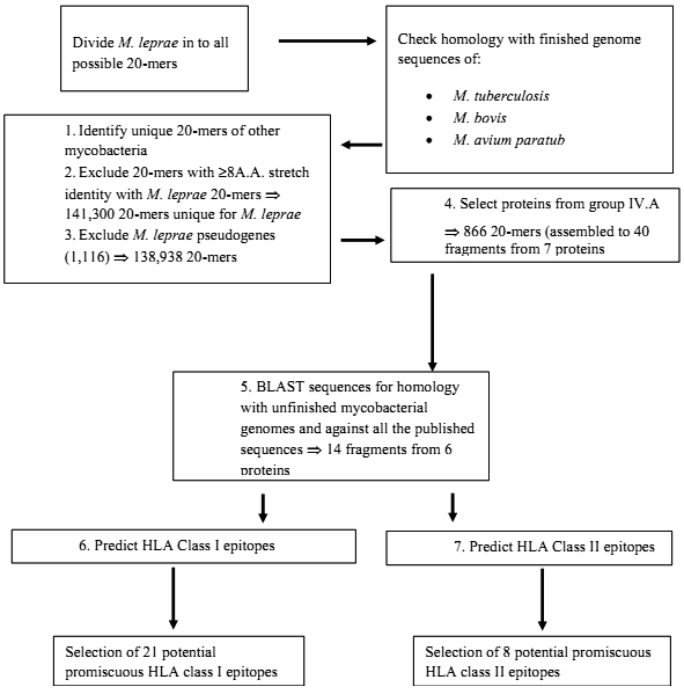


Figure 1: *M. leprae* peptide selection procedure. Identification and selection of predicted *M. leprae* promiscuous HLA class I epitopes (n = 21) and predicted *M. leprae* promiscuous HLA class II epitopes; n = 8) derived from *M. leprae* proteins from functional classification group IV.A (virulence; http://www.sanger.ac.uk/Projects/M_leprae/Ml_gene_list_hierarchical.shtml).

Prediction of CD4 and CD8-restricted T-cell epitopes. NetCTL version 1.2 (www.cbs.dtu.dk/services/NetCTL) was used to predict 9-mer epitopes for $CD8^+$ T-cells from the 14 *M. leprae* unique fragments as described previously for 12 supertypes (HLA-A1, A2, A3, A24, A26 and HLA-B7, B8, B27, B39, B44, B58, B62) [19]. All peptides with a combined score above 1.25 were selected as potential MHC class I ligands. An adapted version of the program TEPITOPE [28] was used in this study to predict CD4-restricted T-cell epitopes from the 14 *M. leprae* unique fragments that were found in a total of 6 *M. leprae* proteins. These 6 protein sequences were submitted to the SubCell 1.0 server (*www.cbs.dtu.dk/services/)* in order to predict the subcellular localization of these proteins for gram-negative and gram-positive bacteria. From the resulting sequences peptides were selected in such a manner that predicted binding sequences were included for most HLA alleles. In this selection, priority was given to peptides predicted to bind promiscuously to multiple HLA alleles (Table I). This selection resulted in 21 potential CD8-restricted T-cell epitopes and 8 potential CD4-restricted T-cell epitopes (Tables I and II). In case of induction of a positive CD4 response by 9mers (possibly suboptimal length), only the strongest inducers will be identified using this approach.

Synthetic peptides. The identified virulence-associated *M. leprae*-derived peptides were purchased from Peptide 2.0 Inc. (Chantilly, VA, USA). Homogeneity and purity were confirmed by analytical HPLC and by mass spectrometry. Purity of all peptides was ≥ 80%. All impurities consist of shorter versions of the peptides caused by < 100% coupling efficiency in each round of synthesis. All peptides were divided in aliquots to allow field testing of identical batches in Brazil, Ethiopia, Nepal and The Netherlands.

Table I
Selected *M. leprae* virulence-associated peptides (HLA class I; n=21)

#	Peptide (9 mer)	Starting-position	ML accession number	Accession number	HLA	HLA	HLA
p15	RAAVVQAAL	262	ML0885	NP_301670.1_245_270	B7	B8	B58
p16	SMDAAVAAL	193	ML1812	NP_302233.1_181_201	A2	B39	
p17	GIAGSASYY	202	ML2055	NP_302372.1_189_211	A1	B62	
p18	HRKGLWAIL	10	ML2055	NP_302372.1_1_78	B27	B39	
p19	QMLEASSSV	210	ML1811	NP_302232.1_209_232	A2		
p20	ALDTFGIPV	73	ML1358	NP_301968.1_64_92	A2		
p21	NGIAGSASY	201	ML2055	NP_302372.1_189_211	A26		
p22	KVTVSSVRK	220	ML1811	NP_302232.1_209_232	A3		
p23	TEAVHSAQL	58	ML0885	NP_301670.1_54_76	B44		
p24	KLMGALDTF	69	ML1358	NP_301968.1_64_92	B58		
p25	VASASAFTM	23	ML2055	NP_302372.1_1_78	B58		
p26	AVVASASAF	21	ML2055	NP_302372.1_1_78	B62		
p27	APLPPSTAT	42	ML2055	NP_302372.1_1_78	B7		
p28	GPVPAVATL	220	ML0885	NP_301670.1_220_250	B7		
p29	IPVAGRCCL	79	ML1358	NP_301968.1_64_92	B7		
p30	RPRRGSVSR	3	ML1812	NP_302233.1_1_20	B7		
p31	LPSADIVPM	172	ML1358	NP_301968.1_158_181	B7		
p32	SASAFTMPL	25	ML2055	NP_302372.1_1_78	B7		
p33	APIPASVSA	274	ML2055	NP_302372.1_257_287	B7		
p34	RPVPVSTAR	204	ML1214	NP_301879.1_173_212	B7		
p35	IPASVSAPA	276	ML2055	NP_302372.1_257_287	B7		

M. leprae recombinant proteins. *M. leprae* genes encoding proteins from which the virulence-associated peptides derived 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) [11] 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 [11]. 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 recombinant protein as tested using a Limulus Amebocyte Lysate (LAL) QCL-1000 assay (Lonza Inc., Basel, Switzerland). 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 (available through the Biodefense and Emerging Infections Research

Resources Repository listed at http://www.beiresources.org/TBVTRMResearch Materials/tabid/1431/ Default.aspx).

Study participants. The following HIV-negative individuals were recruited between August 2008 and February 2011: in Brazil: 10 TT/BT leprosy patients, 10 healthy controls 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 23 leprosy patients (tuberculoid /borderline tuberculoid (10 TT/BT) and borderline lepromatous/lepromatous leprosy (13 BB/BL), 12 HHC of BL/LL patients and 52 healthy controls were tested: 25 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 27 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. From Nepal, 7 TT/BT and 5 BL/LL patients and 20 EC_{high} were enrolled in this study. The national leprosy prevalence in Nepal was 1.1 per 10,000 in 2008/ 2009 (Annual report 2008/ 2009, Leprosy control division, Nepal).

In all settings, leprosy was diagnosed based on clinical, bacteriological and histological observations and classified by a skin biopsy evaluated according to the Ridley and Jopling classification [22] by qualified microbiologists and pathologists. All patients were recruited when newly diagnosed and were untreated and did not develop leprosy reactions within 3 months of MDT initiation. EC were assessed for the absence of clinical signs and symptoms of tuberculosis and leprosy. Staff members working in the leprosy centers or TB clinics were excluded as EC.

Whole blood assays (24 h 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 (100 µg/ ml). After 24 h, 150 µl of supernatants were removed from each well and frozen in aliquots at –20°C until further analysis.

Table II
Selected *M. leprae* virulence-associated peptides (HLA class II; n=8)

#	Peptide (9-mer)	Starting-position	ML number	Accession number	HLA	HLA	HLA
p36	VVRDLRLRA	197	ML1358	NP_301968.1_192_213	1_0301	1_1101	1_1501
p37	WAILAIAVV	15	ML2055	NP_302372.1_1_78	1_0101	1_0801	1_1101
p38	ILAIAVVAS	17	ML2055	NP_302372.1_1_78	1_0301	1_0401	1_1101
p39	VRPVPVSTA	203	ML1214	NP_301879.1_173_212	1_0301	1_1101	
p40	LRADSVLAV	203	ML1358	NP_301968.1_192_213	1_0301	1_0401	
p41	LQQVPTLPA	199	ML1214	NP_301879.1_173_212	1_1101		
p42	LAIAVVASA	18	ML2055	NP_302372.1_1_78	1_1101		
p43	ISLATVLSA	158	ML1358	NP_301968.1_158_181	1_1101		

Lymphocyte stimulation tests (LST). PBMC were isolated by Ficoll density centrifugation from venous, heparinized blood and plated in triplicate cultures (2 x 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). Synthetic peptides, 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) [13]. 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 both laboratories as a reference positive control supernatant.

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 h 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). 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 5.00 for Windows (GraphPad Software, San Diego California USA; www.graphpad.com). P-values were corrected for multiple comparisons. The statistical significance level used was $p<0.05$.

Ethics. This study was performed according to ethical standards in the Helsinki Declaration of 1975, as revised in 1983. Ethical approval of the study protocol was obtained through the appropriate national or institutional ethics committees, namely: Brazilian National Council of Ethics in Research (CONEP), National Health Research Ethical Review committee (NERC) and the Nepal Health Research Council (NHRC). Informed consent was obtained from all individuals before venepuncture.

Results

Post-genomic approach for improved, *M. leprae*-specific CMI test antigens

Owing to the shorter length of CD8-restricted epitopes, reliable bio-informatic methods for their prediction were developed at an early stage (e.g. SYFPEITHI (http://www.SYFPEITHI.de). More recently, prediction methods for the longer CD4-restricted T-cell epitopes were designed by way of better training sets and algorithms. Notably, the PROPRED program (http://www.imtech.res.in) has been shown to accurately predict human T-cell epitopes and many of these have been confirmed experimentally [30;28].

Using the available genomic sequences and these bio-informatic tools, we identified *M. leprae*-unique candidate antigens that were subsequently screened *in silico* for potential T-cell epitopes (Figure 1 and Materials and Methods section). Using this post-genomic approach, *M. leprae*-unique sequences representing epitopes of *M. leprae* proteins derived from functional group IV.A (virulence) were identified. Synthetic peptides encoded by these sequences, designated in this study as ***M. leprae* virulence-associated peptides** (Table I) were used to evaluate cellular responses in leprosy patients and controls from Brazil, Ethiopia and Nepal.

T-cell recognition of *M. leprae* peptides in non endemic controls (NEC)

To exclude the induction of nonspecific T-cell responses by the selected *M. leprae* virulence-associated peptides, they were first tested in 6 days lymphocyte stimulation assays (LST) using PBMC as well as in 24 h whole blood assays (WBA) using undiluted venous blood from Dutch healthy controls (NEC). Most individuals showed high responses to PPD (7/8) and *M. leprae* WCS (5/8). None of the NEC showed detectable IFN-γ responses against any of the peptides tested separately or in pools using PBMC (Figure 2A) or in 24 h WBA (Figure 2B), thereby ensuring the absence of *M. leprae*-non specific T-cell responses.

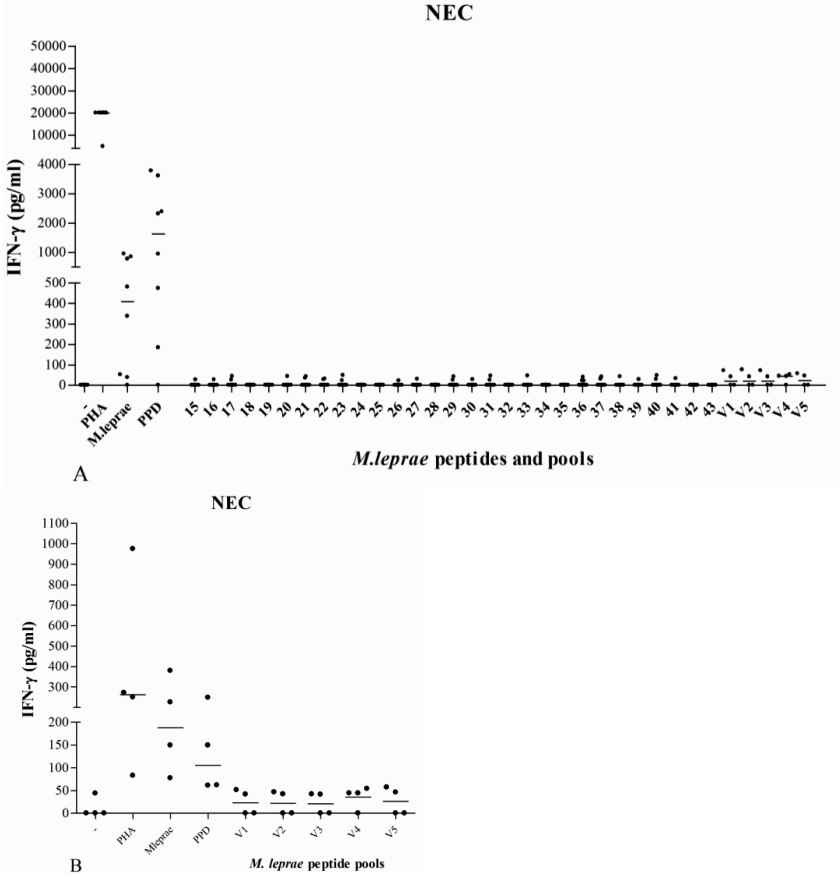


Figure 2: **IFN-γ responses to *M. leprae* peptides in PBMC from NEC.** IFN-γ production (corrected for background values) induced using *M. leprae* virulence-associated peptides or pools thereof (**A**) in 6 day PBMC cultures of Dutch healthy controls (n = 8) or as pools in a 24 h WBA (**B**). Pool V1: 15-20; V2: 21-26; V3: 27-32; V4: 33-35, 37-39; V5: 36, 40- 43 (numbers indicate peptide sequences depicted in Table I and II).

T-cell recognition of *M. leprae* peptides in PBMC from Brazilian test groups
The overall aim of our study was to identify new antigens that specifically indicate *M. leprae* exposure and/ or infection. Thus, *M. leprae* virulence-associated peptides (Table I) were first tested in a Brazilian population using PBMC in 6 days LST. For this study we enrolled tuberculoid or borderline tuberculoid leprosy patients (TT/BT), healthy endemic controls living in an urban area with high leprosy prevalence (P>4/10000; EC_{high}) and endemic controls living in the same city in an area with low prevalence (P<0.2/10000; EC_{low}). In the Brazilian EC_{high} group 50% and 70% specifically recognized ML1358 p20 and ML1358 p24 (Figure 3A), respectively, whereas none of the EC_{low} group nor the TT/BT group produced IFN-γ in response to these two peptides (Figure 3B). In contrast, ML2055 p35 induced considerable levels of IFN-γ (> 1000 pg/ml) in 40% of the TT/BT patients, whereas ML1214 p41, ML1812 p30, ML2055 p31, ML2055 p37, ML2055 p39 and ML2055 p42, induced IFN-γ responses in 30 - 40% of this group, although median values were lower (200 pg/ml; Figure 3C).

T-cell recognition of *M. leprae* peptides in PBMC from Ethiopian individuals
In order to accommodate differences in genetic backgrounds, test groups from different leprosy endemic settings need to be included, thereby allowing analysis of T-cell responses induced by peptides in the context of HLA polymorphism. Thus, the *M. leprae* virulence-associated peptides were also tested using PBMC derived from 12 Ethiopian TT/BT leprosy patients (Figure 3D) and 7 EC (2 EC_{high} and 5 EC_{low}; Figure 3E). The overall IFN-γ concentrations measured in response to the *M. leprae* virulence-associated peptides in both groups were lower compared to those in Brazil, with the exception of ML2055 p35 which, as was the case for Brazilian TT/BT, induced IFN-γ in 50% of the Ethiopian TT/BT patients (Figure 3D). In addition, one of the EC_{high} individuals responded to 8 of the 28 peptides (>100 pg/ml; Figure 3E). Thus, ML2055 p35 is recognized most frequently in TT/BT patients both in Brazil and in Ethiopia.

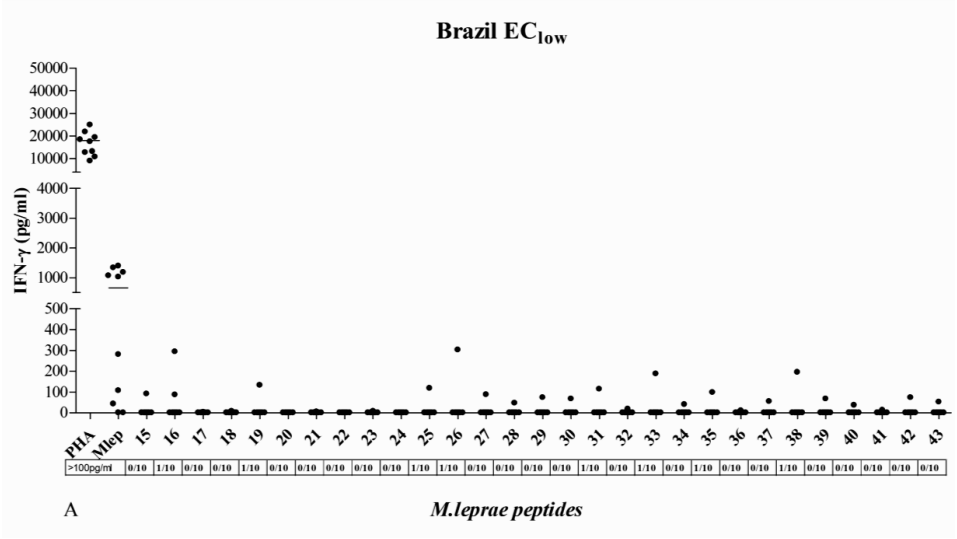
IFN-γ responses in Ethiopian EC_{high} and EC_{low} in WBA
In order to identify peptides with the ability to indicate exposure levels of *M. leprae* in a rapid field-assay, peptides ML2055 p35, ML2055 p42, ML2055 p37 and ML1358 p24 were selected for subsequent comparative analysis of Ethiopian EC_{low} (n = 17) and EC_{high} (n = 18) in a 24 h WBA. Two of these peptides, ML2055 p35 and ML2055 p42, which were recognized by Brazilian TT/BT patients also induced significant levels of IFN-γ in Ethiopian EC_{high} (p= 0.023 and p= 0.020, respectively) compared to EC_{low} (Figure 4). The IFN-γ levels in response to the other *M. leprae* peptides were low and no differences were observed between EC_{high} and EC_{low}.

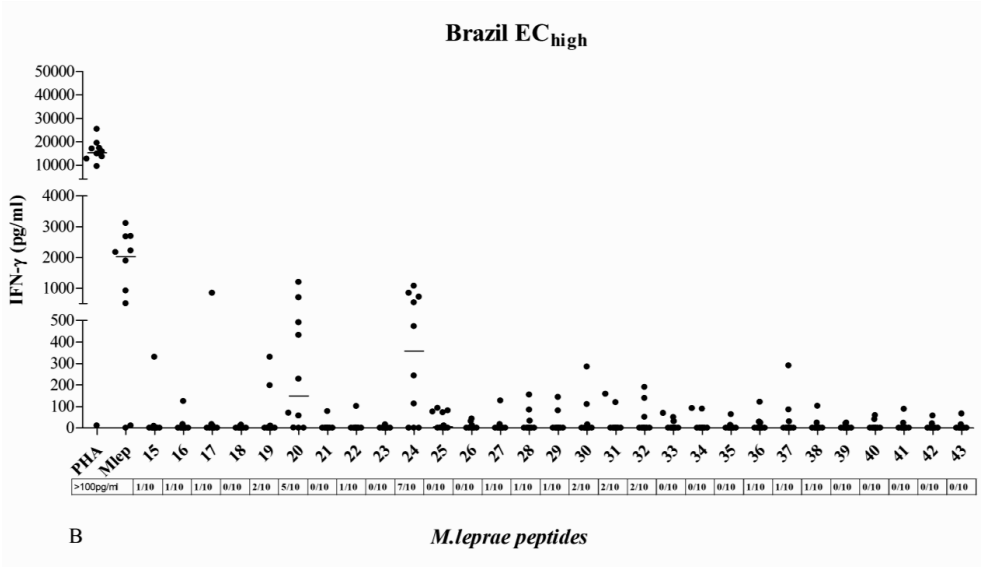
IP-10 as potential biomarker for diagnosis of *M. leprae* exposure
Although IFN-γ is the hallmark effector molecule of Th1 cells and a critical component of the pro-inflammatory immune response, host immunity and immuno-pathogenicity in response to *M. leprae* involves complex interactions between a variety of cells expressing different effector and regulatory molecules. Thus, assessment of multiple rather than single biomarkers may be more representative of the immune status of the host and may identify patterns predisposing to *M. leprae* infection. Therefore, aliquots of the 24 h WBA samples of Ethiopian EC_{high} and EC_{low} were also used for multiplex analysis of 19 additional cytokines/ chemokines. IFN-γ induced protein 10 (IP-10 or CXCL10) has been shown to be a useful biomarker for diagnosis of *M. tuberculosis* infection [28;24]. Interestingly, ML2055 p35 induced significant levels of IP-10 in EC_{high} but not in EC_{low} (p = 0.005; Figure 4E). ML2055

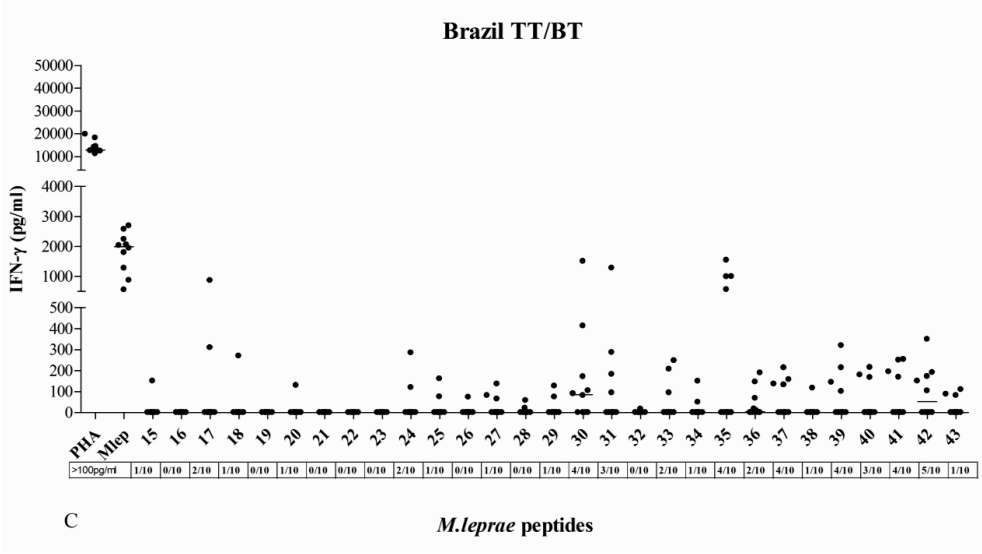
p42 also induced increased levels of IP-10 in EC_{high} compared to EC_{low} although the difference was not significant (p=0.06; Figure 4F). None of the other analytes showed significantly different levels between EC_{high} and EC_{low} (data not shown). Thus, the high IP-10 levels induced by *M. leprae* specific antigens in WBA of EC_{high} shows that this chemokine may have potential as a biomarker for differentiating levels of *M. leprae* exposure in new diagnostic tools in analogy to what has been reported for TB immunodiagnostic assays [23,2].

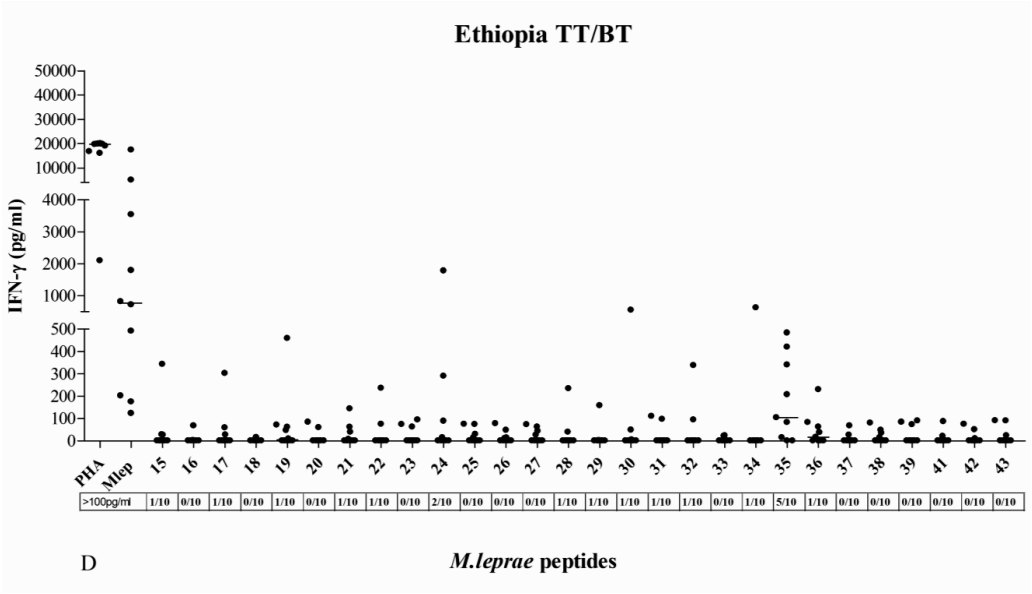
IFN-γ production in response to *M. leprae* recombinant proteins

Strikingly, the *M. leprae* virulence-associated peptides that induced IFN-γ responses in several individuals in this study were derived from only a few proteins, since p35, p37 and p42 were derived from ML2055, p20 and p24 from ML1358 and p41 from ML1214. To investigate the immunogenicity of the whole antigens, recombinant proteins ML2055, ML1358 and ML1214 were tested in 24 h WBA in Ethiopian BB/BL patients, HHC and EC (EC_{high}: n = 7 and EC_{low}: n = 3; Figure 5). ML2055 protein induced IFN-γ response (> 100 pg/ml) in 60% (7 out of 13) BB/BL and 42% (3 out of 7 EC_{high}), but only one HHC responded to this protein. IFN-γ responses against ML1358 were very low in EC and HHC, but significantly present in 60% of BB/BL patients responded to this protein. ML1214 induced IFN-γ response in 69% BB/BL, 66% HHC and 60% EC.




Brazil EC high
IFN-γ (pg/ml)
50000
40000
30000
20000
10000
4000
3000
2000
1000
500
400
300
200
100
0
PHA
MLep
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43
>100pg/ml
1/10 1/10 1/10 0/10 2/10 5/10 0/10 1/10 0/10 7/10 0/10 0/10 1/10 1/10 1/10 2/10 2/10 2/10 0/10 0/10 0/10 1/10 1/10 1/10 0/10 0/10 0/10 0/10 0/10
B
M.leprae peptides


Brazil TT/BT
IFN-γ (pg/ml)
50000
40000
30000
20000
10000
4000
3000
2000
1000
500
400
300
200
100
0
PHA
MLep
15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43
>100pg/ml
1/10 0/10 2/10 1/10 0/10 1/10 0/10 0/10 0/10 2/10 1/10 0/10 1/10 0/10 1/10 4/10 3/10 0/10 2/10 1/10 4/10 2/10 4/10 1/10 4/10 3/10 4/10 5/10 1/10
C
M.leprae peptides



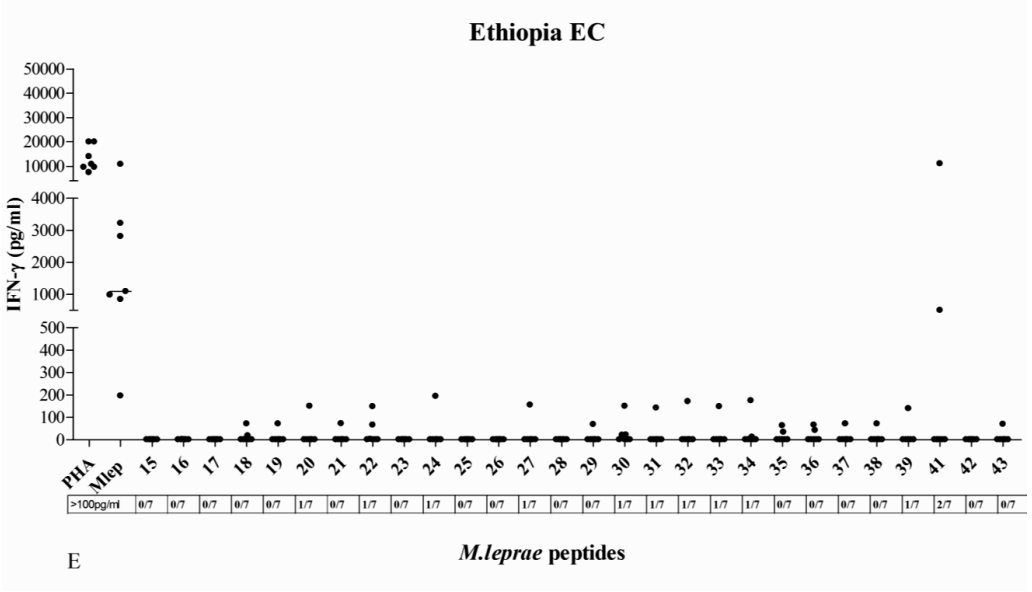


Figure 3: **IFN-γ responses to *M. leprae* peptides in PBMC from Brazilian and Ethiopian individuals.** IFN-γ production (corrected for background values) induced using *M. leprae* virulence-associated peptides in 6 day PBMC cultures of endemic controls from areas of Fortaleza with low (EC_{low}; prevalence <0.2/10,000; **A**; n = 10) and high (EC_{high}; prevalence > 4/ 10,000; **B**; n = 10) leprosy endemicity, Brazilian TT/BT patients (C; n = 10), Ethiopian TT/BT patients (**D**; n = 10) and Ethiopian EC (**E**; n=7). Median values per test group are indicated by horizontal lines. Background values were < 20 pg/ml.

T-cell responses to pools of *M. leprae* virulence-associated peptides in Nepal

To include data from an Asian endemic population, individuals from Nepal were also enrolled in this study. Here, *M. leprae* virulence-associated peptides were tested in 5 peptide pools (V1: p15-p20; V2: p21-p26; V3: p27-p32; V4: p33-p35, p37-p39; V5: p36, p40- p43) using PBMC of Nepali leprosy patients and EC. When peptide pools (V3, V4 and V5) containing ML2055 p35, p37 and p42, were similarly screened in Nepal, IFN-γ production was observed by PBMC of EC, but hardly by PBMC of leprosy patients before treatment (Figure 6). Interestingly, after completion of MDT, 3 out of 7 of the same BT patients showed increased concentrations of IFN-γ in response to the peptide pools (Figure 6C and 6F). Although, these findings will need confirmation in much larger numbers of subjects, they indicated that these peptides may have relevance for monitoring therapeutic intervention.

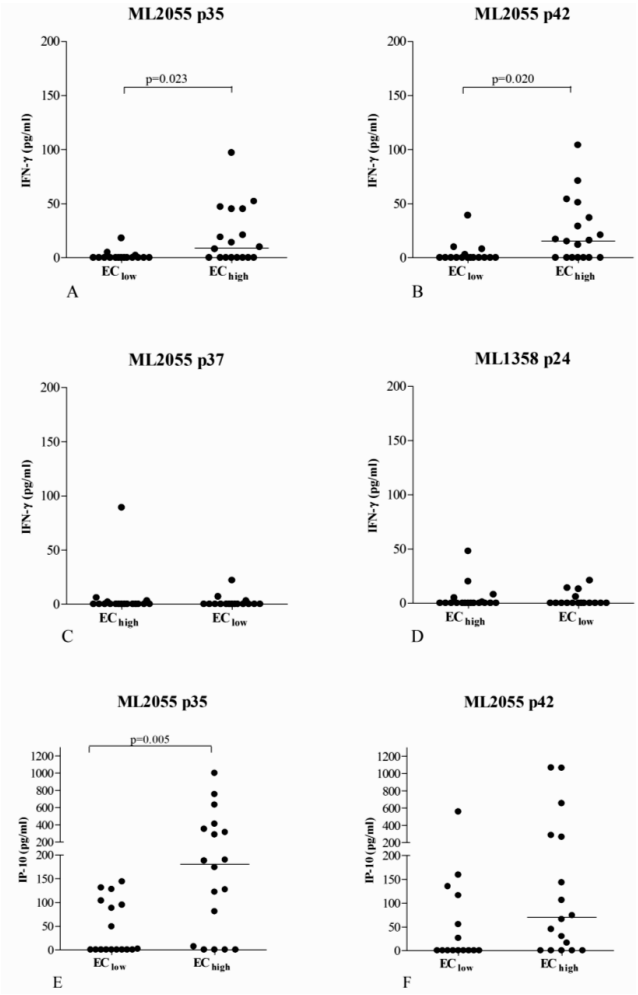


Figure 4: IFN-γ and IP-10 responses to *M. leprae* virulence-associated peptides in 24 h WBA of Ethiopian EC_{high} and EC_{low}. IFN-γ (**A-D**) and IP-10 (**E, F**) production in response to *M. leprae* peptides ML2055 p35 (**A**, **E**), ML2055 p42 (**B**, **F**), ML2055 p37 (**C**) and ML1358 p24 (**D**) in 24 h WBA of healthy individuals from areas in Ethiopia with low leprosy endemicity (EC_{low}; prevalence = 0.36/ 10,000; n=17) and high leprosy endemicity (EC_{high}; prevalence =1.5/ 10,000; n=18). Responses are corrected for background values. Median values per test group are indicated by horizontal lines.

Discussion

Globally, every year more than 200,000 people are newly diagnosed with leprosy at health facilities. The majority of these cases are multibacillary leprosy patients (MB) amongst including a considerable percentage of grade 2 cases as well as children [1]. The lack of tools for early detection of leprosy together with the complications that accompany leprosy reactions, represent the most important challenges still to combat in leprosy research [26;27].

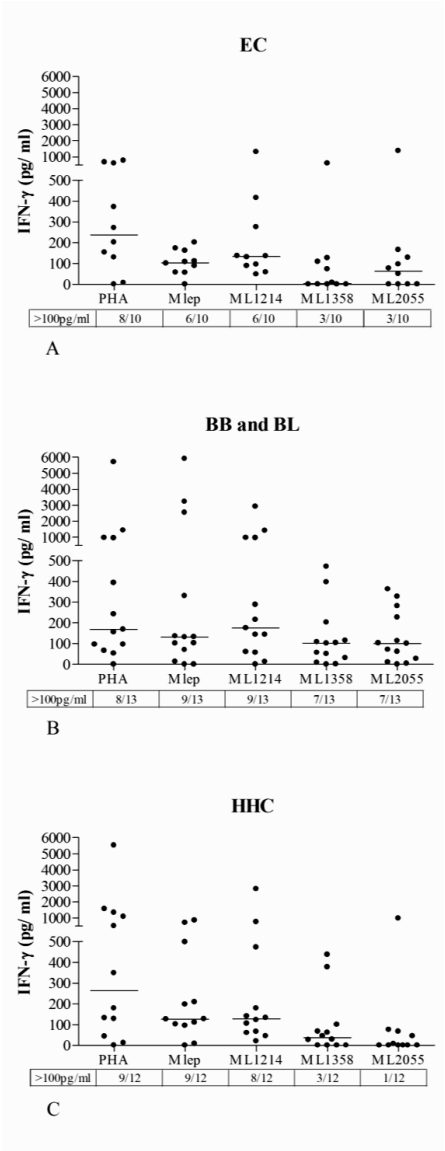


Figure 5: IFN-γ responses to newly identified *M. leprae* virulence-associated proteins.
IFN-γ production (corrected for background values) induced using *M. leprae* recombinant proteins ML1214, ML1358 and ML2055 in 24 h WBA from BB/BL (n=13), HHC (n=12) and EC (n=10) in Ethiopia

The availability of genome sequences of several organisms and the advanced application of bioinformatics has facilitated the search for potential unique antigens in leprosy research [10;29;16;15;6;12]. The current study builds upon our previous studies [13;29;15;17] although instead of aiming at hypothetical peptides derived from group VI (*M. leprae* proteins with unknown functions), this study focuses on peptides derived from virulence-associated *M. leprae* proteins (group IV.A). Twenty-nine *M. leprae* virulence-associated synthetic peptides were selected through bioinformatics-supported prediction of *M. leprae* unique sequences as potential targets of HLA class I and II and subsequently tested in different leprosy endemic areas in three continents for their potential to detect *M. leprae* exposure/ infection.

The peptides that induced T-cell reactivity in leprosy patients or healthy individuals living in areas hyperendemic for leprosy (EC_{high}) but not in NEC were mainly derived from three *M. leprae*-unique proteins: ML2055, ML1358 and ML1214. Consistent with the IFN-γ production observed in response to its single peptides, 7 out of 13 leprosy patients and 3 out of 7 Ethiopian EC_{high} indeed recognized ML2055 recombinant protein as well.

The differences in *M. leprae* peptide recognition patterns observed in this study between PBMC of leprosy patients and EC_{high} on one hand and EC_{low} on the other hand, imply their potential use to estimate the level of *M. leprae* exposure in individuals as described recently for ML1601-derived peptides as well [5]. Interestingly, ML2055 p35 and ML2055 p42 were recognized by Brazilian as well as Ethiopian leprosy patients. Moreover, these two peptides induced significant levels of IFN-γ as well as IP-10 in Ethiopian EC_{high}, suggesting that ML2055 p35 and ML2055 p42 can likely detect *M. leprae* exposure in the context of various HLA-alleles. Also, ML2055 has been described to induce strong serological responses in lepromatous patients [25]. The low responses to ML2055 in Ethiopian HHC compared to EC could have been due to overexposure to mycobacteria, as possible in HHC of MB, may result in T cell downregulation as hypothesized recently [20].

Despite similarities we also observed differences in peptide recognition patterns between Brazilian EC_{high} (ML1358 p20 and ML1358 p24) and Ethiopian EC_{high} (ML2055 p35 and ML2055 p42), reflecting the HLA-polymorphisms in these different areas. Both groups of peptides may be useful to indicate *M. leprae* exposure since neither Brazilian nor Ethiopian EC_{low} responded to these peptides. However, longitudinal analysis of T-cell responses induced by these peptides in a cohort of EC_{high} and household contacts of MB patients at multiple leprosy endemic sites may resolve whether these peptides can be used to predict progression to disease or merely indicate the level of *M. leprae* exposure.

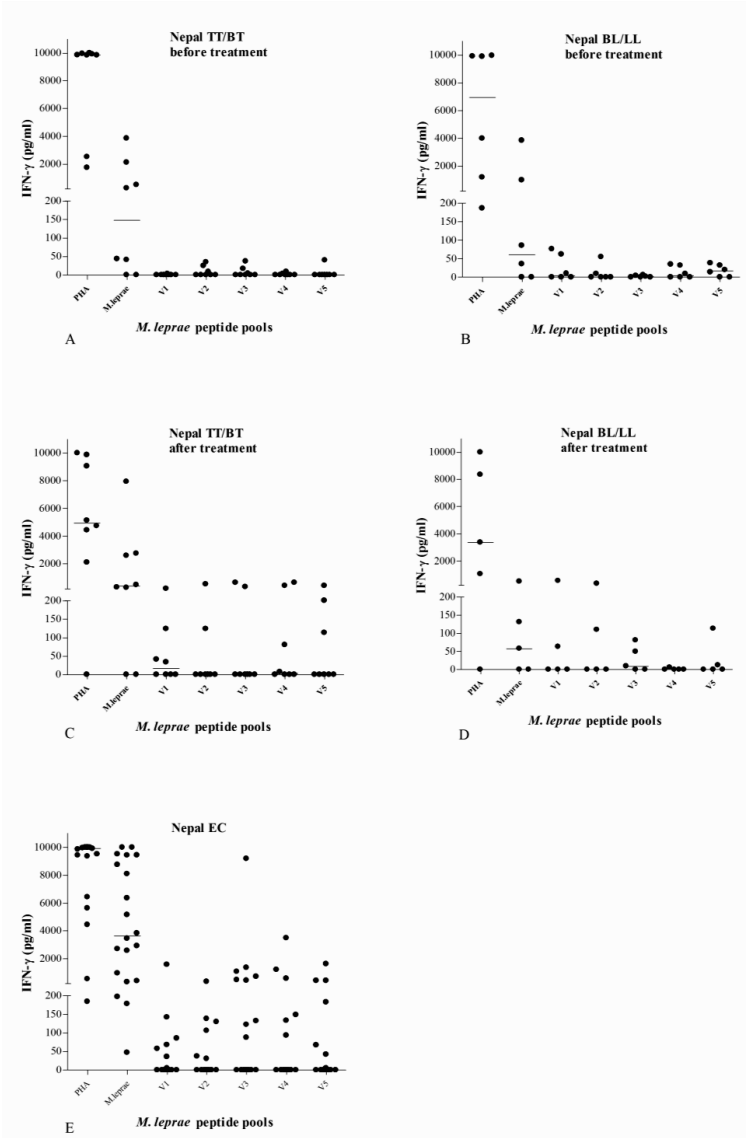


Figure 6: IFN-γ responses to *M. leprae* virulence-associated peptide pools in PBMC from Nepali individuals. IFN-γ production (corrected for background values) induced by pools of *M. leprae* virulence-associated peptides (10 µg/ml each) in 6 day PBMC cultures of newly diagnosed BT (**A**; n = 7) and BL/LL (**B**; n = 5) patients before MDT and after MDT (**C** and **D**) and EC (**E**; n = 20) from Nepal. IFN-γ responses of all leprosy patients before and after treatment in response to pool V4 (**F**); Pool V1: p15-p20; V2: p21-p26; V3: p27-p32; V4: p33-p35, p37-p39; V5: p36, p40- p43.

The benefits of testing peptide pools in detecting potential epitopes among several candidate peptides has been reported previously [16] and combination of peptides, as applied in the QuantiFERON®-TB test for TB diagnostics [8] can cover a wider number of HLA alleles than single peptides [21;18;29;14;15].

Therefore, the *M. leprae* virulence-associated peptides were additionally tested in pools containing 4 - 6 peptides in Nepali EC and patient (TT/BT/BL/LL) groups before and after treatment. Three peptide pools (V3, V4 and V5), which included the immunogenic peptides (ML2055 p35, ML2055 p37 and ML2055 p42) that induced IFN-γ responses in the Brazilian and/ or Ethiopian individuals showed a significant induction of Th1 response in the Nepali EC as well (Figure 6E). In contrast, in leprosy patients before MDT hardly any responses could be detected (Figure 6A and 6B). However, after MDT some BT patients displayed significantly increased IFN-γ responses against the peptide pools as well as *M. leprae* (Figure 6D) thereby reflecting improved cellular immunity against *M. leprae*. The low or absent IFN-γ responses detected for BL/LL patients were in line with their lepromatous phenotype, lacking Th1 cell responses.

Immune responses against *M. leprae* are a collective/ synergistic effect of various cascades involving both innate and adaptive immune cells inducing cytokines and chemokines. IFN-γ has been known to be a potential marker of Th1 response and will remain useful depending on the specificity of the stimulus used. Besides IFN-γ, other cytokines and chemokines such as IP-10 may also have potential to distinguish between different level of exposure and /or infection for leprosy [5] as well as TB [8].

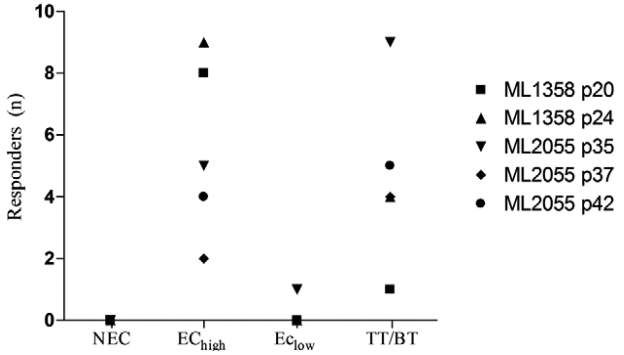


Figure 7: Individuals with IFN-γ responses to *M. leprae* virulence-associated peptides. The total number of individuals that induced IFN-γ production in response to 5 promising *M. leprae* virulence-associated peptides is indicated for each test group: Dutch NEC, combined Brazilian and Ethiopian $EC_{high,}$ EC_{low} and TT/BT.

In the current study, the induction of IP-10 in Ethiopian EC_{high} in response to ML2055 p35 and p42 further confirms the potential of this chemokine as a biomarker to specifically indicate *M. leprae* exposure. Additionally IP-10 can also be used in HIV infected patients since, unlike IFN-γ, IP-10 was not affected by low CD4 counts in TB patients with HIV [2]. Currently further studies on the use of IP-10 as a biomarker for leprosy diagnostics in HIV^+ individuals are ongoing in our Ethiopian test site.

The main advantage of the use of synthetic peptides compared to proteins is the fact that peptides, unlike proteins, less frequently induce T-cell cross reactivity [29;15]. However, due to HLA-restriction of peptide recognition by T-cells, single peptides will not be able to cover a wide population. In this study, we show that ML2055 p35 and ML2055 p42 as well as ML1358 p20 and ML1358 p24 were recognized by patients or EC_{high} individuals in both Brazil and Ethiopia. In addition, these peptides also induced IFN-γ responses in 30% - 40 %

Nepali EC when used in pools. Therefore, as in the case of TB diagnostics, analysis of IFN-γ and other cytokines such as IP-10, after stimulation with combinations of *M. leprae* (virulence-associated) peptides will be helpful in developing new tools for detection of *M. leprae* exposure/ infection.

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

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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.

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.*

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 [2]. 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 [3;4].

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 [6]. Subsequently, these (hypothetical) antigens 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 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* [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* antigens.

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 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. 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 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 [15] 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 (100 µg/ ml). After 24 hour 150 µl 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 x 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.

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 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^{o}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^{TM} software 6.0 (Bio-Rad laboratories, Veenendaal, The Netherlands) [17]. 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^{TM} 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 [10]. 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 (Supplemental 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* [18]. 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 (Supplemental Figure S1B).

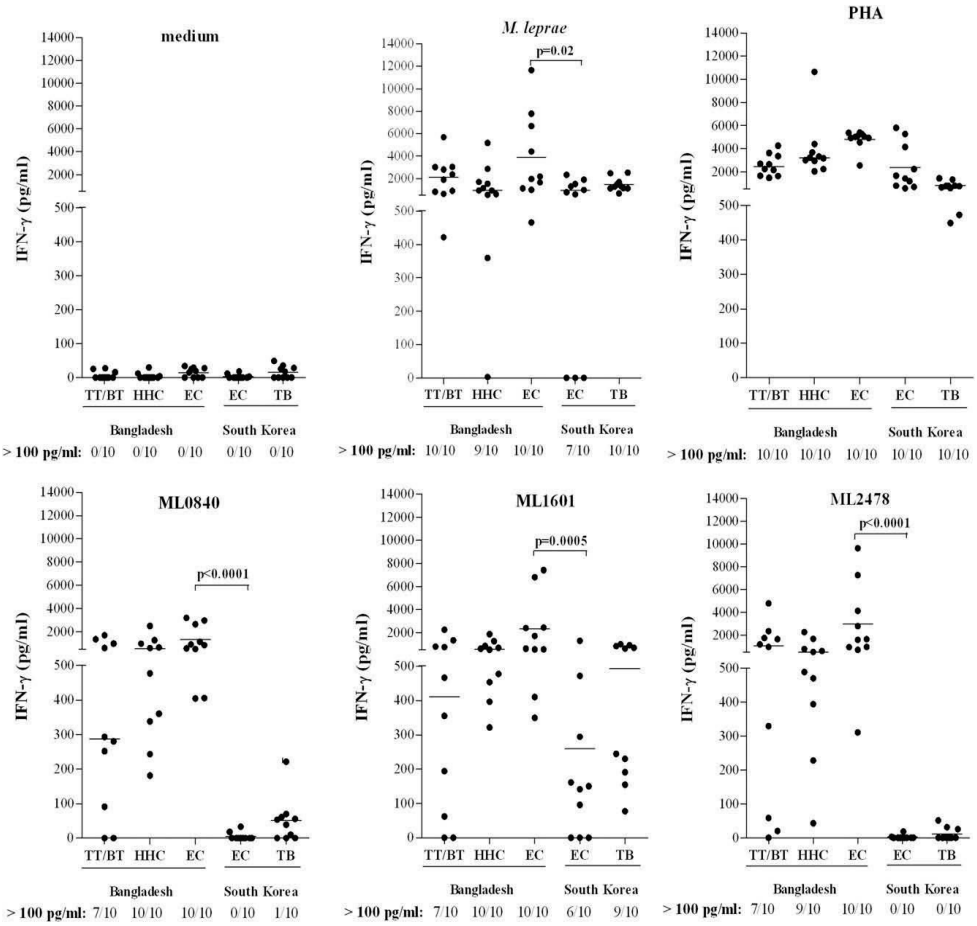

Figure 1: IFN-γ responses in WBA from individuals in Bangladesh and South Korea. IFN-γ production in responses to control stimuli (medium, PHA and *M. leprae* WCS) or to recombinant proteins (ML0840, ML1601, and ML2478) in 24h WBA of leprosy patients (TT/BT; n=10), HHC (n=10), and EC 9n=10) from Bangladesh (prevalence =.45/10,000), or healthy controls (Ec; n=10) and tuberculosis patients (TB; n=10) from Soth Korea (prevalence <1/10,000). For each group, the number of IFN-γ responders (>100pg/ml) versus thetotal number of individuals in the group is indicated below the x-axis. Background values were <50pg/ml. Medium values for each group are indicated by horizontal lines. Significant differences between test groups are indicated by *p* values.

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) [19]. 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 (Supplemental 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.

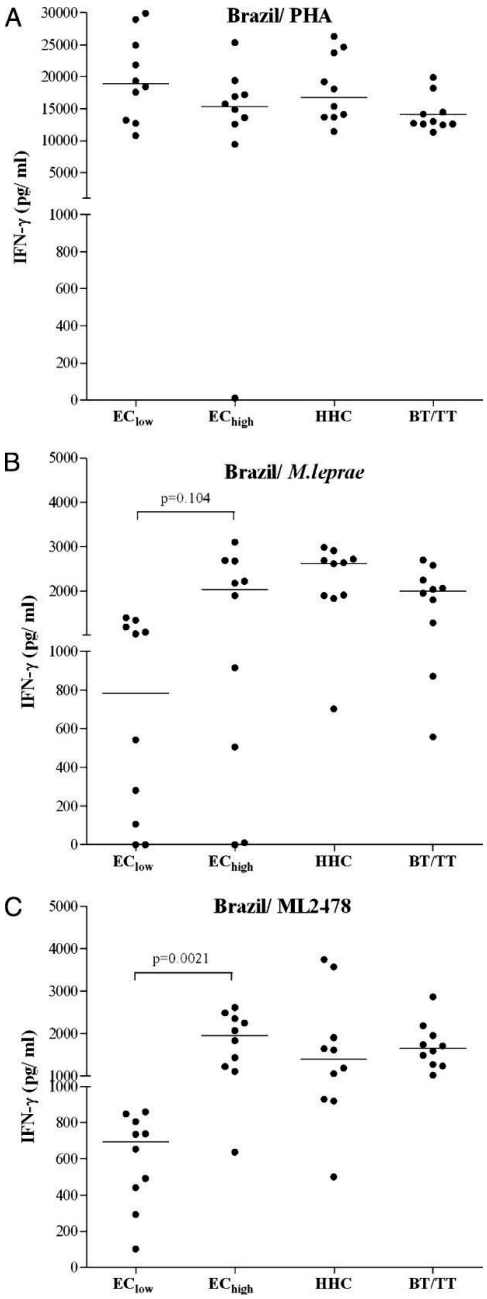

Figure 2. IFN-γ responses to *M. leprae* Ags in PBMC from EChigh and EClow in Brazil. IFN-g production (corrected for background values) induced using PHA(A), M. leprae (B), or ML2478 recombinant protein (C) in 6-d cultures of PBMC from healthy individuals from an area of Fortaleza, Brazil, with low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

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 [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]. 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.

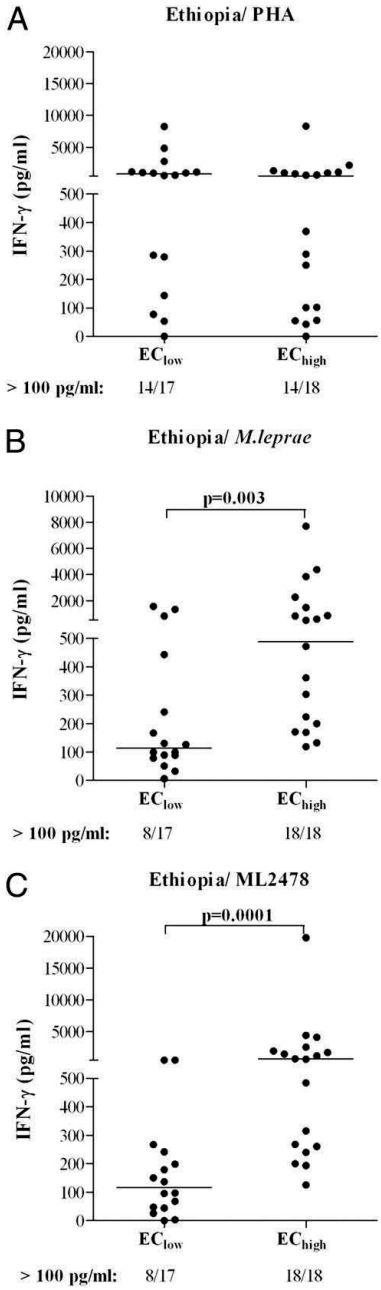

Figure 3. IFN-g responses to M. leprae proteins in WBA from EChigh and EClow in Ethiopia. IFN-g production (corrected for medium values) in response to PHA(A), M. leprae WCS(B), or recombinant protein ML2478 (C) in 24-h WBA of healthy individuals from areas in Addis Ababa, Ethiopia, with low (EClow; prevalence = 0.36/10,000; n = 17) and high (EChigh; 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-g responders versus the total number of individuals in the group is indicated below the x-axis.

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 [21]. 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 (Supplemental 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 [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 (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;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.

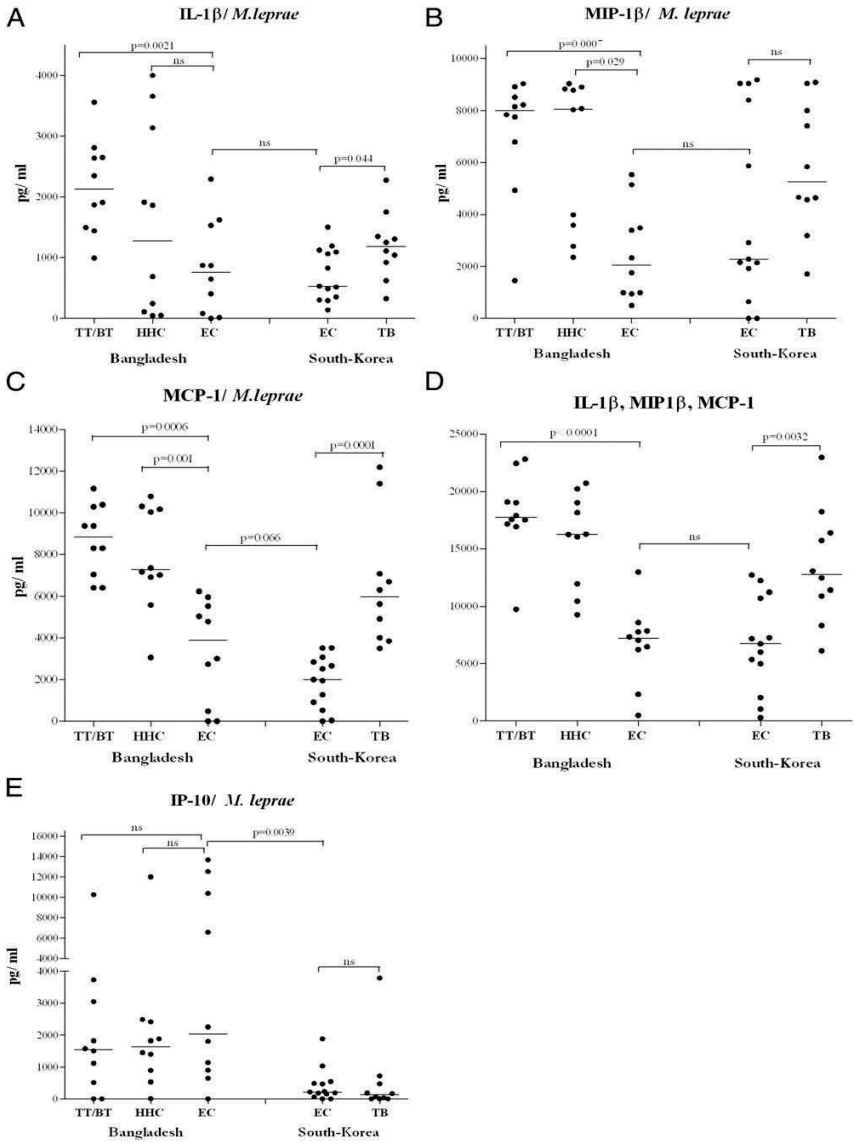

Figure 4. Multiplex cytokine analyses inWBA from individuals in Bangladesh and South Korea. Concentrations (all corrected for background values) of IL-b (A); MIP-1b (B); MCP-1(C); and IL-b,MIP-1b, and MCP-1 combined(D); or IP-10 (E) induced by stimulation with M. leprae WCSin 24-hWBA ofleprosy patients (TT/BT; n =10), HHC(n = 10), and EC (n = 10) from Bangladesh, or healthy controls (EC; n = 10) andtuberculosispatients (TB; n = 10) from SouthKorea. Medianvaluesper testgroup are indicatedbyhorizontal lines. Background values varied from50 pg/ml for IFN-g to 2000 pg/ ml for MIP-1b.

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 [9;11;12]. This was partly based on the initial promising reports on QuantiFERON®-TB, an IGRAs 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 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; [18]. 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 [30]. 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 [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 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 [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 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*.

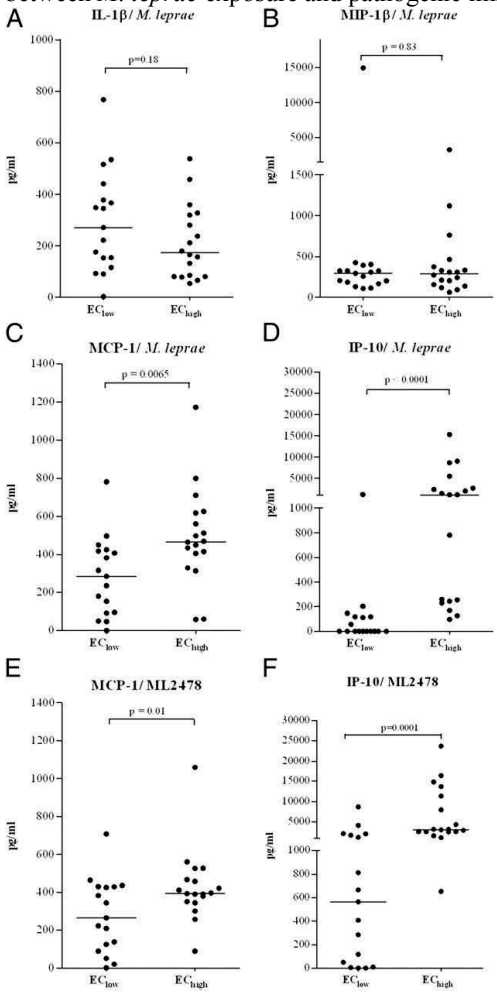

Figure 5. Multiplex cytokine analyses in whole-blood cultures from EC in Ethiopia. Concentrations (all corrected for background values) of IL-b (A), MIP-1b (B), MCP-1(C, E), IP-10(D, F)induced by stimulation with M. leprae WCS(A–D), or ML2478(E, F) in 24-h WBA of leprosy patients (TT/BT; n = 10), HHC(n = 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-g to ,2000 pg/ml for MIP-1b.

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 [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 has been suggested [34]. For TB patients MCP-1 production by *M. tuberculosis*-stimulated PBMC was associated with TB disease severity [35]. On the other hand, for lepromatous leprosy (LL) 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 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 [37]. The exact role of MIP-1β in leprosy pathogenesis is still not clear.

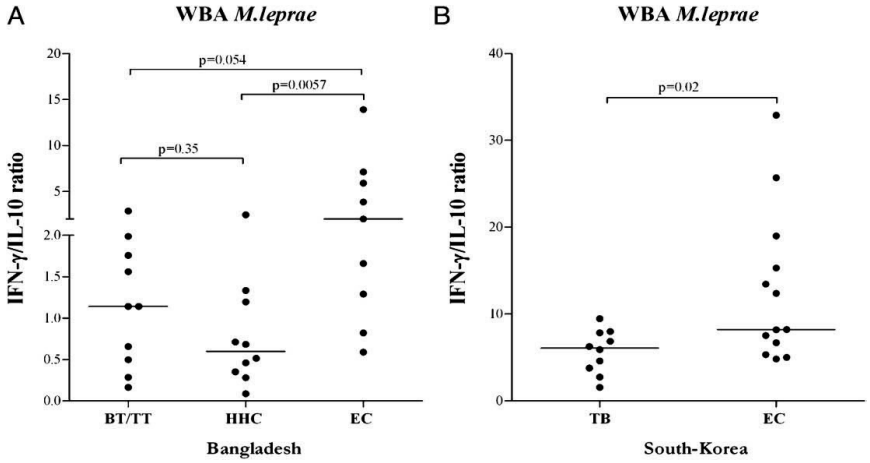

Figure 6. IFN-g/IL-10 ratio in M.leprae-stimulated WBA. Ratios of IFN-gconcentrations (corrected for background values) with respect to IL-10 concentrations (corrected for background values) induced by stimulation with M.lepraeWCS in 24-h WBA in individuals from Bangladesh (A) and South Korea (B).

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* [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 LL 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 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 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 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 [41;42]. 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 [22-25]. 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 [43]. 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 [44;45]. 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*.

Acknowledgements

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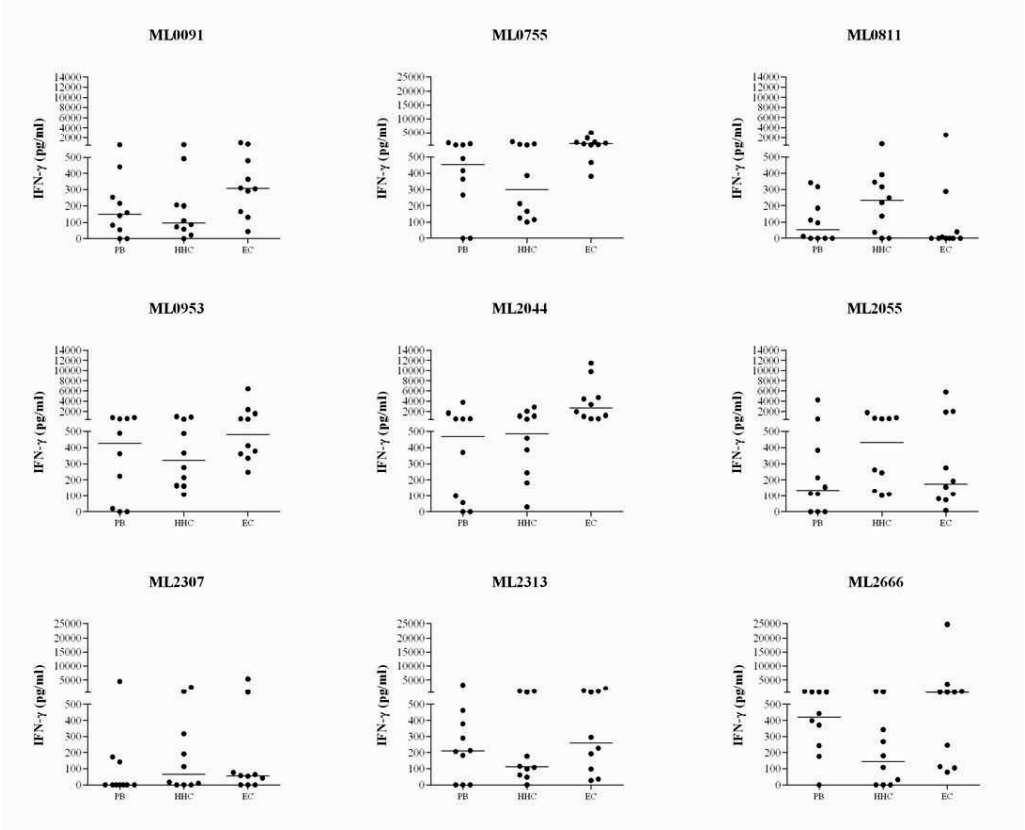
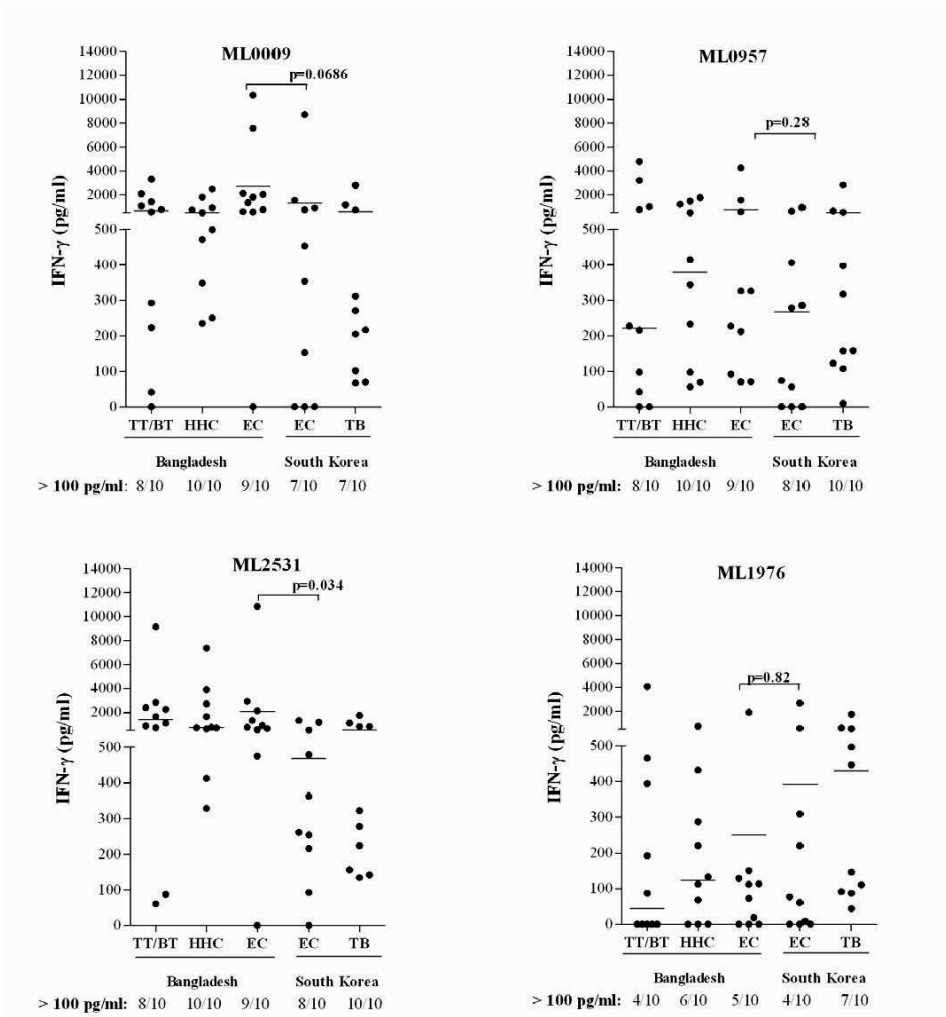
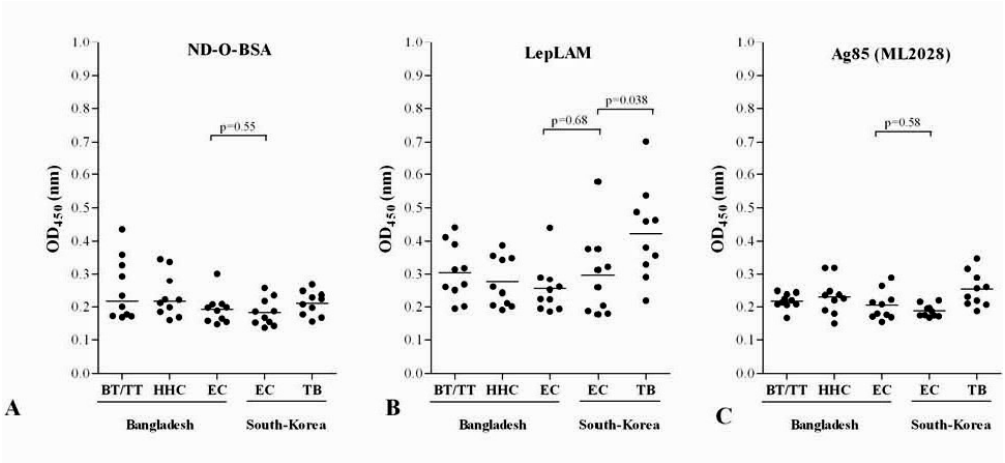
Supplementary files



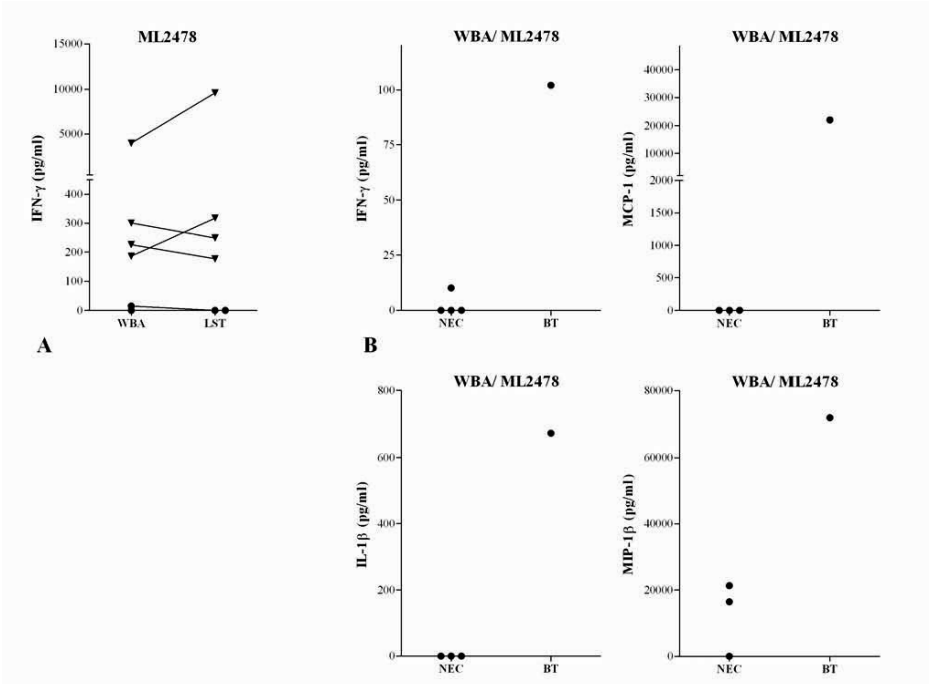
Supplementary Figure S1A: IFN-γ responses in WBA from individuals in Bangladesh. IFN-γ production in response to recombinant proteins (ML0091, ML0811, ML2044 and ML2055) in 24 hour WBA of leprosy patients (TT/BT; n = 10), healthy household contacts (HHC; n =10) and endemic controls (EC; n=10) from Bangladesh. 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).