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

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Dedicated to my mother Gabriela Girmay Woldu
Rest in Peace

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Chapter 1

General Introduction

Chapter 1 General Introduction

Leprosy is a chronic infectious disease that affects hundreds of thousands of people every year. Its prevalence has dramatically reduced from millions in the 1980s to hundreds of thousands in the last 30 years as a result of the introduction of multidrug treatment (MDT) which is a combination of dapsone, clofazimine and rifampicin [7;204]. However, since the last decade, the annual numbers of new leprosy cases have become consistent indicating the continuing disease transmission [183;203;205]. The numbers of new cases in children, and the numbers of patients with grade 2 disability (visible deformity in hands/feet and/or visual impairment) reported every year are good indicators of the ongoing transmission and the delayed detection of cases, respectively. This is mainly due to the lack of early diagnostic tools [83] as well as poor awareness and knowledge of the signs and symptoms of the disease among the public and health professionals.

Leprosy primarily affects the peripheral nerves and skin and its cardinal signs are skin lesions, loss of sensation and nerve thickenings. The disease manifests itself in different clinical forms. For treatment purposes, WHO classifies leprosy cases as multi-bacillary (MB) and pauci-bacillary (PB) where cases with more than 5 skin lesions and high bacterial load are considered as MB and those with less than 5 skin lesions and low bacterial load as PB [1]. The clinical forms are further classified in to five groups based on the host's immunological responses, bacterial load and histopathological features of the lesions into tuberculoid leprosy (TT), borderline tuberculoid leprosy (BT), borderline borderline leprosy (BB), borderline lepromatous leprosy (BL) and lepromatous leprosy (LL). The TT and LL are stable forms whereas the borderlines are immunologically unstable. The cell mediated immunity decreases from TT to LL whereas the bacterial load and antibody level increase [181].

The discovery of *Mycobacterium leprae* (*M. leprae*) as an etiologic agent of leprosy a century ago, and later the development of efficient treatment regimens have proven that leprosy is a curable infectious disease [157;158;247]. The recent whole *M. leprae* genome sequencing project [39] has further opened the way for new avenues in leprosy research. Hence, there are currently more opportunities than ever before to better understand *M. leprae* and leprosy.

Following the availability of the whole genome sequence of *M. leprae*, several unique *M. leprae* proteins have been identified [81;191;218]. These were tested in different endemic sites in Asia, Africa and South America for their immunogenicity and for their discriminating potential of those infected and at risk of becoming infected, such as household contacts [22;86]. The *M. leprae* unique proteins identified as well as the host biomarkers induced by stimulation of blood cells by these proteins (including cytokines, chemokines, growth factors and others) provide important new tools for leprosy disease control, and are currently being validated in large-scale studies for their potential application in the early detection of leprosy (before the onset of clinical signs and symptoms of the disease) [65;68;83].

1.1. The Global Leprosy burden

"*We can endure losing fingers and toes, eyes and nose, but what we cannot endure is to be rejected by those nearest and dearest*"; a leprosy victim from Nepal [174].
The disfiguring character of leprosy and the wrong perception on the cause and transmission of the disease are the main reasons for stigmatizing leprosy affected people [43;174;230]. Leprosy is a curable chronic infectious disease; however this scientific fact is not yet well perceived in minds of many people irrespective of their level of knowledge [15;92]. Some

studies have also shown the strong stigma in leprosy compared to other diseases and its devastating impact on the lives of patients and their close relatives [177].

The dramatic reduction in leprosy prevalence and number of new cases in the post MDT era is undeniable, which has been the result of the determined efforts to eliminate leprosy. However, in the last couple of years, more than 200,000 new leprosy cases including children have been registered each year. Among the 5 WHO regions, the South-East-Asia region has the largest number of new cases (166,445) per year, followed by the Americas (36,000) and the African countries (20,599). Multi-bacillary (MB) new cases dominated in most of the regions and percentages of children affected ranges from 0.6% in Argentina to 24.5% in Cameroon and disabilities from 0.7 % in Marshall Islands to 25.4% in Uganda [7]. Here the major concerns are the considerable number of children and the grade 2 disabilities among new leprosy cases.

Number of new cases per given period of time and death or disability adjusted life years (DALY) are among the many ways to express a disease burden [178]. The disability together with self and society driven psychological distresses leave leprosy affected persons with a life time of misery and pain [212]. Despite the fact that leprosy is the leading cause of disability, leprosy associated DALYs are not registered in many high burden countries except the recent assessment in 3 states in India which showed the loss of 13.4 productive working years due to disability (DAWLY) of the 42 years estimated productive years [178]. Unlike TB or malaria, mortality due to leprosy is not a major concern although some deaths may occur from indirect effects of leprosy [72;138] and there were also a few reports showing the higher risk of death in lepromatous leprosy (LL) patients compared with the general population in pre MDT era [156]. However, the chain of disability to poor quality of life, to inevitable economic and physical dependence on family and society, to noticeable stigma, to mental distress greatly affects the lives of leprosy patients [132;201;235].

Leprosy control activities have been integrated with other health services since 1997 with the intention of better management of leprosy and reducing stigma [5]. However, the emergence of TB in the 1990s [3] and its co-infection with HIV diverted the focus of TB-leprosy control programs in most countries for the last two decades heavily towards TB/HIV. As a result leprosy has been left as poorly managed disease due to lack of knowledge, interest and commitment. The integration of leprosy management in the general health services has had its own advantages [173] and disadvantages. The service is meant to be easily accessible as long as there are health facilities in the vicinity of the patients, but its use depends on their health seeking behavior and the attitude of the community, since active case detection has not been part of the control programs so far. The knowledge, attitude and practice (KAP) of the health professionals at each facility are also main factors determining the quality of the leprosy management. This includes the focus of the control program to keep health professionals updated and committed as there is evidence of below average KAP of health professionals in leprosy [16].

The WHO “leprosy elimination by 2000” goal has also had an impact on the leprosy control programs, leading to less commitment in dealing with the true burden of leprosy. Indeed, it brought budget limitations in most leprosy activities due to the pulling out of donors [129]. However, the WHO leprosy strategy for the years 2011 to 2015 has put renewed emphasis on major issues: early detection of leprosy through active case detection, contact tracing especially in hotspots, and reducing disability [6] which is a step forward towards controlling leprosy.

1.2. Leprosy manifestations

1.2.1. Signs and symptoms of leprosy

Leprosy is caused by *Mycobacterium leprae* (*M. leprae*). It is a unique and challenging pathogen that primarily affects peripheral nerves and the skin through invading and residing in Schwann cells (SC) and macrophages. It also affects the eyes, the testis, the extremities, the hands and feet, and the upper respiratory tract [181;205]. Being primarily a nerve and skin disease, the clinical examination of leprosy is mainly based on the assessment of skin lesions and nerve involvements. There are three basic signs and symptoms to identify leprosy. These are: loss of sensation, presence of skin lesions and nerve thickenings [181]. The degree of sensation loss, the number and features of lesions and the number and level of nerve involvement vary widely in patients depending on the bacillary load and the host immunological capability, which strongly correlate with the different clinical forms of leprosy. In most patients, all three signs of leprosy are present. However, there are also cases with only skin lesions and no or undetectable nerve involvement, while on the other hand there are some cases with only nerve involvement and no skin manifestations, referred to as neuritis. These are the commonly missed cases during diagnosis since they are not fully covered by standard WHO classification methods [169].

In addition, a new mycobacterium species, *Mycobacterium lepromatosis* which can cause a fatal diffuse lepromatous form of leprosy was discovered in 2008 [99]. Preliminary phylogenetic analysis of few genes including the 16S rRNA gene revealed a significant difference of *M. lepromatosis* and *M. leprae* [101;213]. This fatal type of leprosy has been reported as endemic in Mexico and Costa Rica for a century [101;102;237] and according to a recent report it is also found in Singapore [100], Brazil and Myanmar [97].

1.2.2. Clinical forms of leprosy and diagnosis

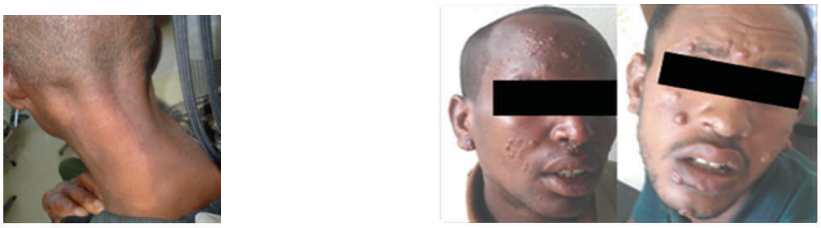
i. *Clinical forms of leprosy*

The diverse clinical manifestations of leprosy are primarily associated with the immunological and genetic variability of the host [181;216]. This renders leprosy an immunologically interesting and challenging human disease [146]. There are three main aspects that have led to the categorization of leprosy into five clinical forms. These are:

1. The level and type of the immunological response.
2. The bacillary load.
3. The histopathological features.

The five clinical forms of leprosy established as standard classification especially for research purposes by Ridley and Jopling [181] are tuberculoid leprosy (TT), borderline tuberculoid leprosy (BT), borderline borderline leprosy (BB), borderline lepromatous leprosy (BL) and lepromatous leprosy (LL). The TT and LL are the two stable forms of leprosy at the opposite extremes or poles. The BT, BB and BL forms are immunologically unstable groups which could possibly downgrade or upgrade, depending on e.g. initiation and efficiency of treatment. The TT and BT forms (Fig 1A) are characterized mainly by few skin lesions and high levels of cell mediated immunity (CMI). This involves the activation and proliferation of T helper 1 (Th1) cells; subsets of effector $CD4^+$ T cells important in activating macrophages via production of pro-inflammatory cytokines like IFN-γ. Along with this, a well characterized granuloma with undetectable or few bacilli are important features characterizing this group [153;181;205]. The "indeterminate" leprosy type is very similar to the TT group except that the lesions can be at an early stage and need to be confirmed histopathologically. Both the TT and indeterminate forms of the disease may spontaneously heal.

On the other side of the spectrum, the BL and LL forms (Fig 1B) are characterized by multiple skin lesions, poor CMI and strong humoral responses. Their granulomas are disorganized and filled with foamy macrophages and numerous bacilli which is a typical histopathological feature for these clinical forms. The BB group which is in the middle of all forms is difficult to clearly define with clinical or histopathological features as it shares characteristics from both BT and BL groups.



A B

Figure 1. A patient with borderline tuberculoid leprosy (BT) (1A) and patients with lepromatous leprosy (LL) (1B). Patients were enrolled in one of the studies in this thesis.
Photo by S/r Genet Amare; AHRI (Posted with permission)

1.3. Diagnostic tools

Leprosy diagnosis requires significant expertise because of the multi-facetted leprosy manifestations and its complications. In most leprosy burdened countries, diagnosis mainly relies on clinical examination, supplemented to some extent by the acid fast bacilli (AFB) staining of the skin slit smear (SSS) reported as BI (Bacterial Index). However, clinical diagnosis can only detect an already manifested and visible stage of leprosy, at which stage often irreversible tissue damage has already occurred. This illustrates the key importance of developing diagnostic tools that can detect leprosy earlier.

Clinical

As mentioned in the previous section, clinical examination includes 1) lesion characterization: number, demarcation, pigmentation, formation (raised, nodular), symmetry and loss of sensation; 2) voluntary muscle testing (VMT) and 3) examining of possible nerve enlargement. It is challenging to clearly diagnose leprosy and classify it into one of the 5 forms based on the clinical signs and symptoms unless it is supported with Acid Fast Bacilli staining (AFB) from skin slit smear (SSS) and with Hematoxylin and Eosin (H&E) staining of biopsy samples. The AFB staining and bacterial index (BI) reporting (Table 1) is within the capacity of many poor resource settings but the histopathological examination needs advanced lab facilities and a pathologist. Hence, classifying leprosy into the five classical forms remains important mainly for research purposes but not for routine diagnosis and treatment. Instead, World Health Organization (WHO) has recommended a more pragmatic, two arm classification of leprosy patients; paucibacillary (PB) patients with less than 5 lesions; and multibacillary (MB) with more than 5 lesions (WHO 1982) which simplifies leprosy management in resource poor settings.

Table 1
BI grading in leprosy diagnosis

BI grading	Definition
1+	At least 1 bacillus in every 100 fields
2+	At least 1 bacillus in every 10 fields
3+	At least 1 bacillus in every field
4+	At least 10 bacilli in every field
5+	At least 100 bacilli in every field
6+	At least 1000 bacilli in every field

Source: WHO (www.who.int/lep/microbiology/en.)

Other than clinical signs and symptoms, there are some serological tests which have been developed but none of these has been used for routine diagnosis; they are of limited value since they mainly detect MB but not PB patients. These tests include the following:

Anti PGL-I IgM antibody ELISA
Phenolic glycolipid I (PGL-I) is the dominant lipid component of the cell wall of *M. leprae* and is "specific" for *M. leprae* although there are some reports of its presence in *Leishmania* parasites [95]. The IgM antibodies level produced against PGL-I in patients is measured using ELISA and is usually high in MB (BL/LL) which have poor CMI and high humoral responses [181]. *M. leprae* has never been grown on artificial media and can only be cultured in armadillos or in the mouse foot-pad, which severely limits its availability and research into disease mechanisms. Therefore, synthetic PGL-I made of natural disaccharide octyl human serum albumin (ND-O-HSA) is used in these ELISAs. This technique clearly detects BL/LL patients and can also be used to monitor their treatment outcome as it is reduced during treatment [168]. TT/BT patients are usually seronegative and do not reproducibly produce detectable circulating antibodies to PGL-I [205]. Clinically, identifying BL/LL patients is not difficult for dermatologists and in that case the anti-PGL-I ELISA may not contribute substantially to the diagnosis as the main challenges are detecting TT/BT patients and sub-clinically infected individuals. Nevertheless, there is some evidence showing its utility in identifying HHC with higher risk of developing the disease [18;60].

Anti PGL-I IgM antibody ML-Flow test
This is an immunochromatographic test which is also based on PGL-I but detects IgM faster [27;28]. It does not require special skills to perform the test and does not need a reader as it can be graded by visual inspection [133] unlike the ELISA. However, it has the same limitations as the anti-PGL-I ELISAs in that it mainly detects MB patients and is not quantitative.

NDO-LID® rapid test
This is a recently developed rapid serological diagnostic test which measures IgM antibodies against PGL-I through the use of NDO-BSA and measures IgG antibodies against LID-1, a fusion protein made of ML0405 and ML2331 [62;65;191]. It is an immunochromatographic test which requires small amounts of serum or whole blood. Like the other serological tests, it detects most MB patients and relatively improved numbers of PB patients (32.3%) compared with NDO-BSA (6.5%) [62]. However, a large scale evaluation of this test in different endemic sites is necessary to evaluate its precise utility in leprosy.

Lepromin Skin Test

This is a skin test using a suspension of whole, killed *M. leprae,* called lepromin, injected intradermally. The reaction to this test is measured as mm induration 4 weeks after injection. Lepromin is not used for diagnosis of leprosy; rather it measures or provides information about the individual's immune response: a positive lepromin reaction indicates the ability of the individual to develop a granulomatous response to *M. leprae* while a negative reaction is commonly seen in lepromatous patients who are incapable to contain or clear the bacilli [103;205].

PCR based M. leprae viability assay

Several attempts have been made since the last 3 decades to establish clinically applicable PCR based leprosy diagnostic tools [192;193;245]. However, the major challenges had been identifying potential target genes for specific detection of *M. leprae* and determination of bacilli viability. In recent years, real time PCR based amplification of the repetitive element of *M. leprae* (RLEP) DNA had shown strong correlation with AFB count and was found reliable for specific quantification of *M. leprae* from mouse and armadillo tissues but with limitations of providing absolute data on viability [48]. In recent development, expressions of number of genes were assessed [135;232] among which *hsp18* (encoding 18kd heat shock protein) and *esxA* (encoding the ESAT 6 protein) were found reliable in detecting viable *M. leprae* [48]. This newly established *hsp18* and *esxA* based viability assay can be used as an assessment tool for early detection of *M. leprae* infection in close household contacts of leprosy patients, in monitoring treatment outcome and in detection of drug resistance by eliminating the tiresome bacterial isolation process. However, the cost and the demand for trained personnel remain limiting factors to implement the assay as point of care (POC) in resource poor settings.

1.4. Treatment and drug resistance

Despite being an ancient disease, treatment for leprosy was not available until the 1940s. The first modern treatment was dapson (diaminodimethyl sulfone) and was given for long-term to life time in case of MB cases. Poor compliance to the long term treatment contributed to the occurrence of dapsone-resistant *M. leprae* isolates in most countries and was a challenge for the leprosy control programs [77;108;167;241]. Several efforts were made to replace dapsone with other monotherapies such as clofazimine, ofloxacin or rifampin but resistance against these antimicrobial agents developed when given as monotherapy. To resolve this, a combined treatment consisting of dapsone, clofazimine and rifampicin which commonly referred as multi-drug treatment (MDT) was recommended by WHO in 1981[1]. Currently, the WHO recommended treatment period is 6 months for PB patients and one year for MB patients (reduced from two years since 1997) [4], however, there are countries which still treat MB cases for two or more years especially those countries with high resources and low leprosy burden). In both PB and MB 100 mg dapsone daily and 600 mg rifampicin monthly is given while for MB cases an additional 50 mg daily and 300 mg monthly dose of clofazimine is given. There is also a third regimen which is recommended to single-lesion PB patients; this is a single dose rifampicin (600 mg), ofloxacin (400 mg) and minocycline (100 mg), commonly referred as ROM. Currently, ROM is being tested for MB patients as 12 month regimen [79;126]. For patients who are unable to take medications because of allergy or suspected complications, fluoroquinolones, ofloxacin, moxifloxacin, or pefloxacin and minocyline, macrolide clarithromycine can be given as second line drugs. MDT is efficient as witnessed by the dramatic reduction of leprosy prevalence since its initiation.

Drug resistance to MDT is not considered as a major issue (ILEP report 2013), however, there are recent reports which identified either mono or multiple drug resistant *M. leprae* strains [244;246]. A study which evaluated samples from 230 new and 3 relapse cases from Venezuela and Brazil using sequencing and real-time PCR Taqman technologies showed drug (rifampicin and dapson) resistance-associated mutations in *fol*P1 and *rpo*B genes in the 3 relapse cases [214]. Similarly, SNPs in biopsy samples of 4 among a total of 92 (4.3%) relapse cases in Brazil were found where in 2 relapse cases multi drug resistance (SNPs in *fol*P1, *rpo*B and *gyr*A) was observed [46]. Although cases with SNPs were small in number, the reports in general indicate the importance of regular drug resistance monitoring especially in relapse cases.

1.5. Vaccines against leprosy

Mycobacterium bovis BCG (Bacillus Calmette–Guérin) is the only vaccine currently available for TB. It protects young infants from severe forms of TB, mainly the miliary and meningeal forms, but its protection wanes over time and varies across age, different countries and the type of vaccine strain used. The BCG vaccine was initially developed for both TB and leprosy but its protective role in leprosy became neglected because of MDT campaigns. There is evidence for higher BCG induced protection in young individuals that wanes overtime and for increased protection through several doses of BCG [41;141;182;208;248].

There were long (up to 8 years) follow up studies that assessed the protection of killed *M. leprae* combined with BCG in comparison with BCG alone. In a Venezuelan study, no better protection was observed after five years follow up [40]. In contrast, in an Indian study, the combined *M. leprae* killed/BCG vaccine showed 64% protection compared to BCG alone which was 34.1% [96].

Further assessment and use of killed *M. leprae* vaccines need large production of *M. leprae* which is very challenging. Therefore, other easily cultivable mycobacteria such as *M. w*, *M. vaccae* and *M. habanna* used in vaccine preparations were tested in household contacts of leprosy patients and showed more than 50% protection for at least 3 to 6 years [209;229;238]. Crude *M. leprae* antigens were also assessed in mice [80;152]. rBCG vaccines that express Ag85 and MMP- II also inhibited *M. leprae* multiplication [134;160]. From recent antigen screenings, those antigens recognized by PB patients were also further assessed for their potential as a vaccine in mice work [63]. So far no successful vaccine has been developed for use except BCG implicating the need for continuous effort to develop potential leprosy vaccines.

1.6. *Mycobacterium leprae* and its unique characteristics

Leprosy has been with humans since ancient times as evidenced by some records in India and China and by more reliable archaeological discoveries of the 2nd Century BC in Egypt [224]. However, *M. leprae,* the etiologic agent of leprosy was discovered only a century ago as the first human pathogen, notably by the Norwegian scientist Gerhard Henrik Armauer Hansen [104;105]. Other human pathogens discovered later have been more intensely investigated than *M. leprae* due to its challenging unique characteristics and the inability to culture *M. leprae* [38;69].

1.6.1. *M. leprae* classification and its cell wall composition

M. leprae is an intracellular obligate, acid-fast, capsulated, rod shaped bacillus which belongs to the order Actinomycetales, family Mycobacteriaceae and genus *Mycobacterium*. As any other mycobacteria it replicates by binary fission but unlike any other species which belong to either fast or slow growers in this genus, *M. leprae* divides in 12-14 days and it has never been grown in artificial media/*in vitro* [179].

The *M.leprae* capsule is an electron transparent zone composed of phthioceroldimycoserosate and phenolic glycolipid (PGL) composed of three sugar molecule linked to phthiocerol (fat) with a phenol component. The outer layer of the cell wall is similar to other mycobacteria containing lipopolysaccharides composed of branched chains of arabinogalactan and long chains of mycolic acids. The inner part of the cell wall is composed of a peptidoglycan layer which is formed from chains of alternating N-glucosamine and N-glycolyl muramyl linked by peptides cross bridges [61;205].

1.6.2. Genome and metabolism

The genome of *M. leprae* was first sequenced in 2001 [38]. The genome size, 3.3Mb, is smaller than that of *M. tuberculosis* (Mtb) and contains 1133 pseudogenes, leaving a total of 1614 genes encoding for functional proteins, and displaying a major reduction of G+C content. The reduced genome size with nearly 50% inactivated genes is the reason for the absence of several metabolic pathways, a feature which distinguishes *M. leprae* from other mycobacteria [38]. As a result of this, *M. leprae* utilizes glucose but no other carbon sources as an energy source, which makes it highly dependent on the host. The *M. leprae* genome project also revealed that the genes encoding enzymes degrading other carbon sources such as acetate and galactose are in fact pseudogenes while in *Mtb* these genes encode functional enzymes capable of degrading these carbon sources [38;69;243]. Being intracellular mycobacteria, *M. leprae* and *Mtb* are also known to utilize host-derived lipids as energy sources through lipolysis. However, *M. leprae* has very few lipase genes as compared to *Mtb*. *M. leprae* has also lost the anaerobic pathways and has less efficient aerobic pathways left due to its incapability to generate ATP by oxidizing NADH which is one possible reason why it is unable to grow in artificial media as it will be difficult to provide appropriate levels of oxygen in the media [243]. It also lost the *mbt* operon, leading to deficiency in iron acquisition from its environment as it is not able to produce mycobactin [113]. However, there are many other genes related to iron storage still active, indicating *M. leprae* is able to use host iron.

1.6.3. Host preference

Besides humans, the wild nine-banded armadillo (*Dasypus novemcinctus)* is a highly susceptible natural host of *M. leprae.* Primates such as Chimpanzee and Mangabey Monkey were reported as hosts as well but not to a major extent [2]. The wild nine-banded armadillos are common in the south central USA [226] and recently, a study in Texas showed genotype similarities of the isolates from US leprosy patients and infected armadillos, indicating the possible existence of zoonotic leprosy in that specific area [228]. Nine banded armadillos and nude mice are very important for leprosy related experiments. The mouse foot pads are convenient to grow *M. leprae* and the nine-banded armadillos also show fully disseminated, MB infection when inoculated with *M. leprae* [205]. These experimental animals are important for drug resistance monitoring, to assess bacilli viability and to culture bacilli for *in vitro* or *in vivo* immunological studies.

1.6.4. *M. leprae* in the environment

M. leprae is known for being an obligate pathogen. However, there are recent reports indicating the presence of viable *M. leprae* in soil mainly in areas with high prevalence of leprosy [121;231]. The genotypes of those isolated from soil were found similar with those isolated from patients in that specific area [231]. MB patients carry very high number of bacilli in their upper respiratory tract and the bacilli can easily be excreted from the nasal area through washing or sneezing [210]. Hence, finding live *M. leprae* in the surrounding soil and water might not be surprising. Previous studies also showed its survival outside the host for 9 days [53] and in an extended study it was found to survive 46 days in wet soil and 60 days in saline at room temperature [54]. *M. leprae* was also found viable and intact after being ingested by a free living pathogenic amoeba for at least 72 hours [119] and a recent study revealed a long term (up to 8 months) survival of *M. leprae* in free living amoebae cyst while retaining its virulence [242]. However, further investigations are required to further understand the capability of the *M. leprae* bacilli found in the surrounding environment including free living amoebae to infect the host and if so these environmental sources can be considered as potential reservoirs for *M. leprae*.

1.7. Host-pathogen Interactions in leprosy

The manifestation of the disease in its different forms, closely correlating with host genetic and immunological factors, has made leprosy an immunological disease model [144;146;147]. Besides its great potential to provide a research model for human immune mediated diseases, extensive knowledge about host-pathogen interactions in leprosy will also help to improve and innovate leprosy control activities.

1.7.1. The route of infection/ entrance of *M. leprae*/ transmission

Untreated lepromatous patients carrying high bacillary load reaching 10^{11} per gram of tissue, are the main sources of infection where the aerosol spreading of nasal droplets from these patients infects healthy individuals through nasal/respiratory routes [107]. Due to lack of active case detection strategy in high burden countries, there is high possibility for untreated MB patients to transmit the infection to their close contacts [175]. Sub-clinically infected individuals as source of infection cannot be ruled out although no clear evidence is available as yet. Transmission through skin contact [55], via nine-banded Armadillos [120;227] and via free living amoebae that contained *M. leprae* [242] also need due attention and further investigation.

1.7.2. Innate Immunity; uptake of *M. leprae* by host cells

M. leprae and Schwann cells

The presence of considerable number of bacilli in the endothelial cells lining the blood vessels and lymphatics revealed that endothelial cells could be sites for *M. leprae* replication and establishment of infection [42;137]. Moreover, they might further assist *M. leprae* to reach peripheral nerve tissues through the blood stream [17] and act as reservoirs to further infect Schwann cells [199;200;207]. A layer of basal lamina which covers the Schwann cell-axon complex is the area where *M. leprae* can interact with laminin-2 as an entrance to infect Schwann cells. In addition, the expression of C-type lectin (CD209) on the surface of Schwann cells also enhances the binding and uptake of *M. leprae* which is regulated by Th2 cytokines like IL-4 [223]. There are also evidences that infected Schwann cells are capable of processing and presenting *M. leprae* antigens to inflammatory type 1 T cells which results demyelination, lyse of infected Schwann cells and nerve function impairment especially in tuberculoid patients with high CMI [161;220;221].

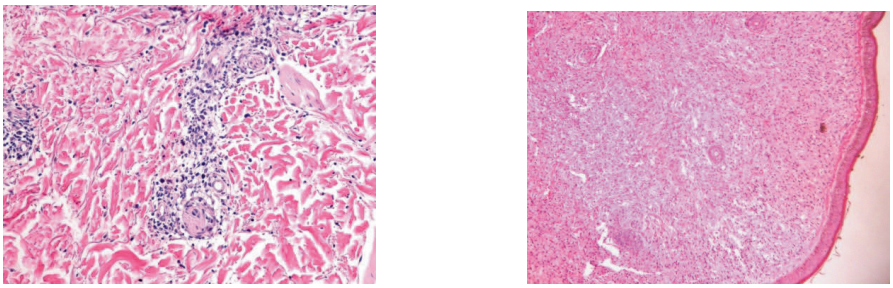
M. leprae and Macrophages

The various pattern recognition receptors (PRR) such as toll like receptors (TLR), C-type lectins, nucleotide-binding oligomerization domain (NOD2) and others recognize pathogen associated molecular patterns (PAMPs) and play a role in determining the function of macrophages during *M. leprae* infection [144]. In mycobacterial infections like TB and leprosy, microbial lipoproteins trigger TLR2, TLR2/1 or TLR2/6 heterodimers and this activation is enhanced by Th1 type cytokines and inhibited by Th2 cytokines.

Macrophages (Mϕ), being mediators of both the innate and adaptive immune systems, show distinct features in different forms of leprosy and determine the outcome of the pathogenesis. In tuberculoid leprosy, Mϕs are activated and their vitamin D dependent antimicrobial function (VDR) is dominant leading to killing of the bacteria. In lepromatous patients, the phagocyte function is more dominant and the Mϕs appear to be foamy, filled with lipid droplets derived from *M. leprae* and the host itself [9;44;146;147;222]. These features are visible in the types of granulomas formed in skin lesions of TT/BT and BL/LL patients (Fig 3 Granulomas). There is also evidence *in vitro* and in tissues that TLR2 and TLR1 are more expressed in TT/BT patients than BL/LL [114]. The mannose receptor (CD206) is mainly expressed on mature macrophages and facilitates phagocytosis through binding to the mannose capped liproarabinomanan.

The dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN); CD209 is also a major receptor on DCs, having similar ligand properties with CD206 for mannose capped liproarabinomanan and mycobacteria target DC-SIGN to suppress DC maturation through induction of IL-10 and inhibition of IL-12 [78;155]. There is also evidence for the expression of CD209 on macrophages of both TT and LL patients, and this is important in recognition of mannose-rich glycoconjugates like mycobacterial lipoglycan mannosylated lipoarabinomanan (ManLam), facilitating the binding and phagocytic process.

Along with this, innate cytokines in the lesions regulate the functions of macrophages in leprosy where IL-10 induces the phagocytic pathway and IL-15 induces the vitamin D dependent antimicrobial pathway [144;146;147]. The complement receptors (CR1, CR3 and CR4) also play a role in facilitating the phagocytosis process; CR3 especially facilitates the uptake of PGL-I by macrophages [197;198]. However, the activated phagocytic pathway in BL/LL patients will not culminate in the fusion of phagosome and lysosome because of the foamy nature of the Mϕs: this impairs lysosomal function and proper antigen presentation due to disrupted HLA-DR rafts [118;144;147]. Similarly, the higher frequency of regulatory T cells with higher production of anti-inflammatory cytokines in lepromatous compared with tuberculoid patients can also be considered as a potential factor modulating the formation of foamy macrophages which eventually form a diffused, unorganized granuloma in BL/LL patients unlike in TT/BT [165;188].



A. BT B. LL

Figure 3. H&E staining of tissues taken from a BT patient skin lesion (A) and LL patient skin lesion (B) showing the differences in granuloma formation.
Image A is taken from www.dermpedia.org and image B is photographed by Dr. Munir Hussien (Dermatovenorologist and Pathologist, AHRI)

Dendritic cells and Langerhans cells in leprosy

Dendritic cells (DCs) are professional APCs and are very efficient in priming and activating naïve T cells. In lesions of lepromatous patients, a deficit in DCs both in the dermis and epidermis was observed [211]. In addition, peripheral monocytes do not differentiate into $CD1^+$ DC following TLR activation in lepromatous patients [115]. These $CD1^+$ DCs ($CD83^+$) are capable of presenting non-peptide components; lipids and glycolipids to $CD1^+$ restricted T cells which in turn produce enormous levels of IFN-γ. Expression of the co-stimulatory molecule B7.1 is also decreased in LL patients [194]. This shows how *M. leprae* is capable of impairing the antigen presenting role of DCs. However, a recent study has indicated that activation of monocytes via NOD2 (Nucleotide-binding oligomerization domain containing protein 2) with its ligand muramyl dipeptide induces their differentiation into DC a process which was dependent on IL-32. Indeed, the expression of NOD2, IL-32 and $CD1b^+$ DC in leprosy lesion was found to correlate with bacterial control, being higher in TT/BT patients [196].

Langerhans cells are known to block dissemination of *M. leprae* at the infection sites and there is evidence showing reduced number of Langerhans cells in lepromatous patients compared to tuberculoid ones, which does not change after treatment [136;143].

1.7.3. Adaptive immunity in leprosy

Leprosy manifests itself in a wide-ranged spectrum where differences among the various forms are characterized by the type and level of immune responses. Cell mediated immunity (CMI) is important in controlling intracellular pathogens like *M. leprae* and *Mtb*. In tuberculoid leprosy patients, *M. leprae* infected macrophages eliminate the bacilli and present antigens in the context of MHC Class II molecules which induces IL-12 and stimulate $CD4^+$ Th1 cells [9]. These dominant Th1 cells, $CD4^+$ T helper and memory T cells in lesions of TT patients produce pro-inflammatory cytokines mainly IFN-γ, IL-2, TNF and other Th1 associated factors [117] and play an important role in activating macrophages to initiate their microbicidal activity and control bacillary multiplication [171]. Simultaneously, the numerous cytotoxic T cells, $CD4^+$ and $CD8^+$ T cells, which are restricted to MHC-Class II and I respectively produce effector molecules such as perforin, granzyme B and granulysin and lyse the infected macrophages.

In lepromatous leprosy patients (LL), suppressor type $CD8^+$ T cells are present, which are distributed in the lesions together with $CD4^+$ T cells. The suppressor $CD8^+$ T cells are

important in down regulating the macrophage activation and suppressing CMI. On the other hand, there is high production of antibodies which leads to accumulation of immune complexes activating the complement system[93]. The IgM level in the circulation facilitates the *M. leprae* evasion through activated C3 which are capable of co-stimulating naive T cells via complement regulation protein CD46, which leads to differentiation of IL-10 secreting regulatory T cells [29]. The Th2 type responses in the lepromatous patients are mainly characterized by the production of cytokines like IL-4, IL-5 and IL-13 and lack of IFN-γ and IL-2 to the extent of *M. leprae* specific anergy in LL patients. In these patients, a higher number of regulatory $CD4^+$ T cells characterized either by their expression of CD25, FoxP3, or production of cytokines such as IL-10 and TGF-β and also higher numbers of anti-inflammatory macrophages (Mϕ2) in the circulation and in skin lesions have been reported in several studies [24;116;165;188]. Along with this, Kumar *et al*., pointed out the importance of the molecular cross-talk of TGF-β, CTLA-4 and Cb1-b and the disruption of HLA-DR rafts leading to *M. leprae* persistence and T-cell hypo-responsiveness in lepromatous patients which they claim is a Th3 type response [117;118]. Other than the classical Th1 and Th2 type responses, a role for Th17 has been uncovered recently in a study in which higher level of IL-17 associated cytokines IL-1, IL-22 and RORC (Th17 transcription factor) were reported in the tuberculoid form of leprosy [187].

1.7.4. Host genetics and susceptibility to leprosy

It has been a century since leprosy was described as not inherited but rather caused by an infectious pathogenic bacterium. Nevertheless, the very different manifestations of leprosy with its distinct clinical presentations and immunological responses has led to investigations which collectively revealed the involvement of various human host genes involved in susceptibility to leprosy [11] and in developing specific forms of leprosy and leprosy reactions [8;11;75;91;236].

Human leukocyte antigens (HLA) are known for their association with several diseases. The association with leprosy was demonstrated three decades ago [49;50] although the presence of genetic susceptibility or predisposition was speculated since 1841 [74]. Later, the major role of the HLA Class II linked genes in determining the type/form of disease to be developed was demonstrated [51;162;236] as for instance shown in previous studies that HLA-DR3 and -DR2 are more associated with tuberculoid leprosy [49] where as HLA-DQ1 is associated with lepromatous leprosy [164]. The polymorphic nature of the peptide binding groove in HLA molecules is the main factor for HLA-peptide binding differences and this basic understanding has led to the development of *in silico* tools that predict immunogenic epitopes relevant in biomarker search for leprosy diagnosis [85].

Two genes highly associated to leprosy susceptibility more recently are PARK2 and PACRG [122;142]. The PARK2 gene is known for its association with Parkinson's disease. The PARK2 and LRRK2 genes have a role in cell apoptosis regulation. Another gene associated with susceptibility or resistance to leprosy and also linked with development of different forms of leprosy is NRAMP1 [8;30;94;139;215]. TLRs, NOD2 and MRC1 are also among the genes which are important in the early phase of host pathogen interaction with roles of bacterial recognition and uptake. The LTA4H gene regulates Lipoxin A4, one of the factors in the formation of macrophages filled with lipid droplets in LL patients. Genes such as TNF, LTA and IFN-γ and other related genes are involved in the formation of granulomas and in the maintenance of adaptive immunity [31]. Since TLR and VDR are important in recognition and killing of the bacilli respectively, polymorphisms in these genes are also important factors in determining the outcome of the leprosy infection. Other than the

previously reported susceptible loci, a recent genome-wide association study of leprosy in a Chinese population revealed six new susceptible loci where BATF3, CCDC88B and CIITA-SOCS1 were reported as new susceptible genes [123].

Genetic studies are powerful tools to decipher host pathogen interactions. Obtaining more detailed insights into the genetic control of infection and disease susceptibility of leprosy affected people, their household contacts and endemic controls may further help in designing strategies for early detection of individuals who are at risk of developing leprosy and contribute towards reducing leprosy transmission.

1.8. Reactions in Leprosy

Leprosy reactions are acute immunologic hypersensitivity episodes which can occur before, during or after MDT in about 30% to 50% of the leprosy patients. These complications are the main reasons of nerve impairment and disabilities in leprosy which increase leprosy related morbidity [26;111;130;131;240].

There are two commonly known leprosy reactions referred to as reversal reaction (RR) or type 1 reaction (T1R) and erythema nodosum leprosum (ENL) or type 2 reaction (T2R) [190;239]. There is also a third category of reactions, called the Lucio phenomenon, but this is less common and is reported mainly in central and south America in non-nodular lepromatous patients[145] as well as in patients with lepromatosis [98].

1.8.1. Reversal Reaction (T1R)

T1R mainly occurs in borderline patients (BT, BB and BL). It is characterized by inflammations in the skin and/or nerves with edema of the hands, feet and face [34;234] (Fig 4A). The diagnosis is mainly clinical and sometimes typical T1R histologic features can help the diagnosis. As T1R are frequently recurrent, close follow up of patients is necessary to avoid additional nerve damage [234]. Although not yet exhaustive, various host and pathogen factors are associated with T1R such as enhanced CMI with expression of IFN-γ, IL-6, IL-8, IL-13, TNF-α and other pro-inflammatory cytokines and chemokines like IP-10 which are typical characteristics of T1R [14;154;166;206]. Recent analysis of T1R has also revealed an increased production of CXCL9, IL-17A and VEGF in addition to IFN-γ and IP-10, and a reduction in IL-10. G-CSF and cytotoxicity associated genes such as granulysins, granzymes, perforins were shown to be up regulated during T1R compared with their levels before T1R [90]. An increased serum level of IL-17F during T1R was also reported which might indicate a role in inflammation [33]. Infiltration of $CD4^+$ T cells and presence of pro-inflammatory cytokines observed in the periphery were reported in skin lesions in patients with T1R. Upregulation of human beta-defensin 3 was also reported during T1R, which later subsided during corticosteroid treatment [37]. The association of certain genes or proteins either being upregulated or downregulated during T1R or after treatment with corticosteroids is an important clue towards a better understanding and management of reactions through developing tools that can possibly predict those at risk of developing reactions.

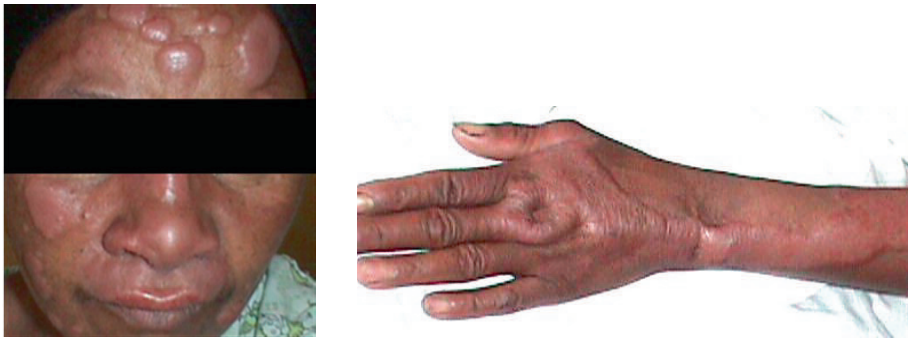


Figure 4 A. Type 1 reaction (T1R) or Reversal reaction (RR)
Photo by Dr. Elizabeth Bizuneh, ALERT (posted with permission)

1.8.2. Erythema nodosum leprosum (ENL)

ENL or T2R is an immune complex mediated episode which is more common in LL patients and some BL because of high level of *M. leprae* antigens and anti-*M. leprae* antibodies in the circulation of these patients [12;111]. Unlike T1R, ENL is characterized by infiltration of neutrophils into the lesions [45]. ENL is commonly diagnosed clinically as patients have tender red papules and nodules associated with fever (Fig 4B). As this type of reaction is a systemic disorder, it may affect organ systems and confer systemic complications. ENL starts as acute but can progress to a chronic phase and can also be recurrent [239]. High levels of serum C-reactive protein, amyloid A protein and alpha-1-antitrypsin are ENL markers [110] but are not commonly tested for.

Figure 4B. Type 2 Reaction (T2R) or Erythema Nodosum Leprosum (ENL)
Photo by S/r Genet Amare; AHRI (Posted with permission)

1.8.3. Risk factors for developing leprosy reactions

Leprosy reactions can occur at any time before, during or after MDT. However, the proportions of patients that develop reactions during and after treatment are higher compared to those before treatment. This is mainly because of the bactericidal effect of rifampicin which kills high numbers of bacilli leading to the release of *M. leprae* antigens in the circulation that trigger inflammatory reactions. Clinically, high BI (or being an MB patient), anti-PGL-I antibody level and being on MDT are among the potential risk factors. A genetic

study revealed that polymorphisms in genes such as TLR1 and TLR2, VDR, NRAMP-1, C4B and IL-6 have associations with developing leprosy reactions [73].
In addition, co-infections, especially oral infections, hepatitis C and hepatitis B are among reported possible risk factors for developing leprosy reactions [149-151]. The majority of leprosy patients co-infected with HIV show T1R [52] but further investigation is required to conclude this with more certainty.

1.8.4. Treatment of T1R and ENL

For both reaction types, corticosteroids (commonly prednisolone) are given to control the inflammation and reverse the nerve impairment. The regimen starts with high dose according to the severity of the reaction and is tailored based on clinical assessments made every 2 weeks. To avoid the long term side effects of corticosteroids, cyclosporine, methotrexate and tacrolimus are also considered as options to manage T1R.

Unavailability of treatment options is a big challenge in management of leprosy reactions but lack of knowledge present among health professionals in identifying reactions properly and prescribing the right dose of corticosteroids and tailoring down or up as to the degree of severity of the reaction is a major knowledge gap as well.

Thus, in addition to the currently available diagnostics tools and treatment, there is a demand to investigate leprosy-specific host or pathogen biomarkers for early detection of leprosy and prediction of leprosy reactions.

1.9. Search for leprosy-specific Biomarkers

1.9.1. Major challenges in leprosy and current opportunities

There have been major achievements in the control of leprosy, especially after the introduction of MDT since the 1980s [205]. However, the presence of pocket areas in different endemic countries contributes to the consistent number of new cases every year, the percentage of patients reporting with grade 2 disabilities and considerable numbers of new pediatrics cases [125;131;202]. This indicates how much effort is needed to "eliminate" leprosy and prevent leprosy associated disabilities. MB patients with high bacillary load and visible deformities stay in the population from months to years due to lack of awareness or being threatened by stigma. Hence, these patients serve as potential sources of infection. On the other hand, preclinical patients without any visible signs and symptoms of leprosy but harboring *M. leprae* in their body also live in the population until the disease is manifest because *M. leprae* has a long incubation period. Although it needs solid evidence, these "sub-clinically" infected individuals might be other sources of infection and there are no clinical or laboratory diagnostic tools to detect them [83].

Leprosy research has provided several key findings in the pre-genomics era. However, the area has benefitted and developed more in the postgenomic era mainly from the whole genome sequencing and bioinformatics which avails all necessary information about *M. leprae,* related *Mycobacterial* species and other species including the human genome. This has shortened the search for unique *M. leprae* antigens and the production of recombinant proteins and synthetic peptides enabling the evaluation of these antigens in a larger endemic population [20;23;83;86] to further use the promising ones in the biomarker search and development of novel tools for early detection of leprosy and prediction of leprosy reactions.

1.9.2. The search for unique and immunogenic M. leprae antigens in post genomic era

In the search for potential and promising antigens, both T and B cell based approaches have been used. As recently reviewed, about 200 *M. leprae* proteins and more than 10 fusion proteins have been tested for CMI and HMI in endemic countries in several studies in Brazil, Bangladesh, Ethiopia, India, Nepal, Pakistan, Philippines, Venezuela, China and in countries with relatively low number of leprosy cases like Japan and South Korea [81] .

T cell-based approaches

Previously, several *M. leprae* antigens like 18kDa, 35kDa, 45kDa or undefined mixtures of *M. leprae* components were tested for their immunogenicity in endemic and non-endemic populations [25;58;59;106;225]. Later our group at LUMC and others, using bio-informatics tools and whole genome sequences of *M. leprae* and other related species, identified *M. leprae* unique regions, with either known or unknown functions in infection biology. Genes with unique sequences were selected and their recombinant proteins were produced by cloning [76]. The recombinant *M. leprae* proteins and/or their synthetic peptides with 9-15 unique sequential amino acids fitting into MHC-I and II binding grooves were first tested for their specificity in non-infected and non-exposed individuals living in non-endemic countries, and next tested in endemic countries for their immunogenicity in whole blood assays (WBA) or in lymphocyte stimulation tests (LST) through analysis of IFN-γ by $CD4^+$ or $CD8^+$ T cells stimulated with these specific antigens in different groups: leprosy patients (TT/BT, BL/LL, patients with reactions), household contacts (HHC), healthy endemic controls (EC) and TB patients.

The main purpose of this evaluation process was to select potential T cell epitopes for diagnostics that could enable to differentiate *M. leprae*-infected from non-infected or exposed individuals, or detecting those who are at risk of developing the disease [84;88;218] or to accelerate leprosy vaccine development [66;67;87;176;191]. For instance, *M. leprae* proteins; ML0049 and ML0050, homologues of *Mtb* ESAT-6 and CFP-10 respectively were tested with the intention to develop similar diagnostic test for leprosy. However, cells of TB patients in endemic countries also recognized these *M. leprae* proteins as measured by IFN-γ response although their sequences are not similar (only 36% and 40% similarity) probably indicating cross-reactivity by the highly similar peptides, although co-infection of the TB patients with *M. leprae* could not be excluded [89]. Other *M. leprae* specific proteins which were initially tested in Brazil [84;218] were further tested in different populations in Africa and Asia [23;86]. Proteins ML1989 and ML1990 were almost fully recognized by all groups which included leprosy patients, household contacts and endemic controls questioning their potential as diagnostic tools. On the other hand, proteins such as ML0126 and ML1601 were found to be immunogenic and showed specific responses in leprosy patients although also nearly half of the healthy endemic controls responded to these antigens [23;86]. Further analysis of these proteins and their peptides including ML1601 and ML2478 in healthy endemic controls with different levels of exposure confirmed the diagnostic potential of these antigens, since endemic controls with relatively higher levels of exposure to *M. leprae* showed increased level of IFN-γ, IP-10, IL-1β, IL-6, TNF-α and CCL2 [22]. Similarly based on the cellular responses to these antigens, biomarkers other than IFN-γ, MCP-1, MIP-1β and IL-1β were shown to differentiate TT/BT patients from HHC [83].

Serological approaches

Antibody responses against *M. leprae*-unique proteins and other components of *M. leprae* have been analysed in several studies for potential use as diagnostic tools to monitor treatment, or disease progression [64;172;191;219]. Assessment of antibody responses

against 12 *M. leprae* specific antigens in leprosy patients and household contacts showed that antibodies to ML2028, ML0286, ML2038, LID-1, ML0405 and ML2055 proteins were detectable in MB patients and antibodies to ML2028, ML0286, ML2038 proteins were detectable in PB patients. The antibody titers against LID-1 and ML2028 were found increasing in two household contacts of MB index for about 15 months and one of these HHC showed progressively higher antibody titers to MLSA (*M. leprae* soluble antigen), LAM and PGL-I and developed borderline leprosy after two years of enrollment [219]. One of these proteins, the fusion protein LID-1 that induces strong IgG responses mainly in MB cases [170;172;180] is also used in the development of NDO-LID rapid test [62] (see page 17).
The fundamental reason for identifying and evaluating *M. leprae* specific antigens based on T cell or humoral approaches is to use these antigens in the development of rapid and field-friendly diagnostic tools to detect *M. leprae* infection earlier and for rapidly monitoring treatment outcome.

1.9.3. Cytokines/chemokines as potential biomarkers in leprosy

The search for biomarkers induced by these antigens, however, has many challenges in itself [82;163]. Several immunological biomarkers for T-cell based assays have now been identified and are discussed below:

Interferon-gamma

IFN-γ is a stable cytokine which has been and will remain a good indicator of pro-inflammatory/Th1 host responses against *M. leprae* and other mycobacteria. If the *M. leprae* protein used to activate its production is specific enough, unexposed individuals will not produce IFN-γ in *in vitro* assays. This would confirm the absence of cross reactivity with other mycobacteria which is important in selecting specific and immunogenic proteins [13;84;86]. As shown in TB diagnostics, two commercially available IFN-γ release assays (IGRAs), QuantiFERON©-TB Gold assay and T-SPOT TB, are developed based on the IFN-γ response to *Mtb* specific peptides of ESAT-6, CFP-10 and TB7.7, and has been successful in detecting latent *Mtb* infection in non-endemic areas and BCG vaccination does not interfere as these peptides are from the RD1 region unique to *Mtb* [35;36]. There is a continuing effort to develop relatively similar diagnostic tests for leprosy and it was shown that IFN-γ can be used to differentiate levels of *M. leprae* exposure as a response to *M. leprae* specific antigens [22;83]. However, prospective longitudinal studies are required to clearly define the different levels of exposure as risk or protective signals [82].

IFN-γ induced protein 10 (IP-10)

IP-10 is a small chemokine produced by many cell types but mainly by antigen presenting cells (APCs). There are several signals that induce IP-10 secretion such as T-cell derived IFN-γ, IL-2, IL-17, IL-23 and others. This makes IP-10 a reliable downstream marker as readout of cytokines like IL-2 and IFN-γ in CMI assays. Its expression is much higher which makes it more preferable for application in diagnostic tests [184]. In TB diagnostics, IP-10 has become a potential alternative to IFN-γ as it was found comparable to QuantiFERON© In-Tube test (QFT-IT) with higher level of expression [186] and also in combination with IFN-γ for detection of *Mtb* infection [185]. In leprosy, it has been decades since Kaplan *et al*. showed the potential of IP-10 in differentiating the different forms of leprosy by measuring its expression in skin lesions of TT/BT and LL patients where more IP-10 level was observed in TT/BT lesions [112]. Recent reports also revealed the potential of IP-10 in leprosy diagnosis by correlating with the level of exposure [22] and its potential in predicting T1R

[90;206]. It has now also been optimized for use in the field friendly UCP-LF assay [21] see chapter 5.

Monocyte chemo attractant protein 1 (MCP-1) (CCL2)
MCP-1 is a CC chemokine which is produced by various types of cells but mainly by monocytes/macrophages. It promotes macrophage infiltration in various inflammatory diseases and there is evidence for its importance in granuloma formation [47] which is an important phenomenon in containment of mycobacterial infections like leprosy and TB. There is evidence in TB studies that plasma levels of MCP-1 associate with TB disease and treatment responses [109]. Its potential to differentiate TT/BT patients from endemic controls was reported [83]. Its association with the PARK2 gene where level of MCP-1 increases along with increased expression PARK2 gene further confirms its involvement in the host defense against *M. leprae* infection [142].

Interleukin 1 beta (IL-1β)
IL-1β is a prominent member of the IL-1 family and is mainly produced by activated DCs and macrophages. Its expression regulates the Th1/Th2 balance such that higher expression of IL-1β leads to Th1 dominated response like in TT/BT patients while lower expression levels of IL-1β were found in LL lesions [124]. It was recently reported by our own group as a potential biomarker in detecting *M. leprae* exposure [22] as well as infection [83]

Interleukin-6 (IL-6)
IL-6 is a pro-inflammatory cytokine associated with inflammation. In leprosy, from previous assessments of plasma cytokines and chemokines in plasma of leprosy patients with type 1 and 2 reactions, IL-6 was reported as being elevated in both episodes as compared with non-reactional patients [19;148;159]. It was also significantly higher in endemic controls living in high leprosy endemic area than in those living in low endemic areas [22]. Recent studies also showed associations of single nucleotide polymorphism (SNP) in the IL-6 encoding gene with susceptibility to T2R [217], indicating the potential of IL-6 as genetic predictive risk marker. Similarly, increased IL6 expression in lesions of leprosy patients with reactions was reported [148] which decreased after prednisolone treatment[14].

1.10. Leprosy and co-infections

Being a mycobacterial infection, leprosy like TB, was predicted to manifest immune reconstitution disease (IRD) in HIV co-infected patients initiating treatment with anti retroviral therapy (ART). IRD or immune reconstitution inflammatory syndrome (IRIS) is an immune restoration disease in immunocompromised individuals such as HIV infected patients usually after the initiation of ART or a change to more active ART. As HIV mainly affects the host Th1 immune arm by affecting $CD4^+$ T cells, failure in containment of the disease in tuberculoid patients, an increase in lepromatous leprosy cases and increased transmission of leprosy was expected [128;233]. However, this has not been the case thus far: lepromatous forms have not increased although some patients on ART are being diagnosed as new leprosy cases, which requires attention [127;233]. Previous and recent reports on cellular and immunological parameters in leprosy and HIV co-infected patients demonstrated low numbers of $CD4^+$ T cells in the periphery but extensive $CD4^+$ T cells infiltration in the lesions of BT [189] with higher $CD8^+$ T cells [32]. In addition, no differences in histopathological features of leprosy were observed in HIV co-infected versus non-HIV patients [52].

In some studies, an association of type 1 leprosy reactions with ART was reported [140;195], suggesting ART and immune reconstitution could be risk factors for the development of leprosy reactions; however this requires further investigation.

In addition, intestinal parasites, mainly helminthic infections (non-protozoan intestinal parasites) are known to immune modulate the host by upregulating Th2 responses [10]. In mycobacterial infections like TB, studies have shown the importance of helminth infestation in weakening the Th1 immunity. For instance, poor immunogenicity of BCG in a helminth infested group compared to a de-wormed group was observed in an Ethiopian cohort [70;71]. The presence of intestinal helminthes may also facilitate the establishment of *M. leprae* infection or the progression to more severe forms of leprosy. A direct correlation between mycobacterial index and the frequency of intestinal helminthes in leprosy patients was also previously observed [56;57]. Therefore, further information needs to be collected in the area of helminth-leprosy co-infections, since this may have impact on clinical diagnosis and management.

Aims and outline of the Thesis

This thesis describes advances in the search for biomarkers relevant to leprosy diagnosis focusing in particular on early detection of the disease. It includes the screening of immunogenic and specific *M. leprae* antigens in endemic countries, identification of potential host biomarkers and development of field applicable diagnostic tools. It discusses the achievements and the unresolved challenges.

The approach for searching potential pathogen-derived biomarkers has changed dramatically after the year 2000 with the availability of the *M. leprae* whole genome sequence. The studies in this thesis selected antigens based on *in silico* algorithms, tested in leprosy patients, household contacts and endemic controls and endemic TB patients for their immunogenicity and specificity. Those unique and immunogenic proteins and peptides were investigated further as potential biomarkers in patients and healthy control groups, and eventually taken further to develop a field friendly diagnostic test based on both T cell and antibody responses. **Chapter 1** provides a detailed introduction into all areas studied in this thesis.

In **Chapter 2,** the screening of recombinant *M. leprae* proteins and synthetic peptides for immunogenicity and specificity in populations with different genetic background is described. T cell responses of stimulated PBMC from MB and PB patients, household contacts of MB patients, healthy endemic controls and TB patients were analysed using IFN-γ as a read-out for antigen-specific T cell activation. The positive responses found in 50% of healthy endemic controls for most of the antigens raised the issue of what level of *M. leprae* exposure was present in that subgroup.

In **Chapter 3** detailed analysis of host biomarker profiles in responses to the selected *M. leprae*-specific and immunogenic recombinant proteins and synthetic peptides is reported. The discriminating potential of these biomarkers among groups based on level of *M. leprae* exposure is an important result, clarifying why 50% of the healthy endemic controls responded to specific antigens. Other than the commonly known pro-inflammatory cytokine IFN-γ, several cytokines, chemokines and growth factors induced by adaptive and innate immunity were studied as well among which several were identified with discriminating potential. **Chapter 4** describes potential biomarker profiles in patients, in a longitudinal assessment, in an effort to predict leprosy reactions. Increased IFN-γ, IP-10, IL-17 and VEGF

were demonstrated whereas IL-10 was decreased in PBMCs simulated with *M. leprae* antigens during the onset of a reaction. This implicates the importance of combined assessments of Th1/Th2 responses in development of potential diagnostic assays. **In Chapter 5**, detection of identified biomarkers using lateral flow tests utilizing Up-converting Phosphor Technology (UPT) is demonstrated. The highly correlating results of the Up-converting Phosphor-lateral flow assay (UCP-LF) and ELISA demonstrated reliability of the assay. The potential of the UCP-LF assay to measure more than one biomarker (cellular and humoral responses) at a time has also revealed its appropriateness for field applicability.
Chapter 6 deals with assessment of the role of Tregs in view of the *M. leprae*-specific T cell non responsiveness in lepromatous patients. Using depletion assays, we demonstrated the recovery of T cell responses in one third of lepromatous patients after depletion of $CD25^+$ T cells. The increased presence of FoxP3 expressing T cells in the vicinity of Mϕ2 in LL lesions further revealed Tregs as one of the key factors responsible for poor CMI in lepromatous patients.

Chapter 7 describes co-infections in leprosy patients. Although the number of leprosy-HIV co-infected patients is small, understanding the influence of one disease on the other is important for proper patient management and for implementing proper control mechanisms. In recent studies including ours, most of the leprosy HIV co-infected patients on ART developed T1R, which requires further investigation to detect the underlying risk factors. In addition, we have also analysed and compared IFN-γ responses in helminth free and helminth-leprosy co-infected patients.

Finally, in **Chapter 8**, the main themes of the thesis are discussed in the broad context of leprosy diagnosis.

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Chapter 2

Post-genomic screening of Mycobacterium leprae proteins for immunogenicity and specificity

2.1 Immunogenicity of *Mycobacterium leprae* unique antigens in leprosy endemic populations in Asia and Africa

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Abstract

Ongoing transmission of leprosy is evident from the stable disease incidence in high burden areas. Tools for early detection of *Mycobacterium leprae* (*M. leprae*) infection, particularly in sub-clinically infected individuals, are urgently required to reduce transmission. Following the sequencing of the *M. leprae* genome, many *M. leprae*-unique candidate proteins have been identified, several of which have been tested for induction of *M. leprae* specific T cell responses in different leprosy endemic areas.

In this study, 21 *M. leprae*-unique proteins and 10 peptide pools covering the complete sequence of five *M. leprae*-unique proteins (ML0576, ML1989, ML1990, ML2283, and ML2567) were evaluated in 160 individuals in Nepal and Ethiopia. These included: tuberculoid and borderline tuberculoid (TT/BT), borderline borderline and borderline lepromatous (BB/BL) leprosy patients; healthy household contacts (HHC); tuberculosis (TB) patients and endemic controls (EC). Immunogenicity of the proteins was determined by IFN-γ secretion via stimulation of PBMC in 6 days lymphocyte stimulation tests (LST) or in whole blood assays (WBA).

In LST, BB/BL patients (40%) responded to ML0573 and ML1601 whereas ML1604 was most immunogenic in TT/BT (35%) and HHC (36%). Additionally, significant numbers of EC displayed IFN-γ production in response to ML0573 (54%), ML1601 (50%) and ML1604 (54%). TB patients on the other hand, hardly responded to any of the proteins except for ML1989. Comparison of IFN-γ responses to ML0121, ML0141 and ML0188 for TT/BT patients showed specific increase in diluted 6 days WBA compared to the undiluted 24 hours WBA, whereas EC showed a reduced response in the diluted WBA, which may indicate detection of disease-specific responses in the 6 days WBA.

In summary, identification of multiple *M. leprae* proteins inducing *M. leprae*-specific T cell responses in groups at high risk of developing leprosy may contribute to improve early detection for *M. leprae* infection.

Introduction

The introduction of multidrug treatment (MDT) recommended by WHO in 1981 aimed at eradication of leprosy by 2000 [17]. The shortened treatment period, 6 months and 1 year for paucibacillary (PB) (which includes TT/BT) and multibacillary (MB) (which includes BB/BL/LL) patients respectively, was one of the major steps forward by WHO which improved adherence and contributed to the dramatic reduction of leprosy prevalence globally. According to the WHO, eradication of leprosy as a public health problem (defined by less than one case per 10,000 people) has been achieved globally. High endemic zones, however, still subsist. The percentages of children among new cases of leprosy, which reflect a country's endemic level, ranged from 0.6 % in Argentina to 30.3 % in Papua New Guinea Moreover, the annual new case detection remains stable in many endemic countries [1]. This steady new case detection and the significant number of patients reporting with grade 2 disabilities, visible and irreversible, urge the need for strong efforts in discovering tools for early detection of *M. leprae* infection [1;5;14;19].

The disabilities and deformities in leprosy affected people along with wrong perception of the disease itself in most societies are the main reasons for stigma and discrimination. Despite the fact that the patients are cured from the disease with MDT, most people left out to live with unbearable social and psychological damage [3;12;13;16;21]. Hence, WHO has developed a new strategy for the next 5 years to mainly work on reducing disabilities, stigma and discrimination through provision of high quality patient care and early detection of new cases is obviously critical for the success of this effort [1]. However, do the available diagnostic tools sufficiently help in detecting leprosy infections at early (pre-clinical) stages?

Multibacillary leprosy patients (MB) are generally known to be main sources of *M. leprae* transmission. Close contacts of these patients who themselves are at high risk of developing the disease are believed to be asymptomatic sources of infection. However, the available diagnostic tools are not fully capable of detecting infections in these groups [5;8-9;11;20]. The clinical signs and symptoms, skin lesions consistent with leprosy having definite sensory loss, with or without thickened nerves and positive skin smears [18] are generally helpful to diagnose already advanced leprosy cases only. The anti-PGL-I (phenolglycolipid) IgM antibody measurement which mainly detects MB cases and which can also be positive for asymptomatic HHC, has been used for epidemiological and other related studies but is not applicable for routine laboratory diagnosis [15]. The lepromin test, on the other hand, measures the potential of an individual in building up granulomatous response to the mixture of *M. leprae* antigens of which lepromin is composed. An individual can develop a positive response to the test without ever having had any exposure to *M. leprae* [19]. This non-specific cellular response is caused by the fact that lepromin shares many proteins with other bacteria thereby inducing cross reactivity.

As in the development of TB diagnostics, the current high-tech era in genetics and bio-informatics has also contributed to the recent progress in the area of searching for potential proteins and peptides specific to *M. leprae* infection as the whole genome of the bacterium is sequenced [6;8-9;11;20]. Recent immunological studies in individuals from endemic countries revealed potential *M. leprae* proteins and peptides that induce T-cell responses. In Brazilian patients, ML1989, ML1990, ML2283 and ML2567 induced IFN-γ responses specific to *M. leprae* exposure [8]. However, further evaluation of these proteins combined with another potential specific protein ML2346 [6] and several *M. leprae* peptides [20] in five leprosy endemic countries (Brazil, Pakistan, Bangladesh, Ethiopia and Nepal) showed

significant responses in patients, but also in endemic controls (EC) as well as in healthy household controls [9]. This significant response in endemic controls raised the question whether reactivity to these proteins depicts exposure to or infection with *M. leprae* and whether the prevalence of leprosy in a region influences the responses induced in EC by *M. leprae* antigens. Thus this indicates the demand for continuous search of new *M. leprae* proteins and peptides that can induce T cell responses specific for *M. leprae* [10;11].

T-cell responses induced by mycobacterial peptides are more specific than those in response to mycobacterial proteins although the level of response in some assays is low [9;20]. The diversity of HLA in the human population will however require screening of large sets of *M. leprae* peptides in different leprosy endemic countries for possible wider application.

In this study, 21 recombinant *M. leprae* proteins, identified previously using a post genomic approach [8], were evaluated in leprosy patients, healthy household contacts, endemic controls and TB patients (completed intensive phase) from two leprosy endemic countries, Nepal and Ethiopia. In addition, pools of peptides covering five specific *M. leprae* proteins (ML0576, ML1989, ML2283, ML2346 and ML2567) previously tested in a Brazilian population [9;11] were further evaluated in the context of these Asian and African populations.

Materials and Methods

General procedure of the study. In this study, Armauer Hansen Research Institute, Addis Ababa, Ethiopia and the Mycobacterial Research Laboratory, Anandaban Hospital, Anandaban, Nepal, both located in leprosy endemic areas were involved in recruitment of patients, endemic controls and healthy household contacts. In both sites, identical standard operating procedures (SOPs) were applied and identical reagents were used to ensure reproducibility of data.
M. leprae recombinant proteins. *M. leprae* genes encoding candidate proteins derived from group VI (unknown function; ref Sanger database) were selected as described in detail previously [2]. The selected genes encoding the candidate proteins were amplified with PCR and cloned in pDEST17 expression vector containing an N-terminal histidine tag using the Gateway technology platform (Invitrogen, Carlsbad, CA). The recombinant proteins were overexpressed in *E. coli* BL21 (DE3) and purified from endotoxins. The endotoxin level in each recombinant protein was measured using a Limulus Amebocyte Lysate (LAL) assay and was below 50IU/mg (Cambrex, East Rutherford, NJ) [7]. All recombinant proteins were tested to exclude antigen non-specific T cell stimulation and cellular toxicity by measuring the IFN-γ level induced during 6 days incubation. Responses to medium and PHA were used as the negative and positive references in the assay. PBMC from *in vitro* PPD unresponsive, Mantoux skin test negative healthy Dutch donors recruited at the Leiden University Medical Center (LUMC) (with no prior contact with leprosy or TB patients) were used in the test.
The *M. leprae* proteins ML0126, ML0840, ML1601, ML1602, ML1603, ML1604, ML0573, ML0574, ML0575, and ML0576 were tested in Nepal and Ethiopia. Other *M. leprae* proteins: ML0121, ML0141, ML0188, ML1788, ML0369 and ML0927 only in Ethiopia. ML0121, ML0141, ML0188 were kindly provided by Dr. JS Spencer 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).

M. leprae whole cell sonicate. Irradiated armadillo-derived *M. leprae* whole cells were probe sonicated with a Sanyo sonicator to >95% breakage (Colorado State University, Fort Collins, USA through the NIH/NIAID Leprosy Contract N01-AI-25469).

Synthetic peptides. Synthetic peptides overlapping the complete sequence of protein ML0576, ML1989, ML2283, ML2346 and ML2567, produced as 20-mers overlapping 10 amino acids, were purchased from Peptide 2.0 Inc. (Chantilly, VA, USA).

Study subjects. The study was approved by the local ethics committees in Nepal (Approval nr. 93 24-08-2006) and Ethiopia (RDHE/163-71/2006). Written informed consent was obtained from each participant before sample collection. A total of 160 HIV-negative individuals were recruited: 50 BB/BL, 35 TT/BT leprosy patients, 22 healthy household contacts of BL/LL patients (HHC), 30 healthy individuals from the same endemic area (EC) and 23 smear positive, pulmonary tuberculosis patients (TB). Leprosy patients recruited were new cases or patients on treatment for less than 3 months with or without leprosy reactions. Clinical, bacteriological and histological examinations were performed to classify the leprosy patients according to Ridley and Jopling [18]. HHC were defined as adults living in the same house as a BL/LL index case for at least the preceding six months. Both HHC and EC were assessed for the absence of signs and symptoms of tuberculosis and leprosy. Staff members working in both leprosy centers were excluded as EC. TB patients had been on chemotherapy for at least 2 months to enable some recovery of T cell function.

Lymphocyte stimulation tests (LST). Venous blood was obtained from study participants in heparinized tubes and PBMC isolated by Ficoll density centrifugation. PBMC (2 x 10^6 cells/ ml) were plated in triplicate cultures in 96-well round bottom plates (Costar Corporation, Cambridge, MA) 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*, SSI, Copenhagen, Denmark) were added at final concentrations of 10 µg/ ml. As a positive control stimulus a final concentration of 1 µg/ ml phytoheamagglutinin (PHA; Sigma, St. Louis, MO) was used. After 6 days of culture at 37°C at 5% CO_2, 90% relative humidity, 75 µl supernatants were removed from each well and triplicates were pooled and frozen in aliquots at –20°C until further analysis.

Whole blood assays (WBA). Venous blood samples from 9 leprosy patients (BT and BL) and 8 EC were collected in heparinized tubes and used for 24 hours undiluted and for 6 days diluted WBA. For the 24 hours WBA, 450µl of blood per well was added in 48 well plate and 50µl of stimuli; *M. leprae* whole sonicate, ML0121, ML0141, ML0188 diluted in AIM-V medium with final concentration of 10µg/ml and PHA; 1µg/ml was added. Plates were incubated at 37°C at 5% CO_2, 90% relative humidity for 24 hours. For the 6 days WBA, blood samples were diluted 1:10 with AIM-V medium (serum free) and 900µl was added per well. The same stimuli with final concentration of 10µg/ml other than PHA (1µg/ml) were added in 100ul volume per well. Plates were incubated at 37°C in 5% CO_2 and 90% relative humidity for 6 days. IFN-γ ELISA was simultaneously done on supernatants of both assays.

IFN-γ ELISA. IFN-γ levels were determined by ELISA (U-CyTech, Utrecht, The Netherlands). 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 Buffy coat (LUMC, The Netherlands) was provided to both sites as a reference positive control supernatant.

TABLE 1
Study population at the two participating sites

Site	P[1]	Category	BI (mean)	Sex ratio[2]	Mean age (yr)
Anandaban (Nepal)	1.56	BB/BL	3.88	35/2	37.0
		BT/TT	0	18/7	36.9
		HHC	-[3]	14/6	32.7
		EC	-[3]	11/7	22.2
		TB	-[3]	9/5	29
Addis Ababa (Ethiopia)	0.60	BB/BL	1.64	9/4	29.2
		BT/TT	0.56	6/4	30.8
		HHC	-[3]	0/2	22
		EC	-[3]	4/8	27
		TB	-[3]	4/5	23

[1] prevalence per 10,000 individuals
[2] male/ female ratio
[3] not applicable

TABLE II
***M. leprae* specific candidate proteins**

Accession gene number*	Functional Classification	Protein product	Mr (kD)	Percentage identity** with *Mycobacterium*:								
				murine and human RT-PCR	*bovis AF2122/97 blastp*** (28)	*tuberculosis H37Rv Blastp*** (28)	*paratuberculosis K10 blastp*** (30)	*microti*# tblastn** (28)	*marinum*# tblastn** (28)	*avium*# tblastn ** (29	*ulcerans* # tblastn ** (31	*smegmatis MC2*# tblastn ** (29)
ML0126	VI	Hypothetical	31	+	<30%	<30%	<30%	<30%	<30%	<30%	<67%	<30%
ML0369	VI	Hypothetical	13	+	<30%	<30%	MAP4250c 38%	<30%	<33%	<39%	<37%	<33%
ML0573	VI	Hypothetical	10	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML0574	VI	Hypothetical	11	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML0575	VI	Hypothetical	7	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML0576	VI	Hypothetical	8	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML0840	VI	Hypothetical	48	+	<30%	<30%	MAP2122 59%	<30%	<30%	<64%	<30%	<47%
ML0927	VI	Hypothetical	11	+	<30%	<30%	MAP1963c 36%	<30%	<36%	<35%	<33%	<30%
ML1601	VI	Hypothetical	13	+	<30%	<30%	MAP3249 33%	<30%	<30%	<33%	<30%	<30%
ML1602	VI	Hypothetical	11	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML1603	VI	Hypothetical	9	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML1604	VI	Hypothetical	14	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%
ML1788	VI	Hypothetical	17	+	<30%	<30%	<30%	<30%	<30%	<30%	<30%	<30%

Functional Classification VI: unknown; ** blast reports were run December 2004; blastp = protein vs. protein; tblastn = protein vs. translated DNA. # unfinished genomes. Ref: (13)

Results

Testing *M. leprae* proteins and peptides in different leprosy endemic sites harbouring variable genetic backgrounds is essential to develop diagnostic tools that can be widely applied. In this study, a total of 160 individuals (50 BB/BL, 35 TT/BT, 22 HHC, 30 EC and

23 TB) from two endemic sites Anandaban in Nepal and Addis Ababa in Ethiopia were enrolled (Table 1).

PBMC or whole blood samples from all individuals were stimulated for 6 days in LST or in 24 hours WBA with 21 *M. leprae* proteins and 10 peptide pools each consisting of overlapping 20-mer peptides covering the sequence of five *M. leprae* proteins (ML0576, ML1989, ML1990, ML2283 and ML2567) previously shown to be specific for *M. leprae* exposure in a Brazilian population [8;11]. The peptide pools were composed of a total of 50 peptides [ML0576 (n = 7), ML1989 (n = 11), ML1990 (n = 7), ML2283 (n = 10), ML2567 (n = 15). Each pool consisted of five peptides (Table 3). Samples with high IFN-γ values (> 200 pg/ml) in the unstimulated negative control wells and with low IFN-γ values (< 100 pg/ml) in response to PHA (positive control for the assay) were excluded from the analysis.

Differential recognition of antigens by individuals within test groups

M. leprae unique proteins ML0573, ML1601 and ML1604 induced IFN-γ responses in EC. These responses clearly showed two distinct subgroups where 50% of the group showed high response and 50% very low (Figure 1).The difference in IFN-γ response against the unique proteins among the endemic controls could be due to the difference in the level of exposure to *M. leprae* (Geluk *et al.,* manuscript in preparation).

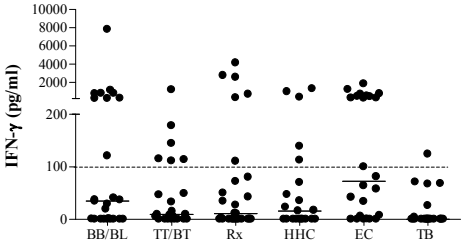
Patients with Type 1 leprosy reactions (RR) recognised ML0573 (48%) more frequently than ML1601 (26%) and ML1604 (30%). Although the median response was not above the cut-off for positive responses (i.e. 100 pg/ml), 40% of BB/BL patients also showed positive responses to ML0573 and ML1601 and 33% HHC and 35% TT/BT to ML1604 (Figure 1).

TABLE III

M. leprae peptide pool composition

Peptide pools	**pool 1 ML0576**	**pool 2 ML0576 & ML1989**	**pool 3 ML1989**	**pool 4 ML1989 & ML1990**	**pool 5 ML1990**
	5 6 7 8 9	10 45 *12 13 14*	*15 16 17 18 19*	*20 21 53* 22 23	24 25 26 27 28
Pool 6	*5*	10	*15*	*20*	24
Pool 7	*6*	45	*16*	*21*	25
Pool 8	*7*	*12*	*17*	*53*	26
Pool 9	*8*	*13*	*18*	22	27
Pool 10	*9*	*14*	*19*	23	28
	pool 11 ML2283	**pool 12 ML2283**	**pool 13 ML2567**	**pool 14 ML2567**	**pool 15 ML2567**
	29 30 31 32 33	*34 35 36 37 54*	*38 39 40 41 42*	*43 44 45 46 47*	*48 49 50 51 52*
Pool 16	*29*	*34*	*38*	*43*	*48*
Pool 17	*30*	*35*	*39*	*44*	*49*
Pool 18	*31*	*36*	*40*	*45*	*50*
Pool 19	*32*	*37*	*41*	*46*	*51*
Pool 20	*33*	*54*	*42*	*47*	*52*

ML0126



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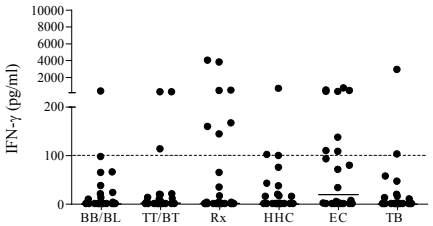

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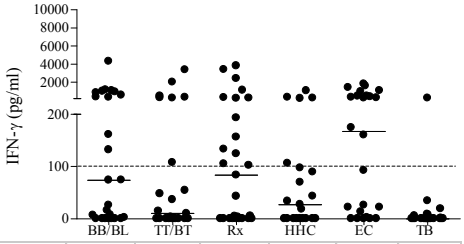
ML0573



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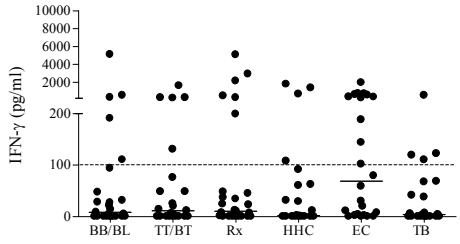
ML0574



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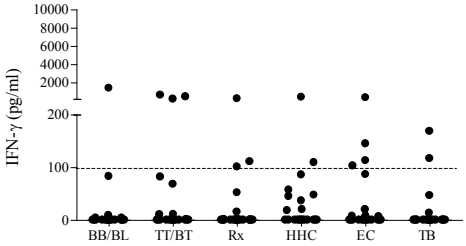
ML0575



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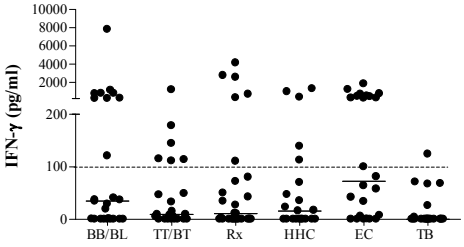

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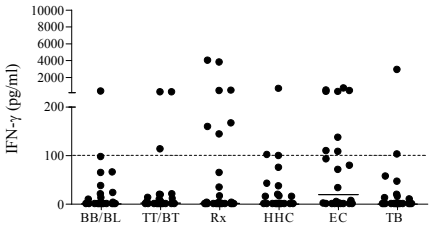

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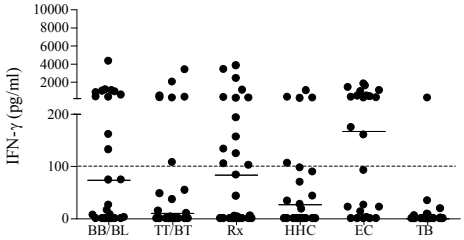
ML0573



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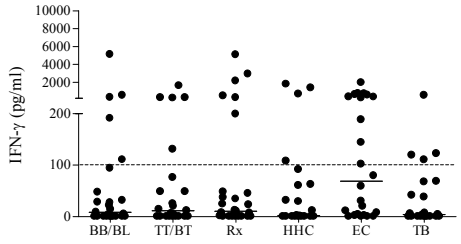
ML0574



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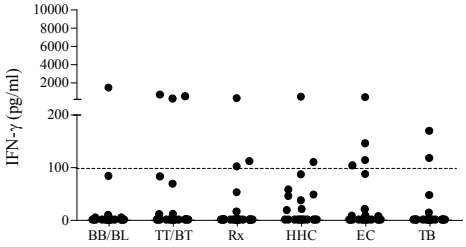

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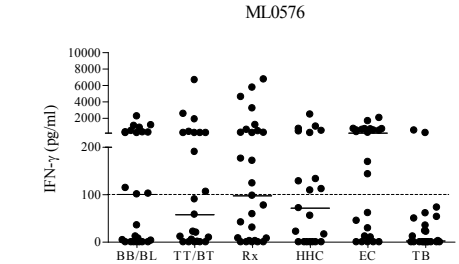

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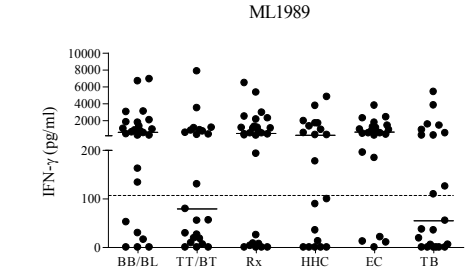

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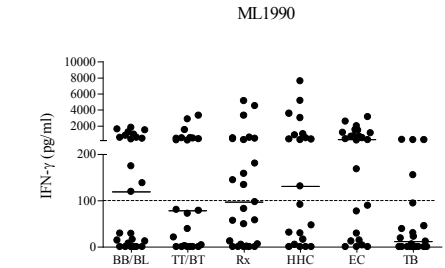

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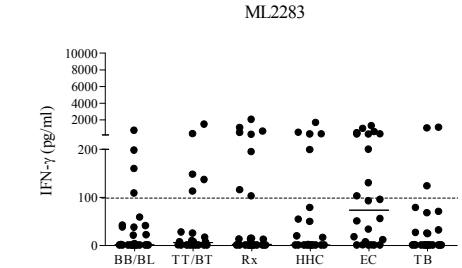

	BB/BL	TT/BT	Rx	HHC	EC	TB
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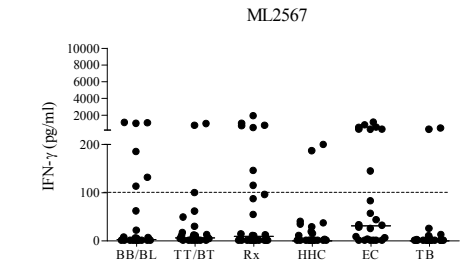

	BB/BL	TT/BT	Rx	HHC	EC	TB
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100-200pg/ml	3/25	0/23	2/27	2/21	1/24	0/23
>200pg/ml	3/25	2/23	5/27	0/21	7/24	2/23

Figure 1: IFN-γ production corrected for medium values induced in response to 14 *M. leprae* proteins in 6 days incubation of PBMC from multibacillary leprosy patients (MB; *n* = 45; 20 with leprosy reaction), paucibacillary leprosy patients (PB; *n* = 30; 7 with leprosy reaction), healthy household contacts (HHC; *n* = 22), healthy endemic controls (EC; *n* = 22) and TB patients (TB; *n* = 23).

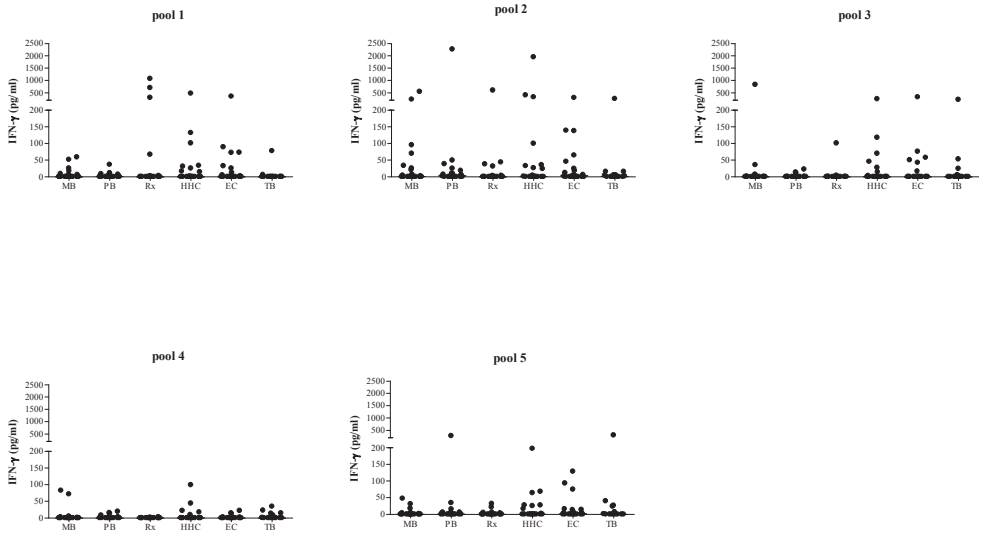
Similarly, ML0126 and ML0574 were more frequently recognised by EC (41%) than by leprosy patients but the median value was slightly below the cut-off. The responses in other groups were low for both proteins except the positive responses in 32% of BB/BL patients for ML0126 (Figure 1). Responses to ML1602 and ML1603 were low in all groups. However,

some individuals from BB/BL and EC group responded well to ML1603 but ML0840 and ML0575 were hardly detected in any of the groups (Figure 1).

Peptide pools induce only low IFN-γ responses in all test groups

The *M. leprae* proteins ML0576, ML1989, ML1990, ML2283 and ML2567 were analysed in five endemic countries previously and IFN-γ production was observed in response to these *M. leprae*-unique proteins in all test groups [9]. The current study analysed T cell reactivity in response to overlapping peptides of these proteins in LST. Peptide pools 1-5 and 11-15 (Table 3) were tested in the cohort from Nepal and peptide pools 6-10 and 16-20 were tested in Ethiopia (data not shown). The median IFN-γ levels in response to each peptide pool in all groups were found to be very low (Figure 2).

However, pool 1, 3, 5, 11, 14 and 15 containing peptides derived from ML0576, ML1989, ML1990, ML2283 and ML2567 respectively, showed some detectable responses in HHC and EC. Pool 2, composed of different peptides derived from ML0576 and ML1989 was recognised by few individuals from all groups except TB patients. Pool 12 and pool 13 contained peptides derived from ML2283 and ML2567 respectively, were recognised by some individuals from BB/BL, TT/BT, HHC and EC. Peptides from these antigens were reported previously also to be specifically recognised by *M. leprae* exposed (patients and HHC) in Brazil [11]. In general, responses induced by peptide pools were found to be low or absent. However, the low but detectable IFN-γ levels indicated the potential of these peptides for inducing specific T cell responses which may be enhanced by using different immunological boosting techniques such as addition of low concentration of cytokines or performing the test at temperatures above 37 °C related to fever [2;10].



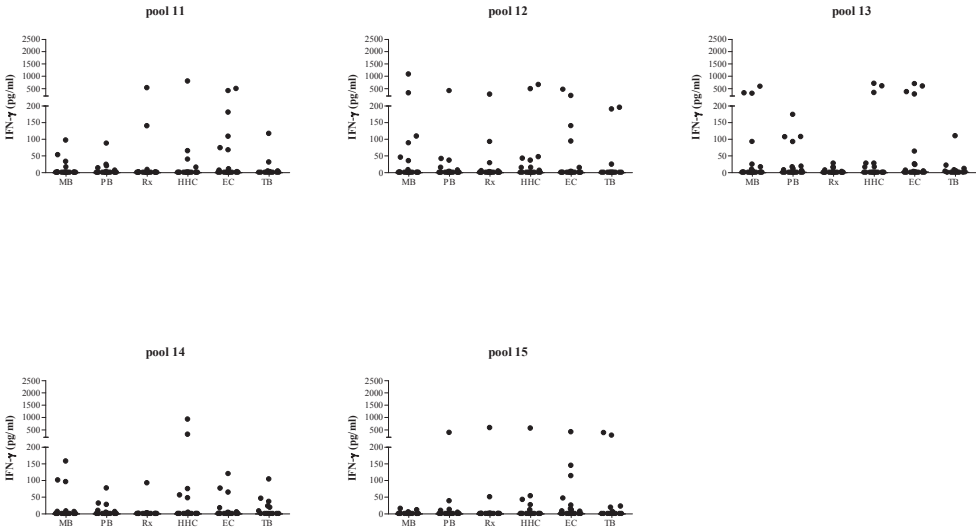


Figure 2: IFN-γ production corrected for medium values induced in response to 10 peptide pools in 6 days incubation of PBMC from BB/BL and TT/BT leprosy patients, healthy household contacts, healthy endemic controls and TB patients from Nepal.

Diluted WBA induced IFN-γ response in patients but not in EC

Finally, in order to compare what type of incubation would be preferable for *M. leprae*-specific IFN-γ detection in WBA, *M. leprae* proteins ML0121, ML0141 and ML0188 were simultaneously tested for their immunogenicity in an undiluted 24 hours WBA and a 1 in 10 diluted 6 days WBA (Figure 3).

The IFN-γ responses induced in whole blood samples derived from EC were significantly reduced (P=0.007 for ML0121 and P=0.049 for ML0141) in 6 days diluted WBA compared to the 24 hours undiluted WBA, whereas an increased IFN-γ response was seen in TT/BT patients in the 6 days diluted WBA.

Discussion

Since transmission of *M. leprae* infection is still ongoing as evidenced by the number of new cases in many endemic countries and the considerable number of patients presenting with Grade 2 disabilities, development of tools for early detection of *M. leprae* infection remains a key priority in combating leprosy [14;19].

The study described here represents the continuation of efforts to identify new *M. leprae* proteins and peptides that induce specific cellular immune responses and may be used in tools for early detection of infection in individuals living in leprosy endemic countries. The availability of whole genome sequences for *M. tuberculosis*, *M. leprae* and other mycobacterial species has tremendously supported this search effort through providing information on potential functions of proteins from unique genes. By selecting sequences that are uniquely found in *M. leprae,* we aimed to exclude T cell cross reactivity caused by homologous sequences. The *M. leprae* proteins tested in this study are hypothetical (categorised in group VI ref Sanger database) and their function is yet unknown except for ML1990, which was classified as a putative integral membrane protein. All proteins tested are unique to *M. leprae* except for ML1601, ML0369, ML0840 which have orthologues in *M.*

avium and ML0126 which has a homologue sequence (67 %) in *M. ulcerans*. Absence of responses in TB patients against the proteins confirmed absence of T cell cross reactivity with *M. tuberculosis*, also observed in Brazilian TB patients.

The IFN-γ responses against ML0573, ML1601 and ML1604 clearly divided the EC in two sub-groups: high and low responders. However, in the previous study, the responses from all Brazilian study groups were low for ML0573 and the responses to ML1604 were not specific as they were also recognised by individuals who did not respond to *M. leprae* whole sonicate *in vitro* [8] The aim of testing proteins in different endemic countries is to eventually design a widely applicable diagnostic tool. Therefore, using combinations of *M. leprae* proteins could be a method to overcome low and high responses in different leprosy endemic areas.

The high responses of EC described above could be due to exposure to *M. leprae* infection. However it is difficult to predict whether it is a sign of protection or infection leading to disease. Similar high responses were observed in EC in a previous study in response to other *M. leprae* proteins [9]. Further evaluation of these *M. leprae* proteins and screening of peptides from ML0573, ML1601 and ML1604 in healthy controls living in high, low and in non endemic areas and in close HHC in a longitudinal study will provide more insight on whether such responses are signs of protection against or susceptibility to disease. In addition, ML0573 may also be used in studies which aim to look for potential reaction predicting markers in leprosy patients as number of patients on reaction responded against this protein. In general, further evaluation of these potential proteins, their peptides and peptide pools in defined high risk groups could contribute in the development of early detection tools of leprosy infection.

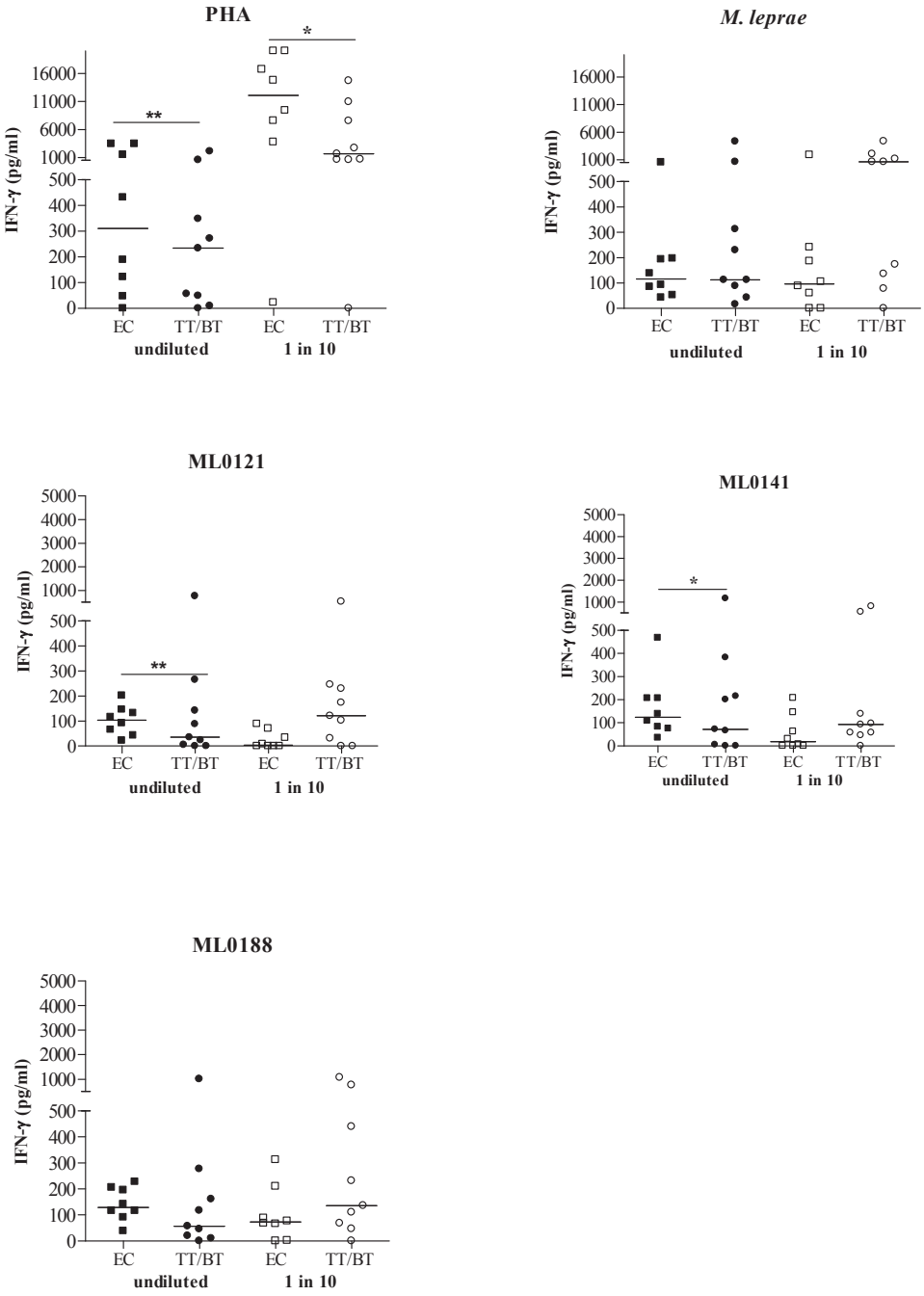


Figure 3: IFN-γ production in response to *M. leprae* recombinant proteins for EC (squares; $n = 8$) and BT/TT (circles; $n = 9$) in undiluted 24 hours WBA (undiluted; black symbols) or in 1:10 dilution 6 days WBA (1 in 10; open symbols). ** indicates P value = 0.007; * indicates P value = 0.05.

The IFN-γ levels produced in response to the peptide pools tested in this study in Nepal and Ethiopia were in general very low except for a few individuals with detectable responses (> 50 pg/ ml). Similarly, low responses against peptides were observed in previous studies [8;9]. However, considering those detectable responses against the peptide pools 2, 12 and 13, it could be useful to further screen single peptides from ML0576, ML2283 and ML2567 and also other potential proteins mentioned above especially in EC and HHC as was done previously in a Brazilian cohort [11]. As peptides are important in inducing specific responses, further boosting techniques should also be examined in order to enhance low but detectable IFN-γ responses [2;10]. Furthermore, identification of cytokines other than IFN-γ will also be necessary.

In determining what type of WBA would be beneficial to detect *M. leprae*-specific responses, we also compared diluted 6 days WBA to the undiluted 24 hour format. Interestingly, IFN-γ responses to *M. leprae* proteins in diluted 6 days WBA were significantly decreased for EC but not for leprosy patients, indicating reduction of some non-specific *ex vivo* responses observed in 24 hours WBA. Since the original aim of testing *M. leprae* proteins in WBA is to develop field-friendly diagnostic tools, 24 hours WBA would be preferred. However, diluted 6 days WBA is still very useful for selecting immunogenic *M. leprae* proteins which may induce specific memory T cell responses in *M. leprae* infected individuals. Therefore, working with diluted blood in 6 days WBA can be used in primary screening of proteins and peptides giving lower load and requiring small amount of blood [4].

The challenges in leprosy, prevention of deformities, disabilities, stigma, and interruption of transmission in leprosy endemic countries, require tools for early detection of *M. leprae* infection. Hence, identifying and screening candidate *M. leprae* proteins and peptides which could potentially induce cellular responses specific for *M. leprae* in individuals at high risk of developing leprosy should remain a continuous effort.

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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).

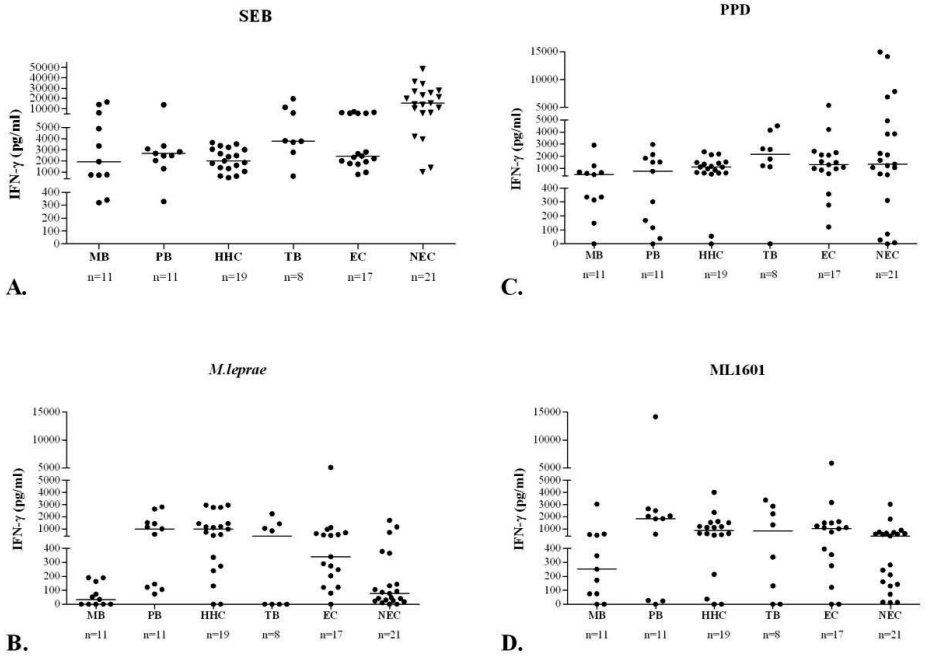

Figure 1 IFN-γ production by PBMC induced by SEB (**A.**), PPD (**B.**), *M.leprae* (**C.**) and ML1601c recombinant protein (**D.**) in MB (n = 11), PB (n = 11), HHC (n=19), TB (n = 8) and EC (n = 17) from Brazil as well as in Dutch NEC (n = 21). For NEC PHA was used instead of SEB. Values were corrected 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}).

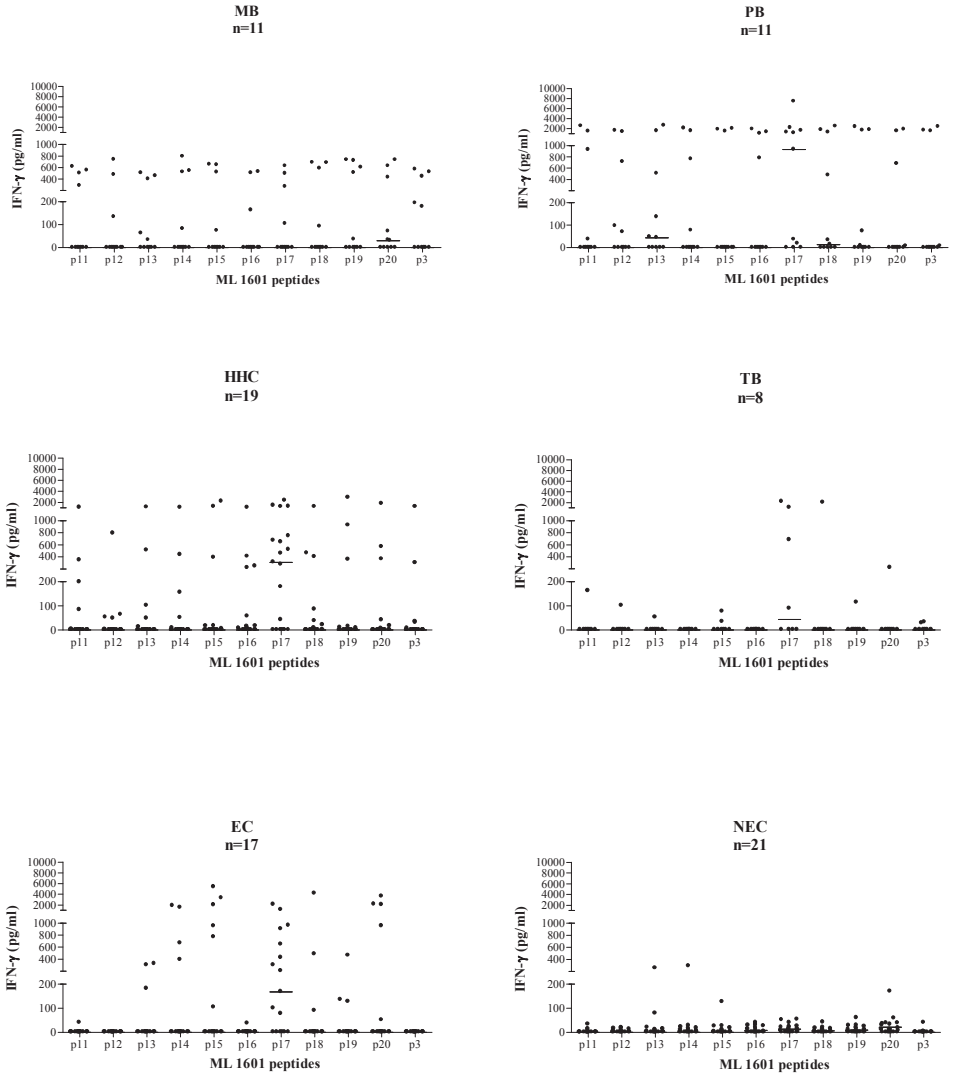

Figure 2 IFN-γ production by PBMC induced by ML1601c peptides (see Table I) in MB (n = 11), PB (n = 11), HHC (n=19), TB (n = 8) and EC (n = 17) from Brazil as well as Dutch NEC (n = 21). Values were corrected for background values. All background values were typically < 20 pg/ml. Horizontal bars indicate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

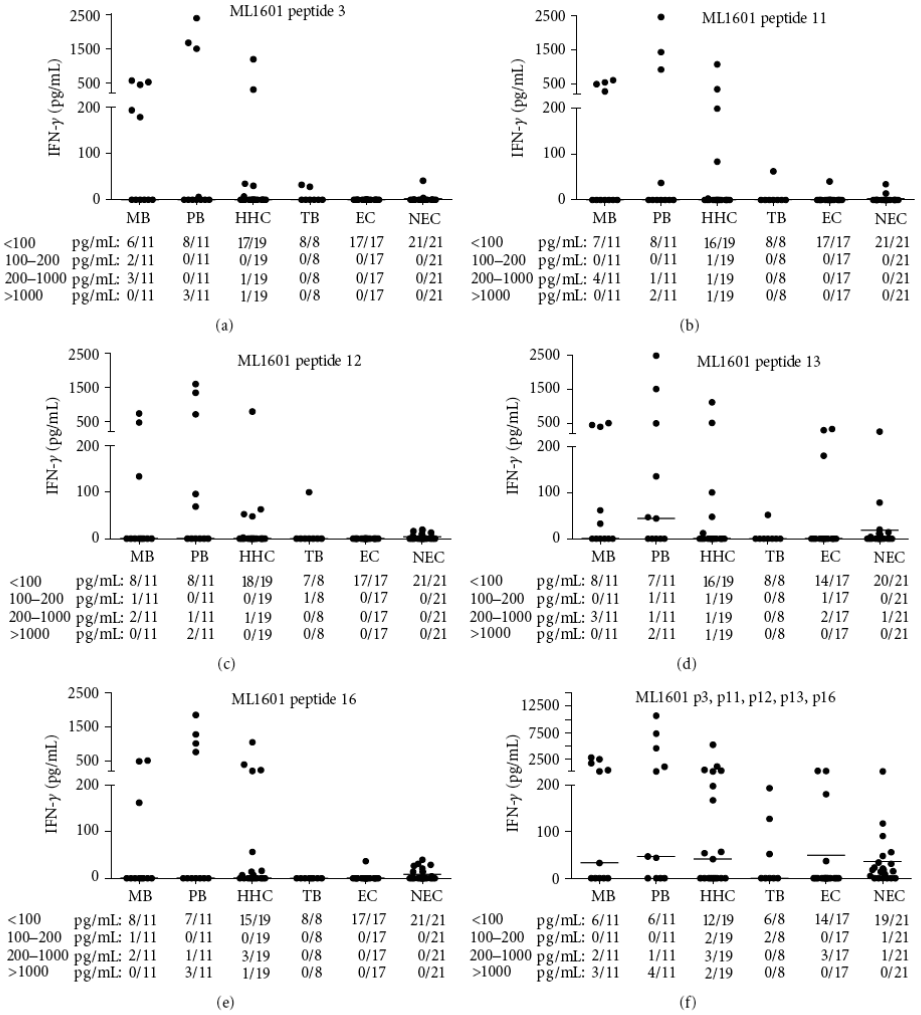

Figure 3: IFN-γ production by PBMC induced in all test groups by ML1601c peptides p3, p11, p12, p13, and p16 and the sum of the IFN-γ values for p3, p11, p12, p13, and p16 combined. The proportions of responders in each test group are indicated below the x-axis.

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.

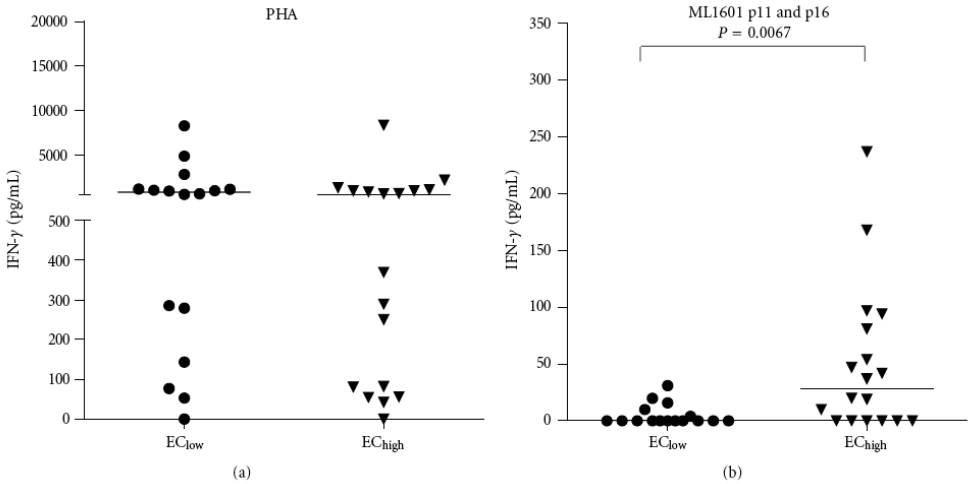

Figure 4: IFN-γ production in response to the positive control PHA (a) or a mix of ML1601c peptides no. 11 and no. 16 (b) measured after 24h culture of undiluted whole blood derived from 34 Ethiopian healthy controls (ECs) derived from areas with low endemicity for leprosy (EC_{low}; *n=16*) or from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

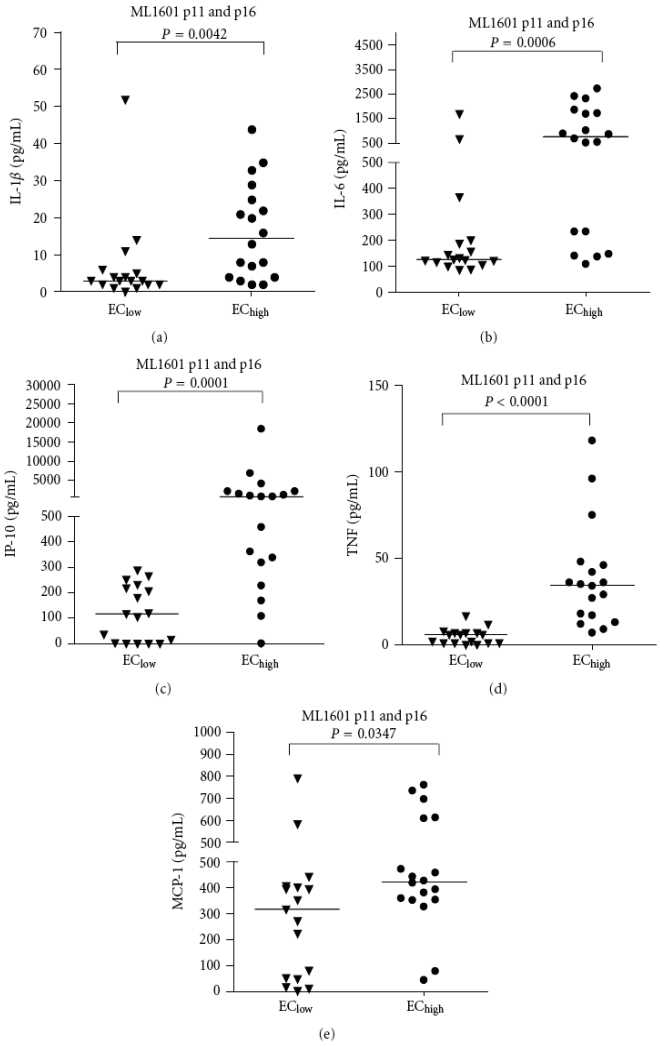

Figure 5: Production of IL-1β (a), IL-6 (b), IP-10 (c), TNF-α (d), and MCP-1 (e) in response to selected ML1601c peptides measured after 24h culture of undiluted whole blood derived from 34 Ethiopian healthy controls (ECs) derived from areas with low endemicity for leprosy (EC_{low}*: n=16*) or from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

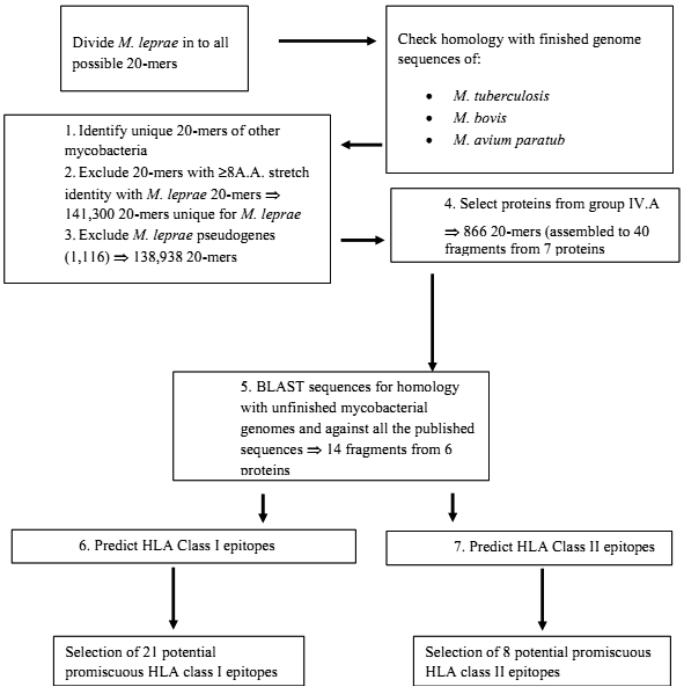


Figure 1: *M. leprae* peptide selection procedure. Identification and selection of predicted *M. leprae* promiscuous HLA class I epitopes (n = 21) and predicted *M. leprae* promiscuous HLA class II epitopes; n = 8) derived from *M. leprae* proteins from functional classification group IV.A (virulence; 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.

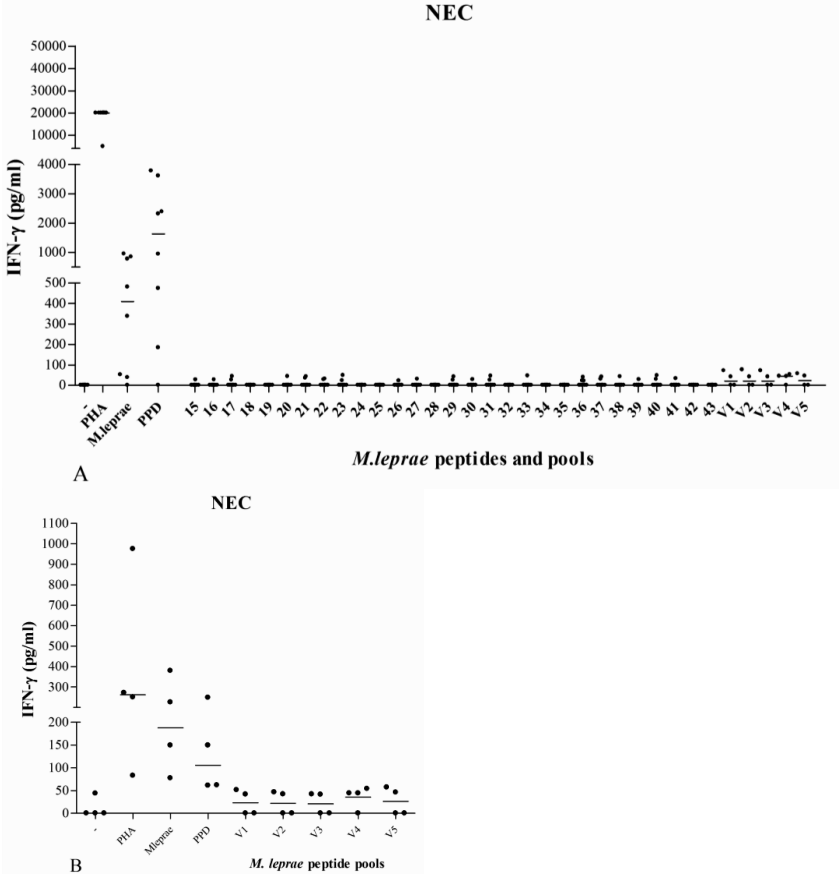


Figure 2: **IFN-γ responses to *M. leprae* peptides in PBMC from NEC.** IFN-γ production (corrected for background values) induced using *M. leprae* virulence-associated peptides or pools thereof (**A**) in 6 day PBMC cultures of Dutch healthy controls (n = 8) or as pools in a 24 h WBA (**B**). Pool 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.

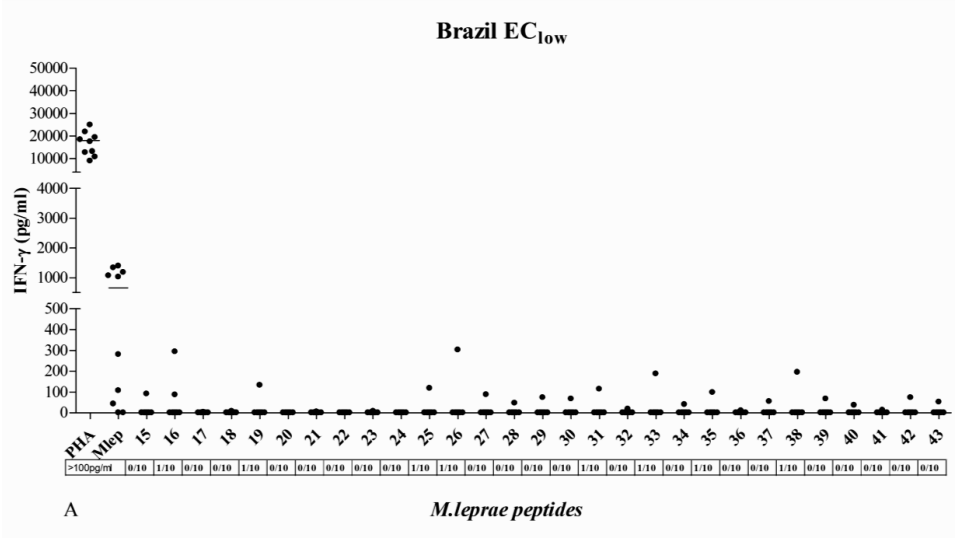
IFN-γ responses in Ethiopian EC_{high} and EC_{low} in WBA
In order to identify peptides with the ability to indicate exposure levels of *M. leprae* in a rapid field-assay, peptides ML2055 p35, ML2055 p42, ML2055 p37 and ML1358 p24 were selected for subsequent comparative analysis of Ethiopian EC_{low} (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}.

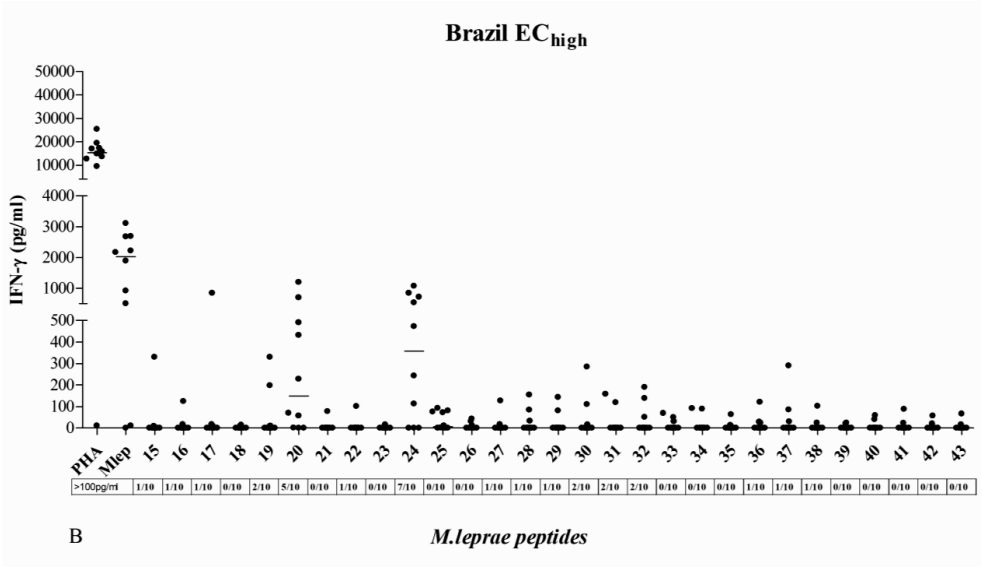
IP-10 as potential biomarker for diagnosis of *M. leprae* exposure
Although IFN-γ is the hallmark effector molecule of Th1 cells and a critical component of the pro-inflammatory immune response, host immunity and immuno-pathogenicity in response to *M. leprae* involves complex interactions between a 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

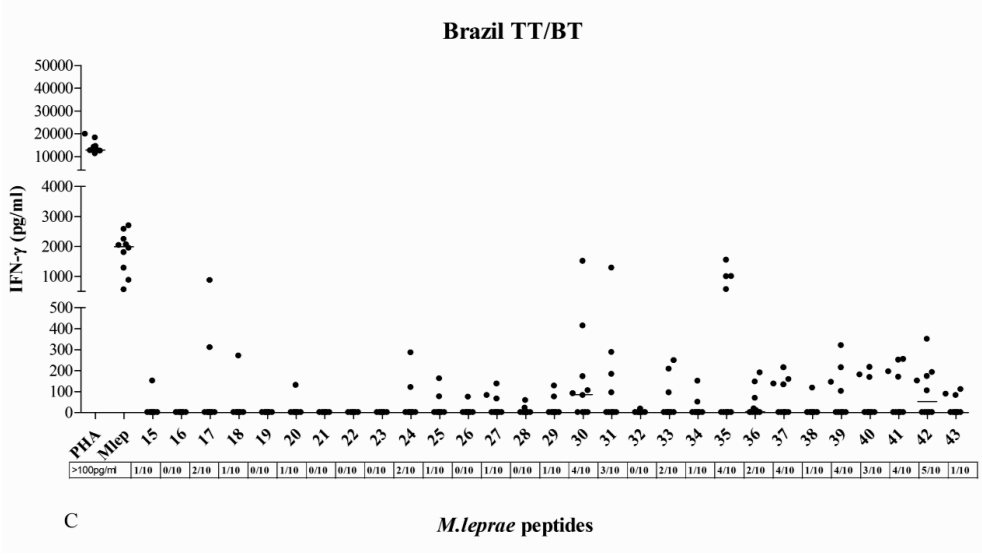
p42 also induced increased levels of IP-10 in EC_{high} compared to EC_{low} although the difference was not significant (p=0.06; Figure 4F). None of the other analytes showed significantly different levels between EC_{high} and EC_{low} (data not shown). Thus, the high IP-10 levels induced by *M. leprae* 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].

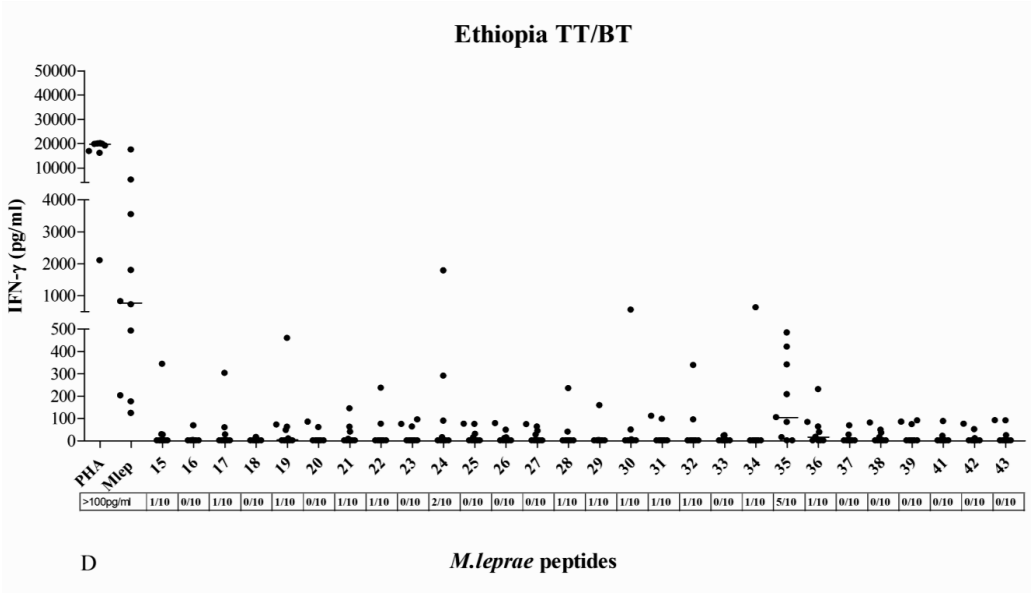
IFN-γ production in response to *M. leprae* recombinant proteins

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



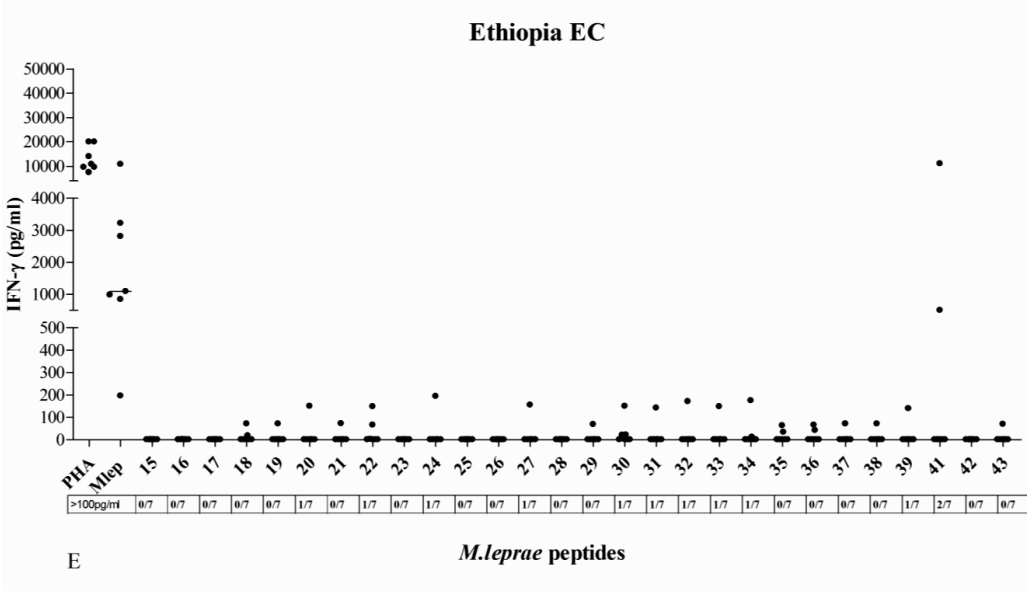


Figure 3: **IFN-γ responses to *M. leprae* peptides in PBMC from Brazilian and Ethiopian individuals.** IFN-γ production (corrected for background values) induced using *M. leprae* virulence-associated peptides in 6 day PBMC cultures of endemic controls from areas of Fortaleza with low (EC_{low}; prevalence <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.

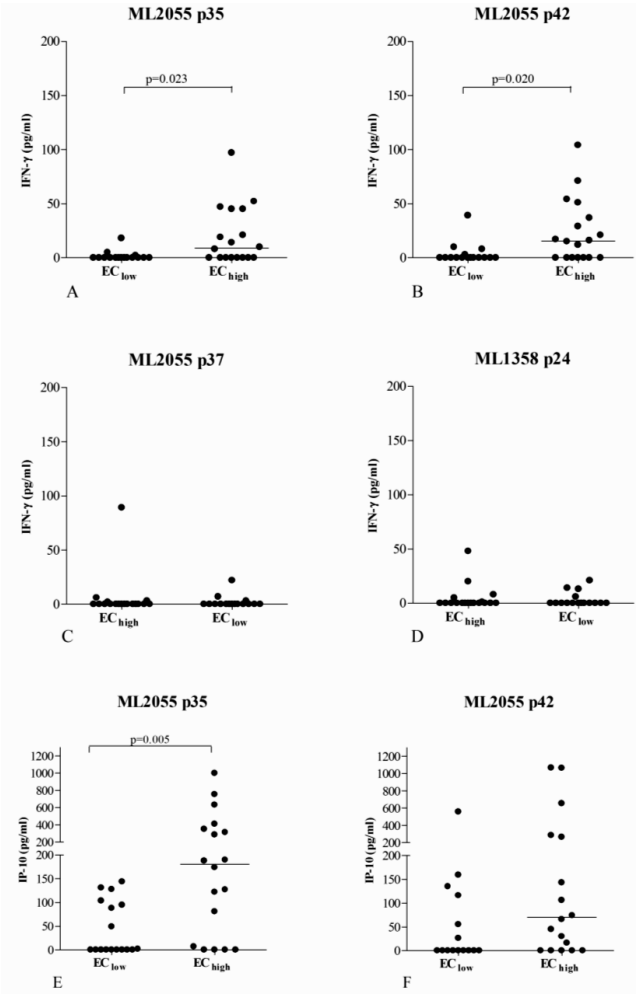


Figure 4: IFN-γ and IP-10 responses to *M. leprae* virulence-associated peptides in 24 h WBA of Ethiopian EC_{high} and EC_{low}. IFN-γ (**A-D**) and IP-10 (**E, F**) production in response to *M. leprae* peptides ML2055 p35 (**A**, **E**), ML2055 p42 (**B**, **F**), ML2055 p37 (**C**) and ML1358 p24 (**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].

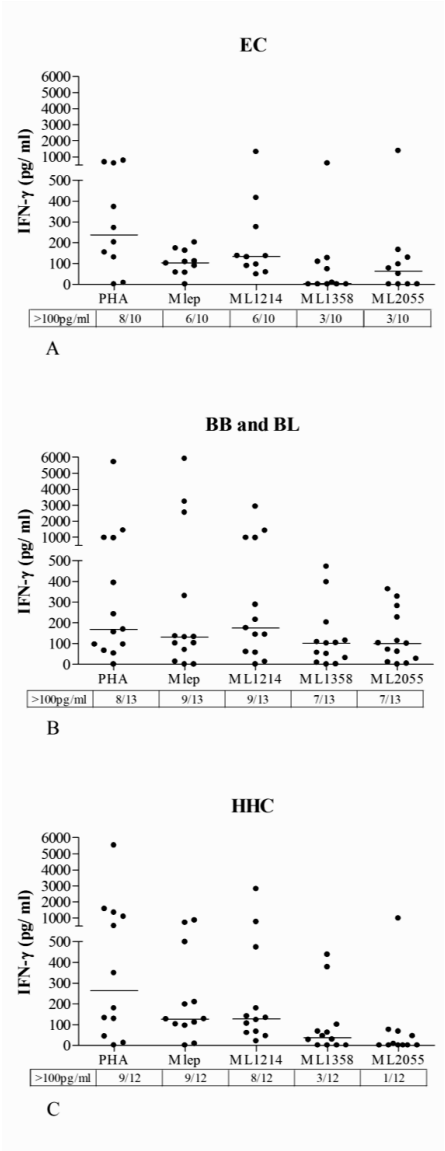


Figure 5: IFN-γ responses to newly identified *M. leprae* virulence-associated proteins.
IFN-γ production (corrected for background values) induced using *M. leprae* recombinant proteins ML1214, ML1358 and ML2055 in 24 h WBA from BB/BL (n=13), HHC (n=12) and EC (n=10) in Ethiopia

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.

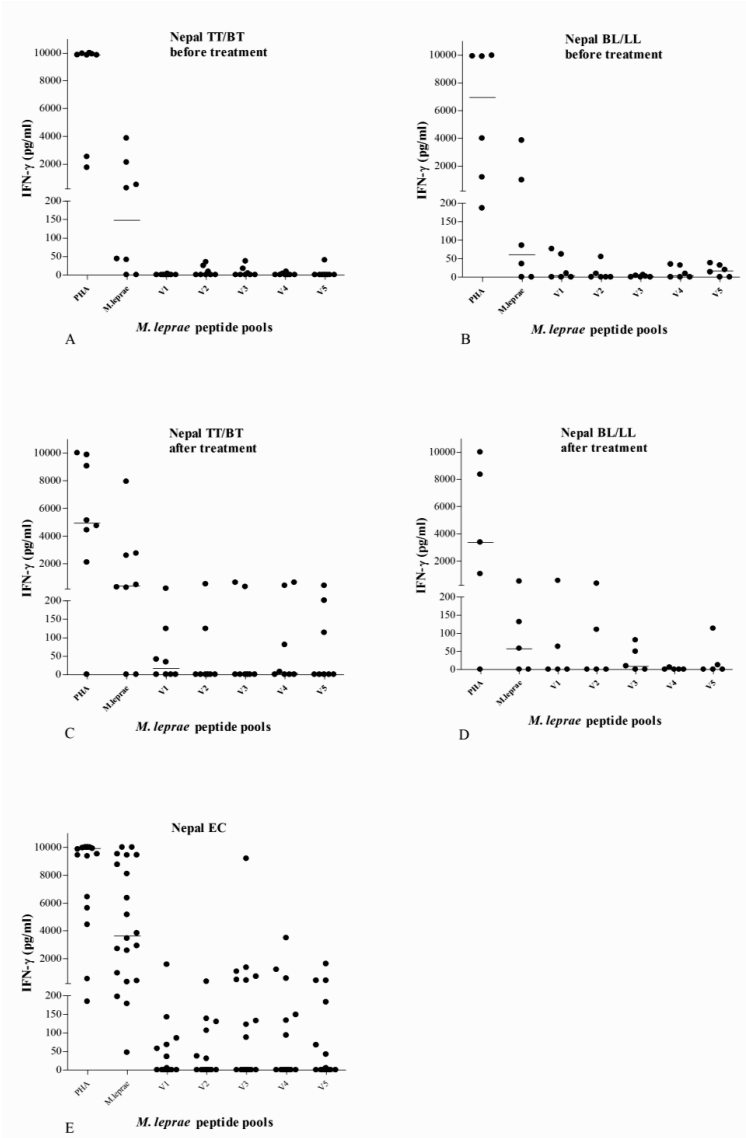


Figure 6: IFN-γ responses to *M. leprae* virulence-associated peptide pools in PBMC from Nepali individuals. IFN-γ production (corrected for background values) induced by pools of *M. leprae* virulence-associated peptides (10 µg/ml each) in 6 day PBMC cultures of newly diagnosed BT (**A**; n = 7) and 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].

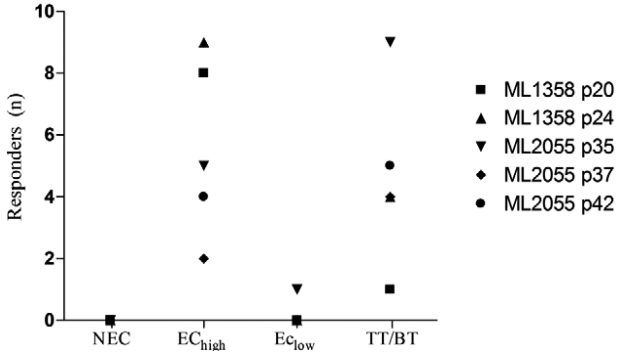


Figure 7: Individuals with IFN-γ responses to *M. leprae* virulence-associated peptides. The total number of individuals that induced IFN-γ production in response to 5 promising *M. leprae* virulence-associated peptides is indicated for each test group: Dutch NEC, combined Brazilian and Ethiopian $EC_{high,}$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

Acknowledgements

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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).

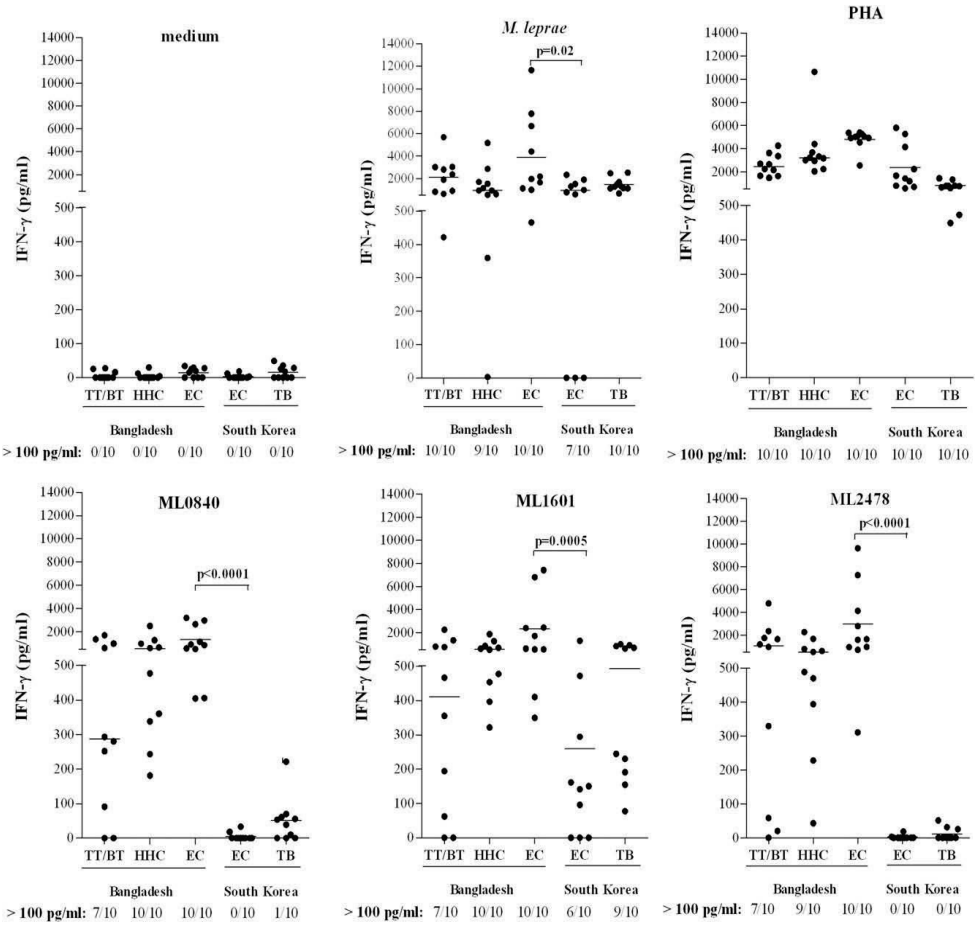

Figure 1: IFN-γ responses in WBA from individuals in Bangladesh and South Korea. IFN-γ production in responses to control stimuli (medium, PHA and *M. leprae* WCS) or to recombinant proteins (ML0840, ML1601, and ML2478) in 24h WBA of leprosy patients (TT/BT; n=10), HHC (n=10), and EC 9n=10) from Bangladesh (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.

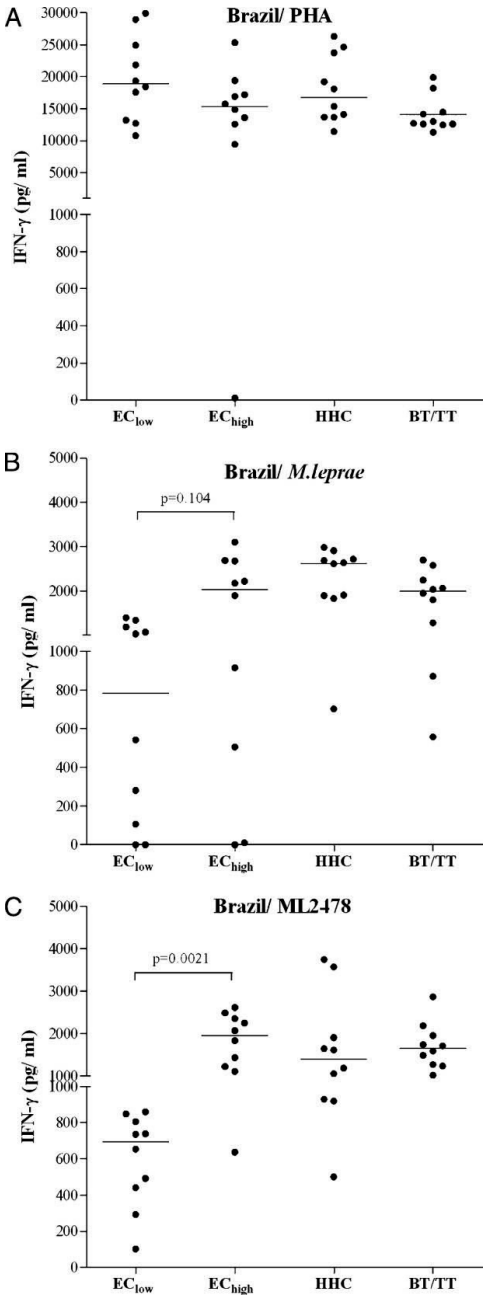

Figure 2. IFN-γ responses to *M. leprae* Ags in PBMC from EChigh and EClow in Brazil. IFN-g production (corrected for background values) induced using PHA(A), M. leprae (B), or ML2478 recombinant protein (C) in 6-d cultures of PBMC from healthy individuals from an area of Fortaleza, Brazil, with low (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.

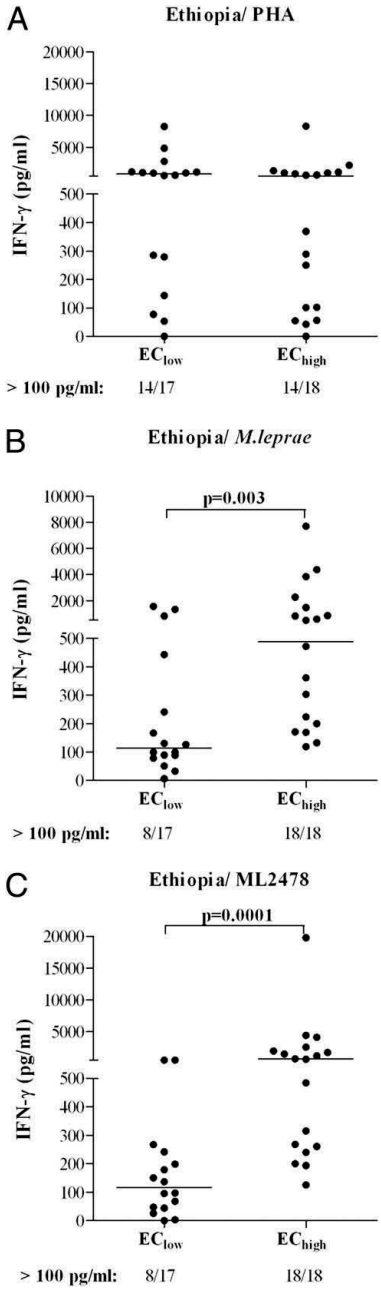

Figure 3. IFN-g responses to M. leprae proteins in WBA from EChigh and EClow in Ethiopia. IFN-g production (corrected for medium values) in response to PHA(A), M. leprae WCS(B), or recombinant protein ML2478 (C) in 24-h WBA of healthy individuals from areas in Addis Ababa, Ethiopia, with low (EClow; 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.

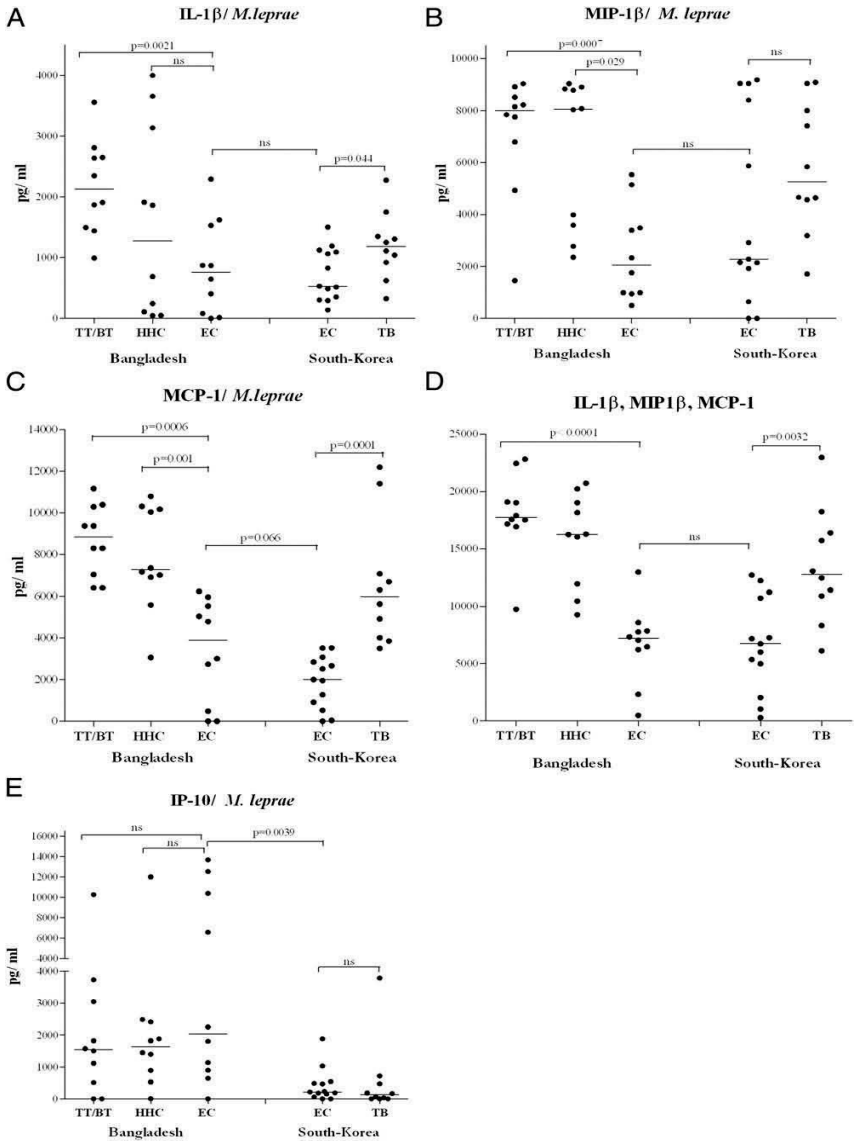

Figure 4. Multiplex cytokine analyses inWBA from individuals in Bangladesh and South Korea. Concentrations (all corrected for background values) of IL-b (A); MIP-1b (B); MCP-1(C); and IL-b,MIP-1b, and MCP-1 combined(D); or IP-10 (E) induced by stimulation with M. leprae WCSin 24-hWBA ofleprosy patients (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*.

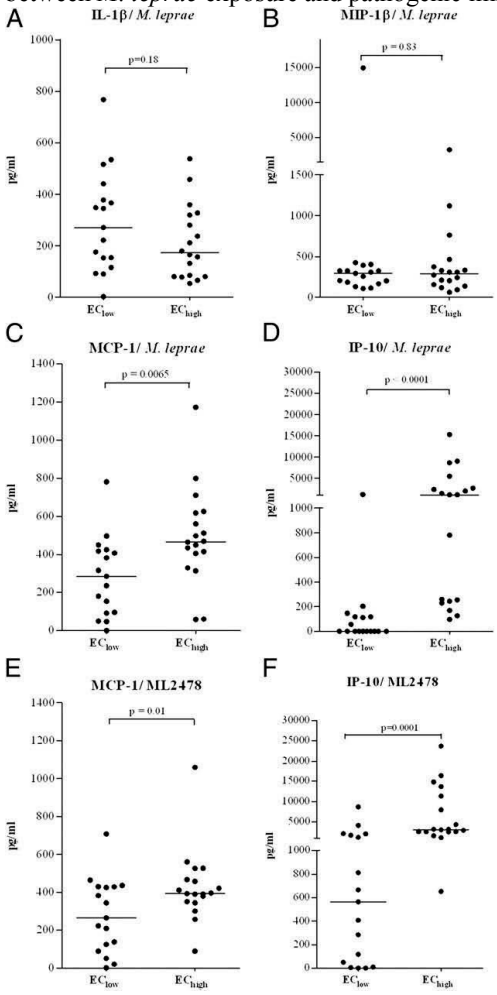

Figure 5. Multiplex cytokine analyses in whole-blood cultures from EC in Ethiopia. Concentrations (all corrected for background values) of IL-b (A), MIP-1b (B), MCP-1(C, E), IP-10(D, F)induced by stimulation with M. leprae WCS(A–D), or ML2478(E, F) in 24-h WBA of leprosy patients (TT/BT; n = 10), HHC(n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

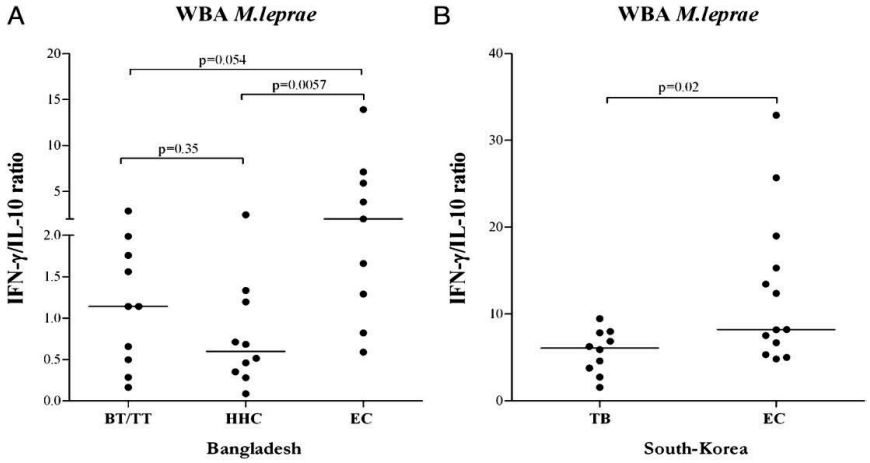

Figure 6. IFN-g/IL-10 ratio in M.leprae-stimulated WBA. Ratios of IFN-gconcentrations (corrected for background values) with respect to IL-10 concentrations (corrected for background values) induced by stimulation with M.lepraeWCS in 24-h WBA in individuals from Bangladesh (A) and South Korea (B).

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

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

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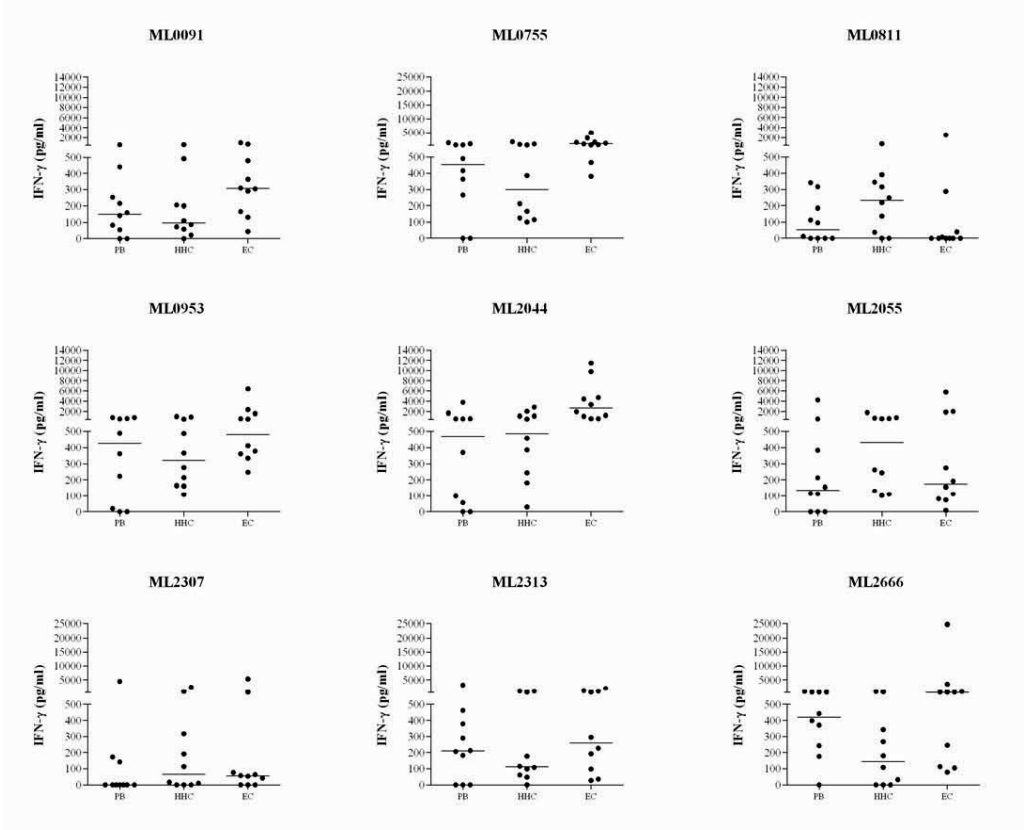
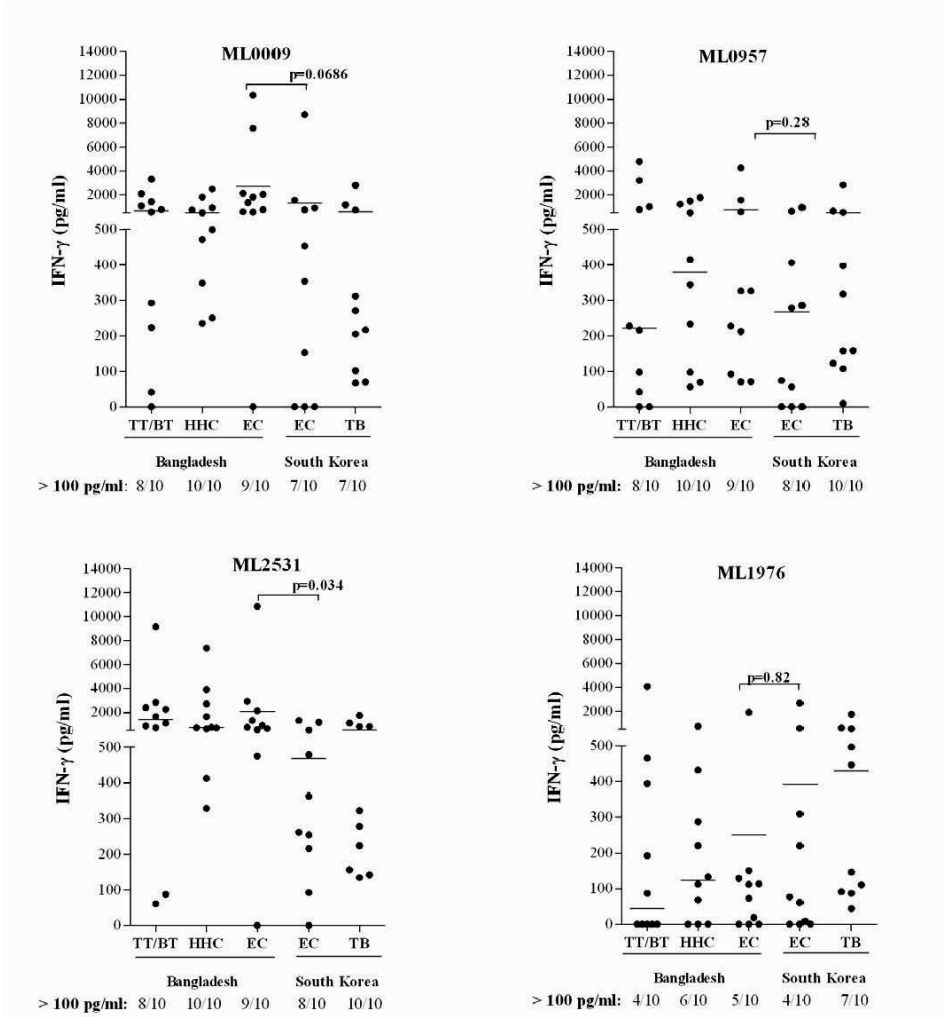
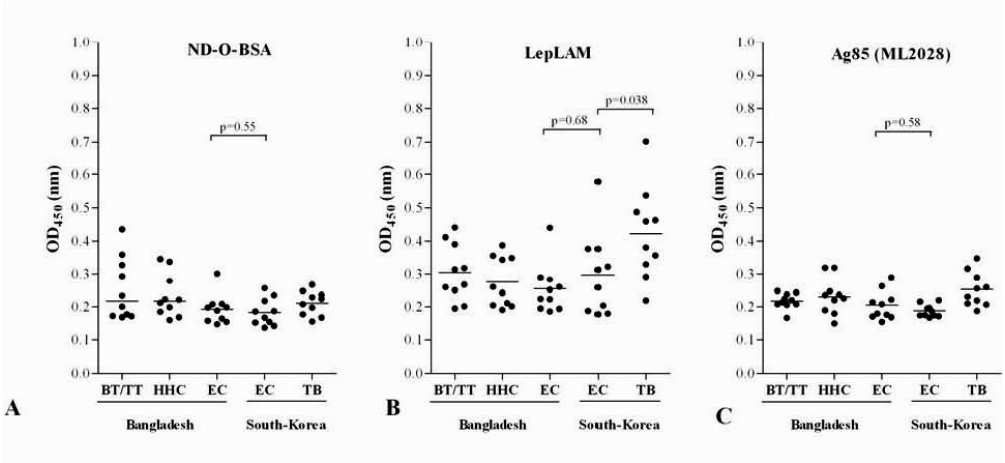
Supplementary files



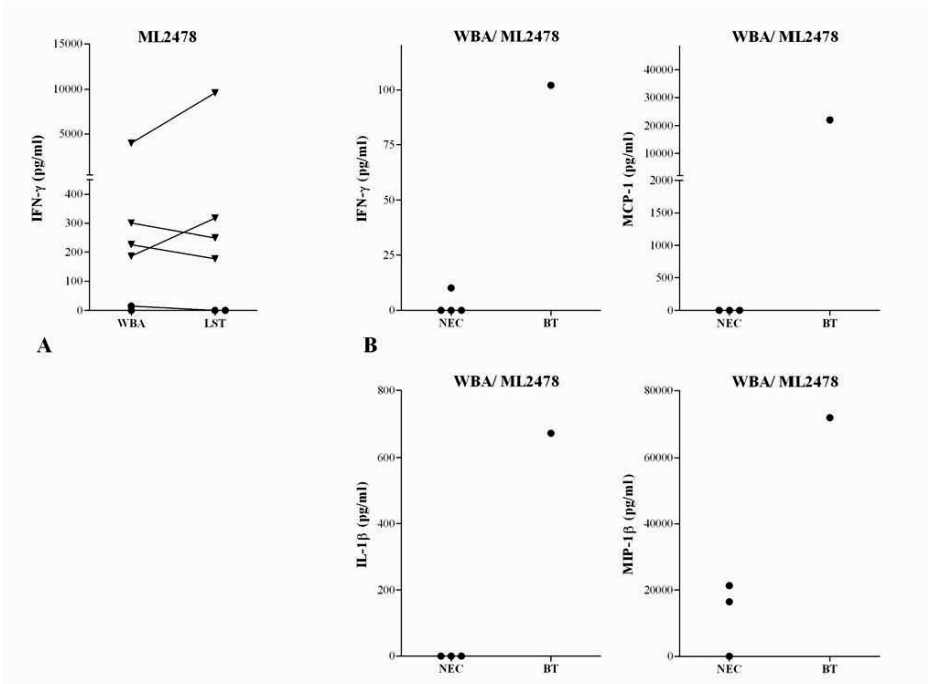
Supplementary Figure S1A: IFN-γ responses in WBA from individuals in Bangladesh. IFN-γ production in response to recombinant proteins (ML0091, ML0811, ML2044 and ML2055) in 24 hour WBA of leprosy patients (TT/BT; n = 10), healthy household contacts (HHC; n =10) and endemic controls (EC; n=10) from Bangladesh.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

Chapter 4

Longitudinal immune and metabolic profiles of type 1 leprosy reactions in Bangladesh, Brazil, Ethiopia and Nepal

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Abstract

Background: Acute inflammatory reactions are a frequently occurring, tissue destructing phenomenon in infectious- as well as autoimmune diseases, providing clinical challenges for early diagnosis. In leprosy, an infectious disease initiated by *Mycobacterium leprae* (*M. leprae*), these reactions represent the major cause of permanent neuropathy. However, laboratory tests for early diagnosis of reactional episodes which would significantly contribute to prevention of tissue damage are not yet available.
Although classical diagnostics involve a variety of tests, current research utilizes limited approaches for biomarker identification. In this study, we therefore studied leprosy as a model to identify biomarkers specific for inflammatory reactional episodes.
Methods: To identify host biomarker profiles associated with early onset of type 1 leprosy reactions, prospective cohorts including leprosy patients with and without reactions were recruited in Bangladesh, Brazil, Ethiopia and Nepal. The presence of multiple cyto-/chemokines induced by *M. leprae* antigen stimulation of peripheral blood mononuclear cells as well as the levels of antibodies directed against *M. leprae*-specific antigens in sera, were measured longitudinally in patients.
Results: At all sites, longitudinal analyses showed that IFN-γ-, IP-10-, IL-17- and VEGF-production by *M. leprae* (antigen)-stimulated PBMC peaked at diagnosis of type 1 reactions, compared to when reactions were absent. In contrast, IL-10 production decreased during type 1 reaction while increasing after treatment. Of further importance for rapid diagnosis, circulating IP-10 in sera was significantly increased during type 1 reactions. On the other hand, humoral immunity, characterized by *M. leprae*-specific antibody detection, did not identify onset of type 1 reactions, but allowed treatment monitoring instead.
Conclusions: This study identifies immune-profiles as promising host biomarkers for detecting intra-individual changes during acute inflammation in leprosy, also providing an approach for other chronic (infectious) diseases to help early diagnose these episodes and contribute to timely treatment and prevention of tissue damage.

Background

Leprosy is a chronic, immunoregulatory infectious disease caused by *Mycobacterium leprae* that particularly affects the skin and peripheral nerves and often results in severe, life-long disabilities and deformities [1;2]. The number of new cases has plateaued at 220,000–250,000 annually, but many linger undetected [3;4]. Leprosy remains endemic in Africa, South America and Asia and with increasing migration, new cases are detected in developed countries, where initial misdiagnosis is likely to occur [5-7].

The inter-individual variability in clinical manifestations of leprosy closely parallels the ability of the host to mount an effective immune response to *M. leprae*. This is depicted by an immunological and clinical spectrum in those who progress to disease, ranging between two completely different poles i.e. tuberculoid (TT) and lepromatous (LL) leprosy [8]. Host resistance to *M. leprae* is associated with the emergence of a protective Thelper-1 (Th1)-based response characterized by the secretion of the innate and adaptive cytokines IL-12p70, IFN-γ, lymphotoxin-α/β, and (moderate levels of) other pro-inflammatory cytokines such as TNF-α. LL patients secrete predominantly anti-inflammatory mediators such as IL-10, accompanied by the absence of Th1-associated cytokines in response to *M. leprae* but characterized by high anti-*M.leprae* antibody titers. Conversely, TT patients produce exacerbated levels of pro-inflammatory cytokines, including those produced by Th17 rather than Th1, and frequently driven by strong innate immune activation resulting in the release of IL-1β and/or IL-6, TGF-β and IL-23 [9;10].

Although leprosy can be treated effectively with multidrug therapy (MDT), it is complicated by persisters [11] as well as acute inflammatory episodes called leprosy reactions. These immunological complications, occurring before, during and after MDT treatment in 30-50% of the patients, represent the major cause of leprosy-related neurological damage [12;13]. Two types of reactions are recognized: type 1 or reversal reactions (RRs) and type 2 or erythema nodosum leprosum (ENL). RRs are considered a delayed hypersensitivity reaction with characteristic infiltrations of skin and nerve lesions by $CD4^+$ T-cells producing IFN-γ and TNF-α [14-16]. Up to 30% of leprosy patients are affected by RRs, which most commonly occur in borderline forms of leprosy (borderline-tuberculoid (BT), borderline-borderline (BB), borderline-lepromatous (BL)) in which concomitant immunological fluctuations can generate significant neuropathology [17]. Prompt diagnosis and anti-reactional treatment contributes to recovery significantly thus reducing risks for permanent tissue damage [18;19]. Unfortunately, reactions are frequently misdiagnosed due to decreased expertise within integrated health services [17]. Therefore, reliable tests for early diagnosis of RR could make huge differences in clinical outcomes. A major obstacle to developing such tests is the lack of dependable biomarkers for reactions across endemic populations.

For the complex host immuno-pathogenicity of leprosy [2;14], assessment of multiple rather than single biomarkers is more informative of the hosts' immune status. Therefore, we aimed to identify relevant host immune-biomarkers for early diagnosis of type 1 reactions. We recruited newly diagnosed leprosy patients longitudinally and studied *M. leprae*-specific cellular- and humoral immunity in blood of patients 1) in the absence of any clinical signs of reactions at least three months before reactions, 2) very early after clinical presentation of reactions and 3) after completion of treatment. Non-reactional patients (before and after treatment) as well as healthy individuals from the same area were analyzed similarly. To accommodate worldwide applicability, independent of the genetic and environmental background, this study was executed similarly in four distinct, prospective cohorts in Asia, Africa and South-America.

Materials and Methods

General study-procedure. Recruitment took place in Bangladesh (International Centre for Diarrhoeal Disease Research Bangladesh, Dhaka), Brazil (National Reference Centre for Sanitary Dermatology and Leprosy, Uberlandia), Ethiopia (ALERT hospital and Health Centre,) and Nepal (Mycobacterial Research Laboratories, Kathmandu). Experiments were performed according to standard operating procedures and each site was provided with identical reagents.

Study participants. Patients and endemic controls (EC) were recruited on a voluntary basis between February 2008-March 2013 (Table 1). Leprosy was diagnosed based on clinical, bacteriological and histological observations and classified by skin biopsies according to Ridley and Jopling [1]. Leprosy patients were treated according to WHO standards. Clinical monitoring for reactions was performed during monthly clinic visits. Clinical and demographic data was collected in clinical research forms (Supplementary file 2) and subsequently transferred in databases with special emphasis on standardizing data collection and definition of reaction between all cohorts [20;21]. For patients who presented with reactions the type, severity, skin- and/or nerve involvement, number of lesions and relapse were noted, according to state-of-the-art clinical expertise and international consensus scoring [21;22]. EC were assessed for the absence of clinical signs and symptoms of leprosy and TB. Staffs of leprosy- or TB clinics were excluded.

TABLE 1

Participating study sites and study groups

Site	Category[1]	Mean BI[2]	Sex ratio (M/F)	Age range (yr)	Total[3]
Bangladesh	EC	na[4]	0.9	20-40	20
	BL/LL	2.20	5	18-61	31
	RR	1.68	2.5	21-63	20
Brazil	EC	na[4]	1.3	24-76	23
	BL/LL	1.51	1	22-26	25
	RR	1.95	3.3	25-68	20
Ethiopia	EC	na[4]	1.8	18-45	11
	BL/LL	1.25	1.7	18-52	25
	RR	0.46	2.8	18-60	15
Nepal	EC	na[4]	3.6	19-28	20
	BL/LL	2.96	2	35-58	13
	RR	1.45	2.5	27-50	20

[1]EC: endemic control; BL/LL: borderline leprosy/ lepromatous leprosy; TT/BT: tuberculoid leprosy/ borderline tuberculoid leprosy; RR: reversal reaction (type 1 reaction);
[2]BI: bacterial index (mean);
[3]Total number of recruited individuals is indicated; samples for multiple time points were not always included. For multiplex cytokine analysis or UPLC-ESI-TOF MS a selected sample size was used for analysis.
[4]not applicable.

Leprosy prevalence: Dhaka, prevalence: 2.45/10,000, new case detection rate (NCDR): 0.31/10,000 (Annual Reports of Leprosy Control Institute & Hospital, Dhaka); Uberlandia, prevalence: 0.96/10,000, NCDR: 1,12/10,000 (National Disease Surveillance System,

Secretariat of Health Surveillance, Ministry of Health Brazil); Addis Ababa, prevalence: 0.6/10,000 in 2010-2011, 0.4/10,000 in 2012, NCDR: 0.35/10,000 (FMOH reports); Kathmandu, prevalence: 1.1-0.79/10,000, NCDR: 1.67- 1.15/10,000 (Annual Report 2012-2013, Leprosy Control Division, Department of Health Services, Kathmandu).

Recruitment: Newly diagnosed, untreated leprosy patients without clinical reactions were enrolled and blood was drawn before MDT (t=0). Patients who presented reactions within three months of the start of therapy were excluded to avoid profile analyses of patients with latent reactions. If patients presented with reactions after more than three months of MDT, blood was drawn before initiation of anti-reactional therapy (t=x). Newly diagnosed leprosy patients who visited clinics with RR were recruited (t=x) but consequently lacked t=0 samples. From all patients, blood was collected after MDT and/or steroid therapy (t=end). For patients with RR this was done at least one month after completion of steroid therapy to avoid assessment of the effect of steroids. All patients were assessed for the absence of reactions three months after t=end. For patients showing clinical signs of reactions within three months after t=end, this time point was excluded. In case patients died, moved or withdrew from the study, preventing follow-up, their samples were excluded. Blood was used for isolation of peripheral blood mononuclear cells (PBMC). Supernatants and sera were stored at -20°C.

Antigens. *M. leprae* recombinant proteins were produced as described [23]. *M. leprae* whole cell sonicate was provided through the NIH/NIAID "Leprosy Research Support" Contract N01AI-25469 (http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx).

Cytokine/chemokine analysis. PBMC, freshly isolated from venous blood, were cultured for 6 days with antigens as described [23]. IFN-γ was determined by ELISA (U-CyTech, Utrecht, The Netherlands) [24]. A positive, reference supernatant was provided to all laboratories. 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, G-CSF, GM-CSF, MCP-1, MIG, MIP-1β and TNF in supernatants or sera were measured using the Bio-Plex-suspension-array-system (Bio-Rad,Veenendaal, NL)[23]. IFN-β was determined in undiluted sera (25ul) using Procartaplex IFN-β simplex-kit (eBioscience, Hatfield, UK) and CCL18 was determined (1:10 dilutions; 100µl) by ELISA (DY394 DuoSet, R&D Systems, Minneapolis, MN) according to manufacturers' instructions.

Serology. Antibodies against ML2028 (Ag85B) and ND-O-BSA, a synthetic analogue of phenolic glycolipid I (PGL-I), were determined as described [25].

Ethics. This study was performed according to the Helsinki Declaration (2008 revision). Participants were informed about the study-objectives, the samples and their right to refuse to take part/ withdraw from the study without consequences for their treatment. Written informed consent was obtained before enrolment. All patients received treatment according to national guidelines. Ethical approval of the study-protocol was obtained through appropriate ethics committees: Ethical Review Committee of ICDDR,B (#PR-10032; #PR-2007-069); Brazilian National Council of Ethics in Research (CONEP) and UFU Research Ethics Committee (#499/2008); National Health Research Ethical Review committee Ethiopia (NERC # RDHE/127-83/08); Nepal Health Research Council (NHR #751).

Statistical analysis. Differences in cytokine concentrations were analysed with two-tailed Mann-Whitney U tests (unpaired samples) for non-parametric distribution and Wilcoxon matched-pairs signed rank test or paired t test for longitudinal analyses using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego,CA, USA; www.graphpad.com). The statistical significance level used was $p<0.05$.

Results

Recruitment of four prospective cohorts

To identify biomarkers for early type 1 reactions, blood of newly diagnosed, untreated leprosy patients was obtained longitudinally in Bangladesh, Brazil, Ethiopia and Nepal (Table 1). The analysis included two samples of patients without reactions [1. before treatment (t=0); 2. after treatment (t=end)] and three of patients who developed RR during the study [1. in the absence of clinical signs of reactions, at least 3 months before RR diagnosis (t=0); 2. at RR diagnosis, before steroid-treatment (t=x); 3. after RR and at least one month after ending steroid-treatment (t=end)]. Since patients were frequently diagnosed with RR at their first clinic-visit, it became clear that it was not always feasible to include these first samples. Initially, patients who developed RR within 3 months of recruitment were excluded to avoid measuring markers for RR already at t=0. Similarly, patients showing clinical signs of reactions within 3 months after ending MDT and/ or steroid treatment were excluded to prevent measuring biomarkers of RR at t=end. For longitudinal analysis (Fig. 1) only patients entering the study without reactions were utilized. Due to the low frequency of untreated cases without RR at their first clinic visits who developed RR during this study, we also included patients with RR at their first clinic visits (as initial RR cases) consequently lacking the first time point (t=0). Patients included in the analysis after database cleaning at each site are indicated in Table 1 and Figure 3 and 4. For healthy individuals from these areas with identical socio-economic background, one sample was collected.

Longitudinal *M. leprae*-induced cytokine/chemokine production during reaction development

First, we analysed *M. leprae*-induced cytokine production by blood cells of RR patients for whom valid samples were available at three time points (Bangladesh: n=3; Brazil: n=4; Nepal: n=3). All patients produced significantly higher IFN-γ and IL-17 at RR diagnosis than before or after treatment (Fig.1). Also, levels of IP-10, VEGF and IL-1β peaked at RR-onset (Fig.S2). In contrast, IL-10 was virtually not produced at RR diagnosis, compared to before diagnosis and after treatment. Cytokine responses to *M. leprae*-unique proteins, in particular ML2478 [23], corresponded well with responses to *M. leprae* (Fig.S1).

Since cytokines modulate each other's effects, we considered ratios as markers for disease-status. Indeed, the differential cytokine production at RR onset was even more evident from the ratios of IFN-γ/IL-10 and IL-17/IL-10 (p=0.0032; p=0.0033; Fig.1), whereas IFN-γ/IL-10 for patients who did not develop reactions remained similar before and after treatment (Fig.S3D) due to the simultaneous increase of both IFN-γ and IL-10 after MDT treatment in non-reactional patients (Fig. 3 and Fig. 4). The potential of cytokine ratios for discrimination between RR and its absence was also evident from the ROC (receiver operating characteristics) with AUC (areas under curve) ranging from 0,955 to 1. IP-10/IL-10 ratios showed a similar profile, with slightly less significance (AUC: 0.79; Fig.S3B-C). Thus, cytokine ratios proved valuable, RR-associated markers as well as markers for reactional treatment efficacy.

Longitudinal serological analysis during reaction development

For detection of *M. tuberculosis* infection [26] and to indicate *M. leprae* exposure [23;27], IP-10 was reported a useful marker. Notably, IP-10 is produced in large quantities facilitating its use in field-friendly test-platforms such as lateral flow [28]. IP-10 analysis of longitudinal sera of reactional patients showed increased levels during RR (Fig.2: p=0.0059; Fig.S4: AUC: 0,79) consistent with previous studies [6;29]. Upon anti-reactional treatment, serum IP-10 decreased (p=0.002; Fig.2A). In contrast, longitudinal sera from patients without reactions or

healthy donors, as control for RR-specificity, showed no significant difference in IP-10, clearly designating IP-10 as a serological marker for RR (Fig.2).

The dynamics of CCL18 (chemokine (C-C motif) ligand 18) serum levels, elevated in lepromatous leprosy [30], were also investigated for patients experiencing reactions (Fig.2B), showing a decreasing trend at RR, increasing after treatment for most patients. CCL18 in healthy controls were much lower than for borderline lepromatous patients in line with recent findings [30].

In view of the reduction of IL-10 during RR, these sera were also analysed for the presence of IL-10-inducing IFN-β [31]. Although no significant differences were detected at RR compared to before onset, IFN-β decreased significantly after treatment ($p=0.006$; Fig.S6).

Cross-sectional analysis of cytokine production

Cytokine profiles produced by blood cells cultured with *M. leprae* sonicate/ -proteins [23] were analysed cross-sectionally as well (Fig.3, S1, S2). In line with our longitudinal results, patients who developed RR produced significantly higher IFN-γ levels in response to *M. leprae* proteins at RR diagnosis than before onset of reaction or after reaction treatment regardless of their ethnic origin (blood at t=0 from Ethiopian RR patients was not available). As found previously for leprosy-endemic areas, EC produced high IFN-γ levels to *M. leprae* [23;27;32;33].

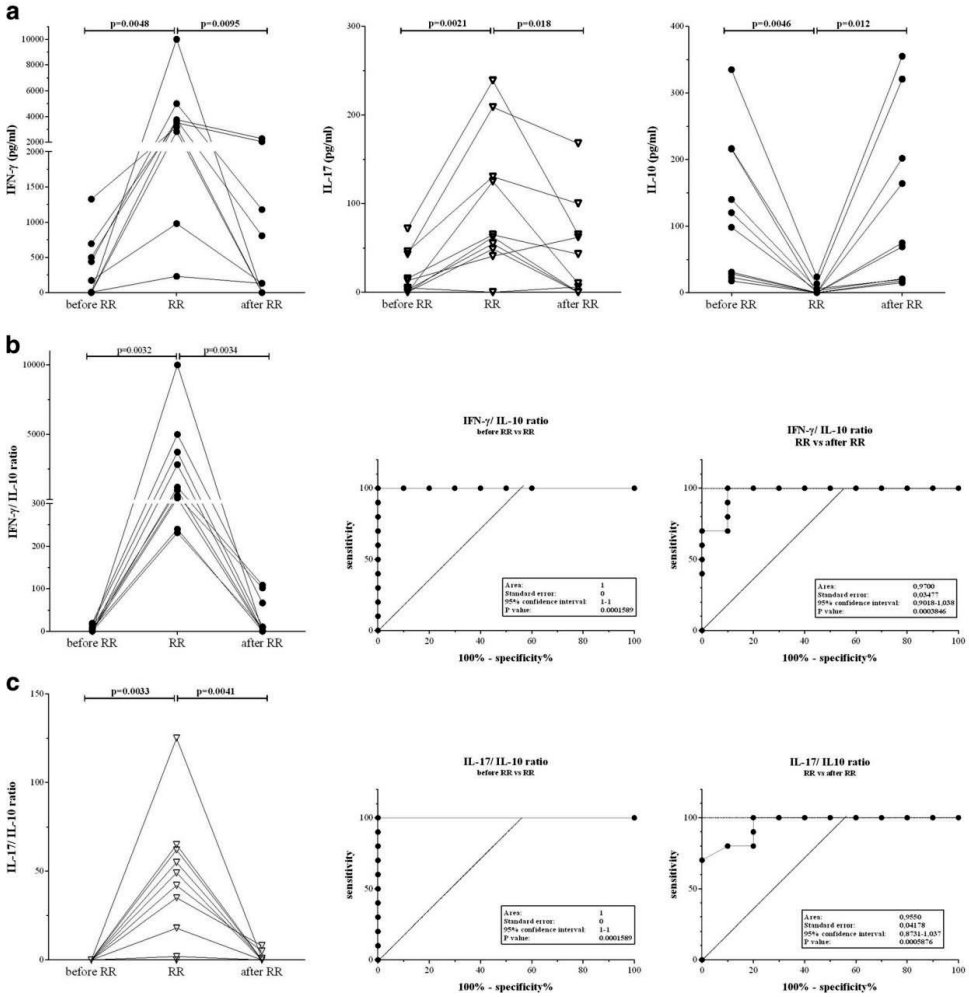

Figure 1. Longitudinal pattern of cytokine ratios for patients with reversal reaction (RR) IFN-γ, IL-17 and IL-10 production was induced by stimulation with *M.leprae* a. for 10 patients who developed RR during this study (Bangladesh:n=3; Brazil;n =4; Nepal:n =3) at leprosy diagnosis before MDT in the absence of any clinical signs of reactions and at least three months before reaction (before RR),at diagnosis of reaction before steroids (RR) or after MDT and RR, at least one month after end of steroids (after RR). IFN-γ/IL-10band IL-17/IL-10 c ratios and ROC (receiver operating characteristics) curves are shown. For calculations of ROC values, time points before RR versus at RR diagnosis (B, C middle panels) or at RR diagnosis versus after RR (B, C right panel) were considered

IL-10 levels in response to *M. leprae* were again in striking contrast to IFN-γ levels (Fig.4). Virtually no responses were seen at RR diagnosis, compared to elevated IL-10 levels before diagnosis and after treatment. IP-10, IL-17, VEGF and to a lesser extent IL-1β levels followed those of IFN-γ, whereas G-CSF trended towards a decline at RR (Fig.S2). High levels of IL-5, IL-6, IL-8, MCP-1, MIP-1β, GM-CSF and TNF were observed for all groups but lacked a distinct longitudinal pattern, whereas induction of IL-2, IL-4, IL-7, IL-12p70 and IL-13 was low (data not shown).

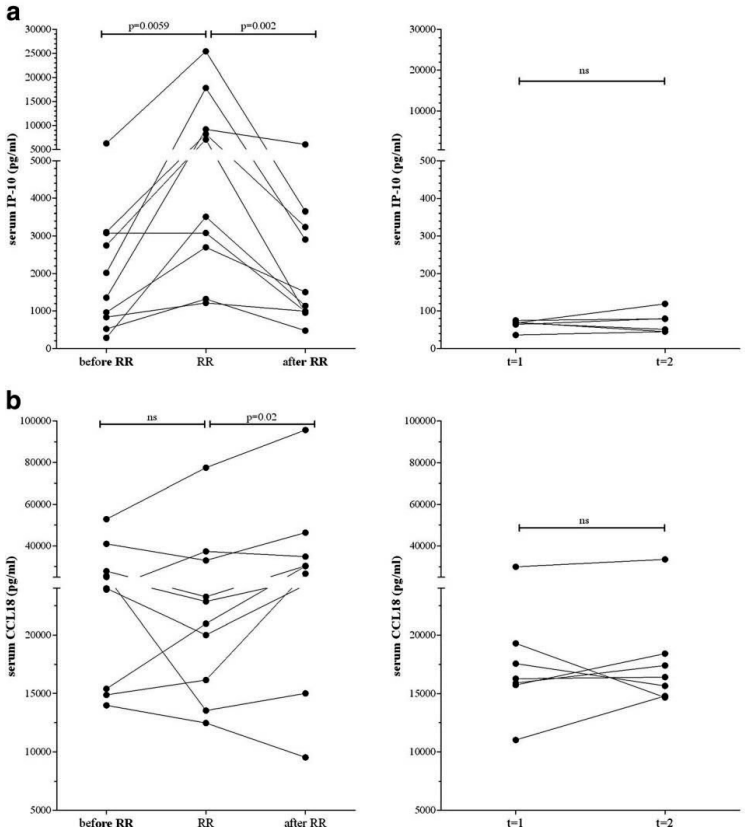

Figure 2. Longitudinal serum analysis of patients with reversal reaction (RR) Levels of IP-10 a and CCL18 b in unstimulated sera derived from 10 leprosy patients (left panels) developing RR (Bangladesh: n =4;Brazil;n =3; Ethiopia:n =1;Nepal:n =2) in the absence of any clinical signs of reactions and at least three months before reaction (before RR),at diagnosis of reaction before steroids (RR) or after MDT and RR, at least one month after end of steroids (after RR), or from healthy Dutch controls (n= 10) at two sequential time points with six months intervals (right panels). For calculations of the ROC values, time points at least three months before RR and at RR diagnosis before steroids were considered. IFN-β levels for controls were not detectable

Biomarkers to monitor treatment efficacy

Besides biomarkers associated with reactions, biomarkers to monitor treatment efficacy provide practical tools as well. Thus, we analyzed the effect of treatment on immunemarkers: IFN-γ responses to *M. leprae* antigens of patients without reactions increased after treatment (Fig. 3), whereas IL-10 increased slightly, but not significantly with treatment (Fig.4). Treatment-induced increasing trends were also observed for VEGF, IL-1β and IL-17A levels (Fig.S2) thereby contributing to the biomarker profile for RR. As observed for RR patients, IFN-γ levels also increased in patients without RR after MDT treatment. In contrast to reactional patients, however, IL-10 levels were higher after MDT which renders the drop in IFN-γ/IL-10 ratio (Fig.1 and Fig.S3D) specifically associated with RR.

Finally, cross-sectional screening of sera for the presence of antibodies to ND-O-BSA and ML2028 was performed (Fig.5 and Fig.S5). Anti-PGL-I IgM levels, but not anti-ML2028 IgG levels were generally lowest in EC. In patients without RR, treatment significant

decreased antibodies (p = 0.0003 – 0.01), confirming that these serological markers add to host profiles useful to estimate treatment [25]. However, *M. leprae*-specific antibody detection did not identify RR, but allowed treatment monitoring (p = 0.0001. – 0.02; the Ethiopian cohort did not reach significance), suggesting that humoral immunity could serve as auxiliary tool for monitoring reactional treatment in addition to serum IP-10 and IFN-β as well as cytokine ratio's.

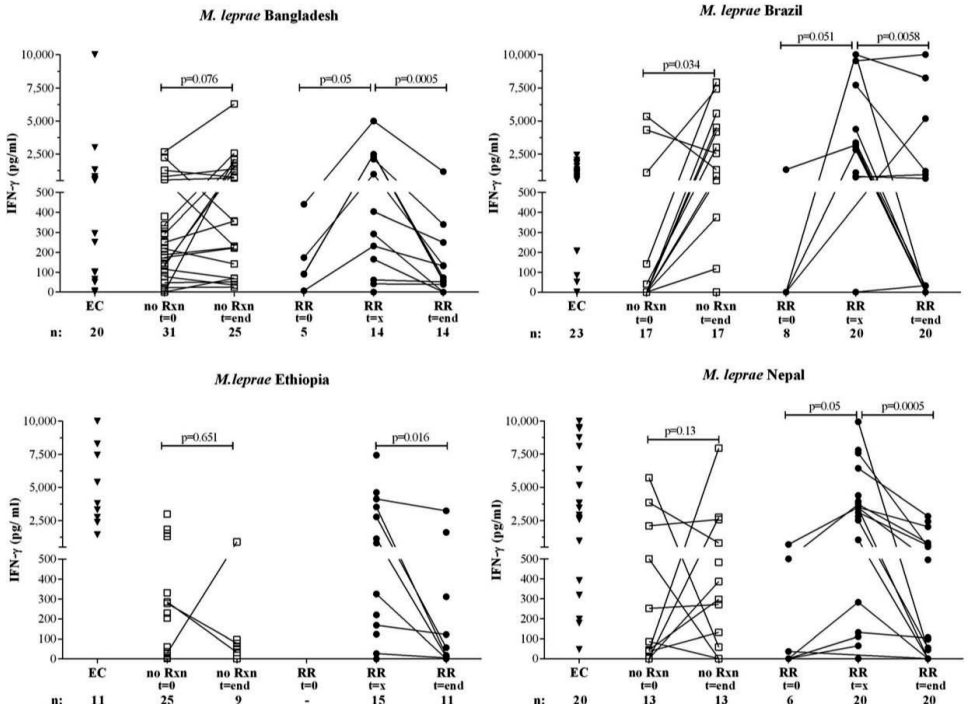

Figure 3. Longitudinal cross-sectional pattern of IFN-γ and IL-10 secretion. IFN-γ (Fig.3) or IL-10 (Fig.4) production (corrected for background values) in response to *M. leprae* sonicate (10 µg/ml) in 6day cultures of peripheral mononuclear cells (PBMC) of endemic controls (EC;▼), newly diagnosed leprosy patients without reactions (noRxn;) before treatment (t=0) and after treatment (t= end) and leprosy patients (•) in the absence of any clinical signs of reactions and at least 3 months before RR (t=0), at RR diagnosis before steroids (t=x) or after MDT and RR (t= end), at least one month after end of steroids (after RR) inindividuals from Bangladesh, Brazil, Ethiopia, and Nepal. All patients were assessed for the absence of reactions three months after t =end. Background values were typically <50pg/ml. The number of individuals per group and the timepoint are indicated below the x-axis for each site

Discussion

Biomarkers as reliable correlates of disease complications and response to therapy are essential tools for early diagnosis of disease states in chronic infections. Generally, the performance of one biomarker can be significantly enhanced by using instead a custom-made grouping of independent biomarkers, called a profile or signature. In the current situation of leprosy elimination, the availability of sensitive and specific biomarkers that aid early diagnosis of leprosy reactions as well as monitor therapy, would be a strategic advantage enabling health care workers to identify, treat and possibly prevent these episodes at early stages, thereby reducing nerve damage. Since the immunopathology of leprosy, particularly in reactional states, is linked to temporal changes in the immune response to *M. leprae*, leprosy represents a uniquely suitable model to study immune-biomarker changes in relation to clinical disease manifestations.

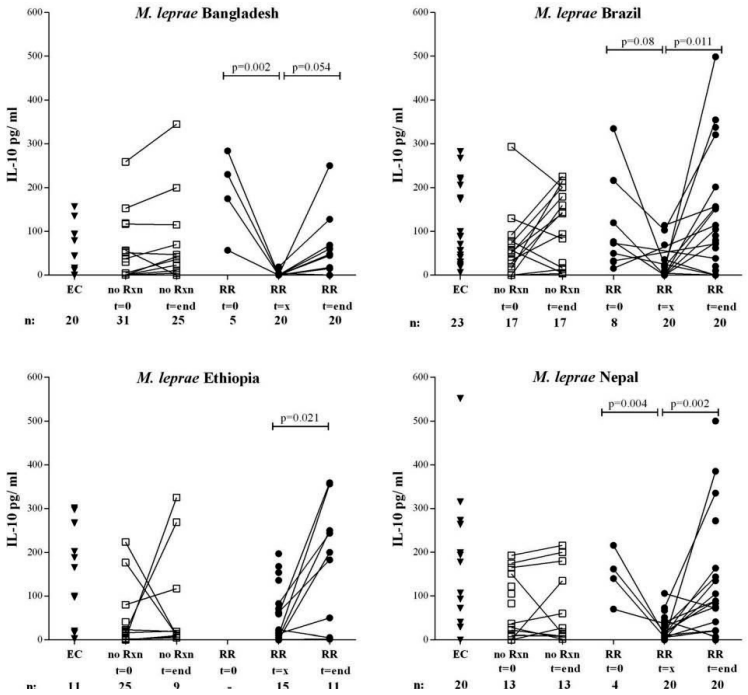

Figure 4. Longitudinal cross-sectional pattern of IFN-γ and IL-10 secretion. IFN-γ (Fig.3) or IL-10 (Fig.4) production(corrected for background values) in response to M. leprae sonicate (10 µg/ml) in 6day cultures of peripheral mononuclear cells (PBMC) of endemic controls (EC;▼), newly diagnosed leprosy patients without reactions (noRxn;) before treatment (t=0) and after treatment (t= end) and leprosy patients (•) in the absence of any clinical signs of reactions and at least 3 months before RR (t=0), at RR diagnosis before steroids (t=x) or after MDT and RR (t= end), at least one month after end of steroids (after RR) in individuals from Bangladesh, Brazil, Ethiopia, and Nepal. All patients were assessed for the absence of reactions three months after t =end. Background values were typically <50pg/ml. The number of individuals per group and the time point are indicated below the x-axis for each site

This is the first study in which cellular- and humoral immunity specific for *M. leprae* in leprosy patients within the three main continents reporting leprosy were monitored longitudinally during treatment. Although previous studies have analyzed circulating cytokines and chemokines [29] around the time of leprosy reactions, the addition of an *M. leprae* antigen-specific component, as utilized in this study provides more specificity to this approach.

The data demonstrate translational importance since similar intra-individual trends were observed for development of RR in different endemic areas, allowing global application of these biomarkers in tests for early diagnosis of RR. In this respect, the importance of the combined effect of *M. leprae*-induced cytokine production (IFN-γ, IL-17, IP-10, IL-1β, VEGF), determined by their ratios versus IL-10, was highlighted, providing valuable tools for diagnosis of reactional states.

The biomarker profiles identified in this study for RR can be used in blood-based diagnostic tests [28] to detect (intra-individual) changes during these acute inflammatory periods but also provide an approach for other chronic diseases with acute inflammatory states such as tuberculosis [34] and buruli ulcer [35] (paradoxical reactions) and Crohn's disease [36;37], to

help early diagnose such episodes thereby contributing to timely treatment and prevention of disease-specific tissue damage.

The acknowledged immunosuppressive role of IL-10 in lepromatous leprosy [38] as well as in *M. leprae* infected mice [39;40] was also evident from its reduction at RR-onset [41]. Thus, during RR the breakdown of regulation, in favour of inflammation, seems to underlie the aetiology of reactional tissue damage, whereas balanced ratios of these immune responses, as present in nonreactional leprosy patients, are protective against RR [42]. This is in line with the associations of IL-10 genetic variants with development of leprosy and leprosy reactions [6;43-46]. Suppression of IL-10 in a borderline tuberculoid-like murine model significantly augmented $CD4/44^+$ and $CD8/44^+$ longitudinal infiltrative responses specific to *M. leprae* antigens and permitted $CD4^+$ T-cells to penetrate and fragment nerve [47], in line with our current field findings and supporting monitoring patient IL-10 levels in ratio to cytokines proven to escalate during RR as a potential early indicator of impending clinical RR.

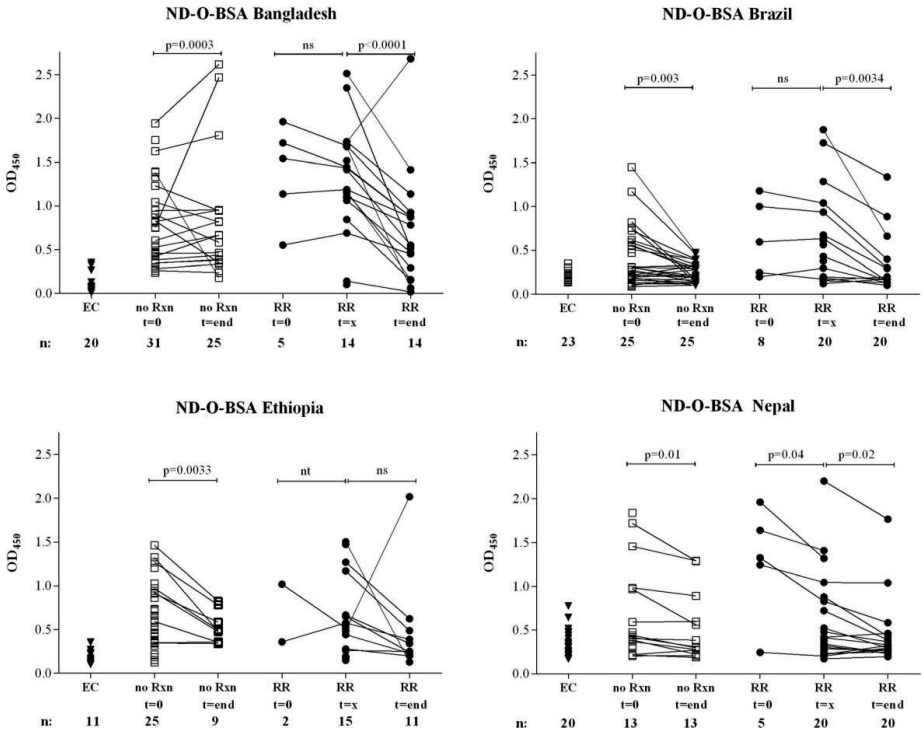

Figure 5. Humoral immunity to *M. leprae* antigens Antibodies against synthetic PGL-I (ND-O-BSA, a synthetic analog of the *M. leprae*-specific PGL-I) by ELISA. Sera were derived from Bangladesh, Brazil, Ethiopia, and Nepal and included endemic controls (EC;▼), newly diagnosed leprosy patients without reactions (noRxn;) before (t=0) and after treatment (t= end) and leprosy patients (•) in the absence of any clinical signs of reactions and at least 3 months before RR (t=0), at RR diagnosis before steroids (t=x) or after MDT and RR, at least one month after end of steroids (t= end).Optical density readings were performed using a 1:200 serum dilution. Median values for each group are indicated by horizontal lines. P-values <0,05 indicate significant differences. The number of individuals per group and the timepoint are indicated below the x-axis for each site

As a second biomarker for RR in multiple ethnic backgrounds, increased serum IP-10 levels were identified, whereas CCL18, which is elevated in lepromatous leprosy [30], decreased at early RR in 6/10 patients who developed RR. Since CCL18 is secreted by dendritic cells upon recognition of *M. tuberculosis* [48] and has been implicated in differentiation of macrophages into an alternative phenotype [49] this suggests that decreased CCL18 levels lead to fewer alternatively activated macrophages and less T-cell regulation [6;50]. These data therefore indicate that new biomarker discovery approaches for RR also contribute to our understanding of the RR-associated immunopathologic mechanisms, suggesting new opportunities for therapeutic interventions.

Since RRs are considered delayed hypersensitivity reactions caused by overreaction and/ or dysregulation of host defence mechanisms, conscientious (personalized) treatment monitoring is vital similar to other diseases with acute inflammatory states such as psoriasis and Crohn's disease which share specific susceptibility genes with leprosy [51] [36]. Our data showed that pro-inflammatory cytokine/ IL-10 ratios, serum IP-10 can be used for monitoring treatment while not on steroids. Therefore, besides for early diagnosis of reactions, tests to monitor efficacy of treatment are useful as well, especially in the light of the reoccurrence of these episodes.

To allow access to diagnostic test at resource-poor field settings, we recently developed low-tech, robust lateral flow assays (LFAs) for (simultaneous) detection of inflammatory (IP-10) and regulatory (IL-10) immune responses together with anti-PGL-I IgM antibodies in short term whole blood assays [28;52]. In the light of the currently identified immune markers for RR, field-friendly LFAs measuring these cytokines for leprosy patients on MDT at each clinic-visit may be helpful to early detect RR if used for intra-individual testing. Thus, to provide a rapid test, the diagnostic potential of the cytokine ratios defined here, need to be determined in future studies using whole blood assays as well.

Conclusions

Type 1 or reversal reactions (RRs) are a major cause of leprosy-related nerve impairment and bear similarities with acute inflammation induced episodes in other (infectious) diseases. Since there is no laboratory test for the early diagnosis of these episodes, this multi-continental, longitudinal study on the occurrence of RRs in leprosy patients, showed for the first time that both *M. leprae*-specific cellular- as well as humoral host immune-profiles, correlating with early onset of these inflammatory episodes, can be identified. Biomarkers associated with diagnosis or efficiency of treatment of type 1 reactions were identified based on intra-individual changes rather than single values. In particular, ratios of cytokines secreted by *M. leprae* stimulated blood cells as well as circulating cytokines in sera, contributed to these biomarker profiles. Thus, these profiles can be applied for the early diagnosis and to monitor reactional episodes and contribute to timely treatment and reduction/ prevention of tissue damage.

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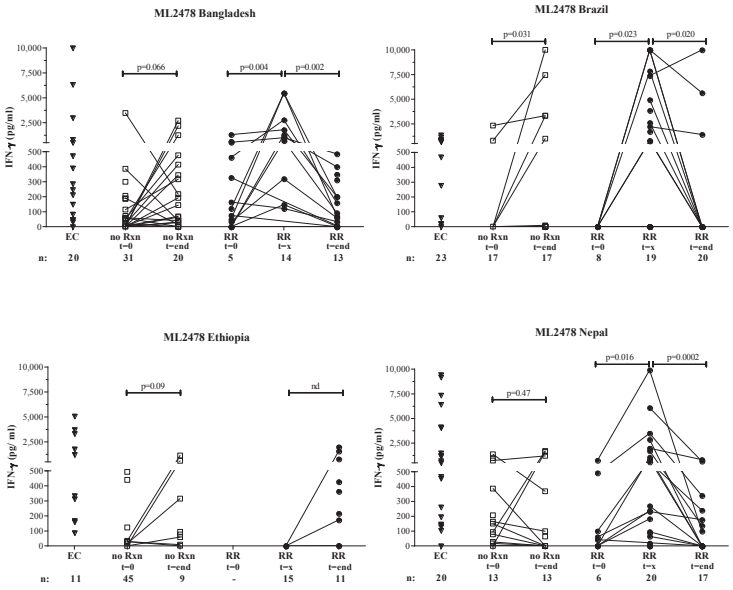


Figure S1: IFN-γ in response to *M.leprae*-unique protein ML2478 in 6 day cultures of PBMC (see Figure 3). Simultaneously, PBMC were cultured with proteins: ML0009, ML0121, ML0141, ML0188, ML1601, ML1976, ML1989, ML1990, ML2283, ML2478, ML2531, ML2532 and ML2567 (data not shown).

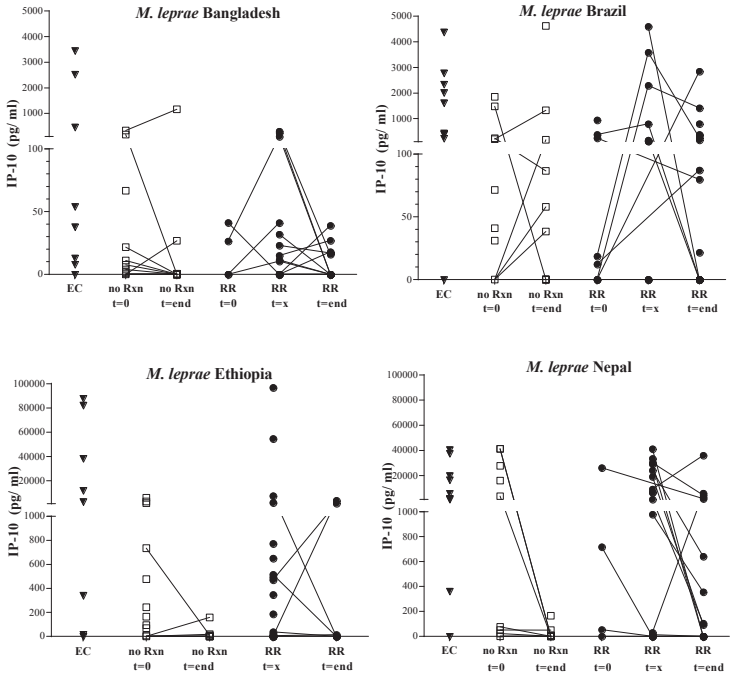


Figure S2A (IP-10)

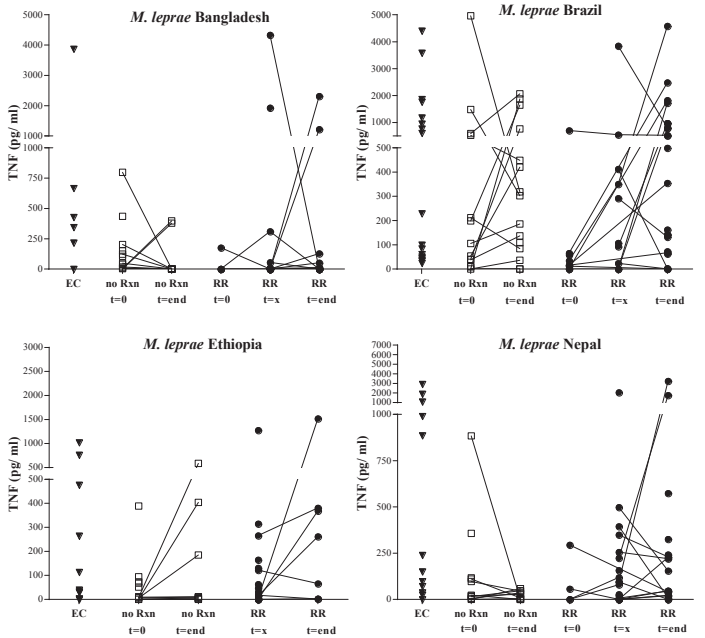


Figure S2B (TNF)

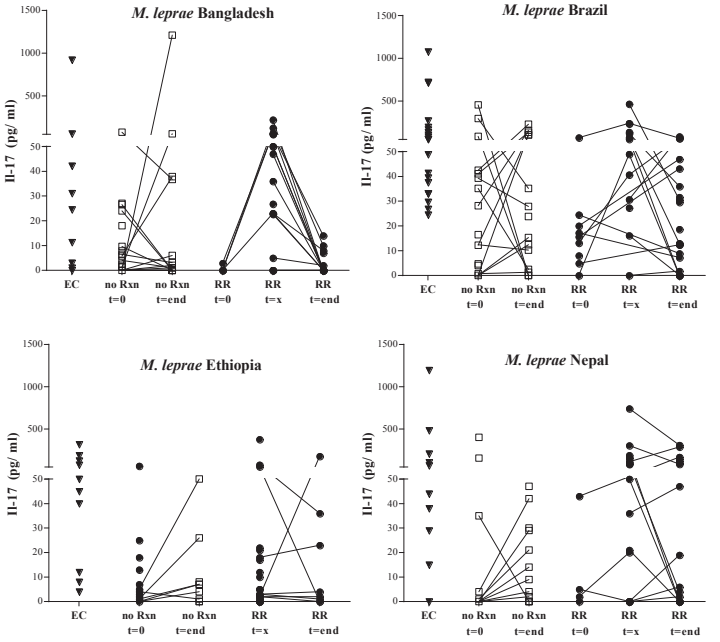


Figure S2C (IL-17)

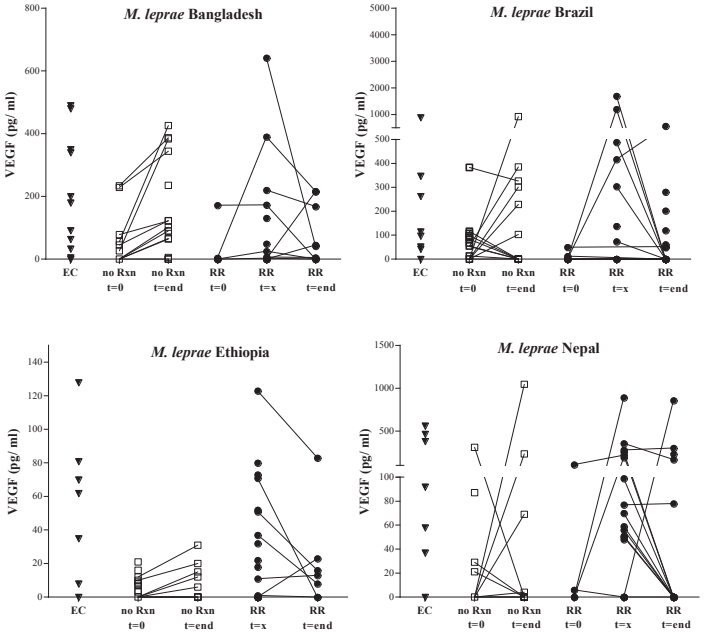


Figure S2D (VEGF)

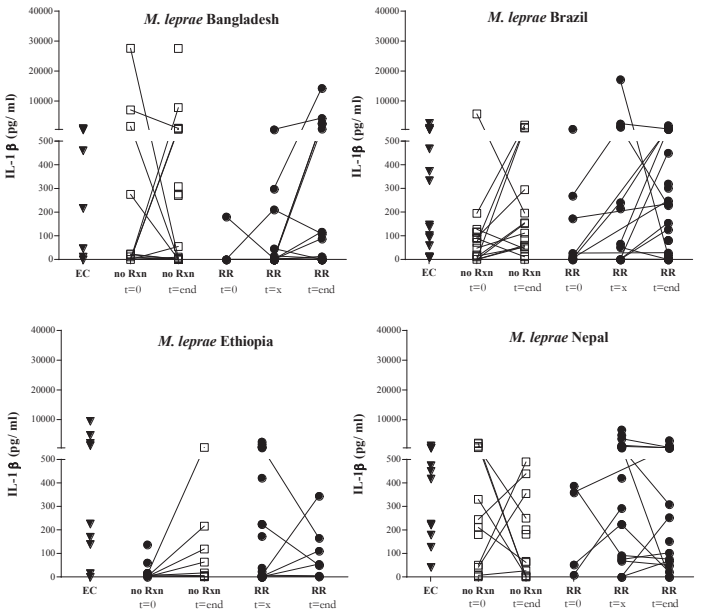


Figure S2E (IL1-β)

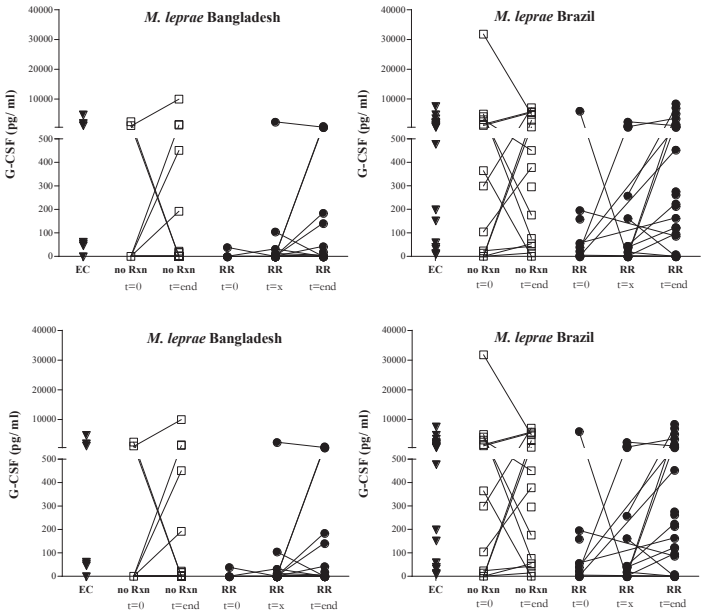


Figure S2F (G-CSF)

Figure S2: IP-10 (**A**), TNF (**B**), IL-17 (**C**), VEGF (**D**) , IL1-β (**E**) and G-CSF (**F**) production in same cultures as described in Figure S1.

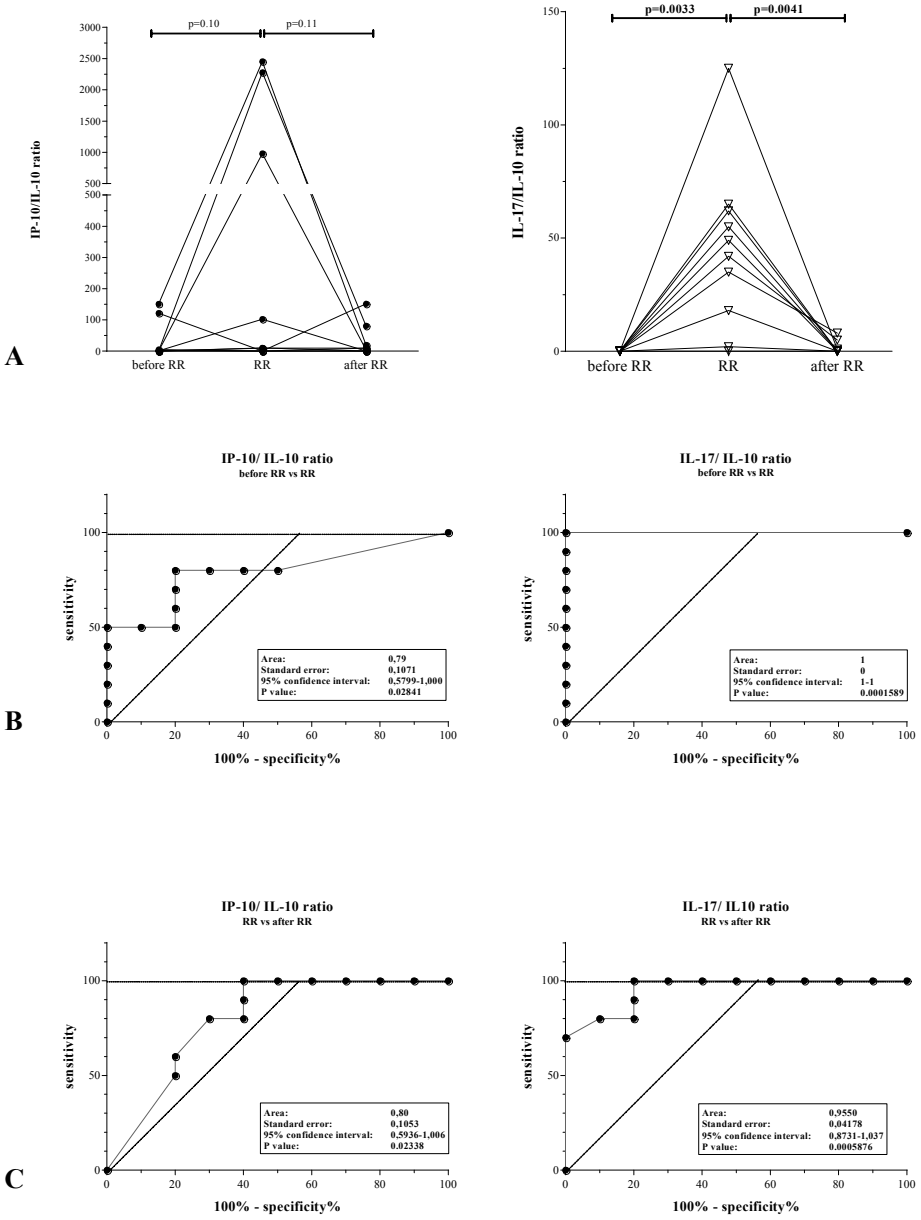

A
IP-10/IL-10 ratio
p=0.10
p=0.11
before RR
RR
after RR
IL-17/IL-10 ratio
p=0.0033
p=0.0041
B
IP-10/ IL-10 ratio
before RR vs RR
sensitivity
100% - specificity%
Area: 0,79
Standard error: 0,1071
95% confidence interval: 0,5799-1,000
P value: 0.02841
IL-17/ IL-10 ratio
Area: 1
Standard error: 0
95% confidence interval: 1-1
P value: 0.0001589
C
IP-10/ IL-10 ratio
RR vs after RR
Area: 0,80
Standard error: 0,1053
95% confidence interval: 0,5936-1,006
P value: 0.02338
IL-17/ IL10 ratio
Area: 0,9550
Standard error: 0,04178
95% confidence interval: 0,8731-1,037
P value: 0.0005876

Figure S3A-C

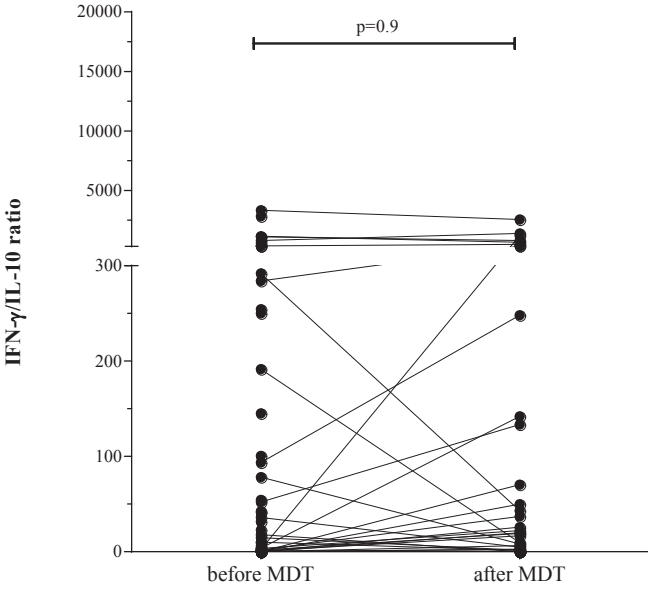
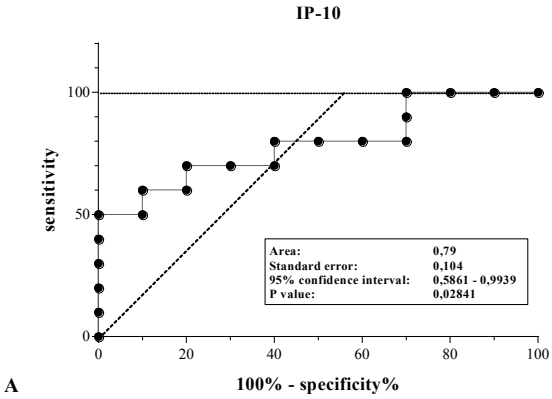
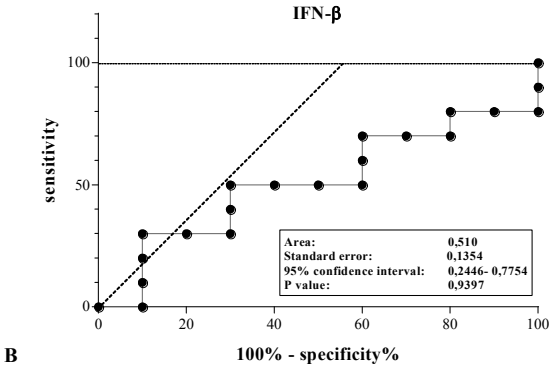


Figure S3D

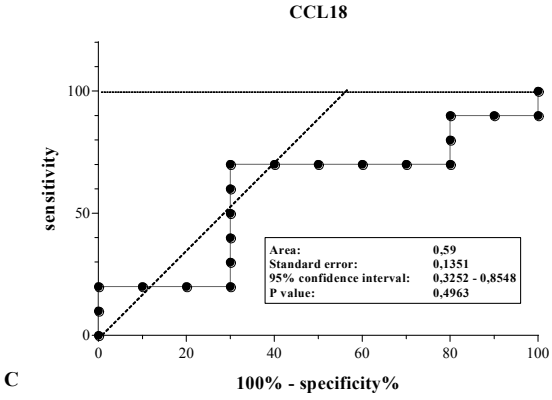
Figure S3: IP-10 and IL-17 (**A**) after stimulation with *M. leprae*. IP-10/ IL-10 and IL-17/ IL-10 ratios are indicated (**B, C**). ROC curves were calculated for IP-10/ IL-10 and IL-17 /IL-10. Ratios for patients without reactions are shown as controls (**D**).



A



B



C

Figure S4: IP-10 (**A**), IFN-β (**B**) and CCL18 (**C**) in sera.

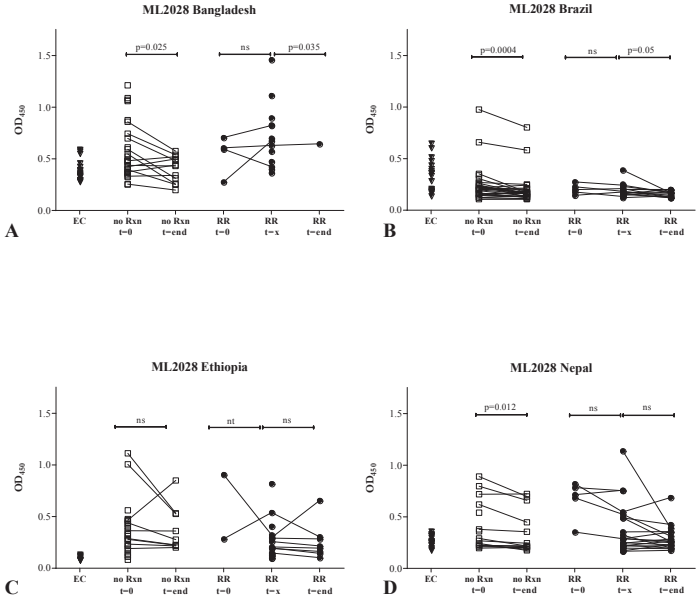


Figure S5: Antibodies against *M.leprae* protein ML2028 in sera determined by ELISA. Optical density readings were performed using a 1:200 dilution. Median values are indicated by horizontal lines.

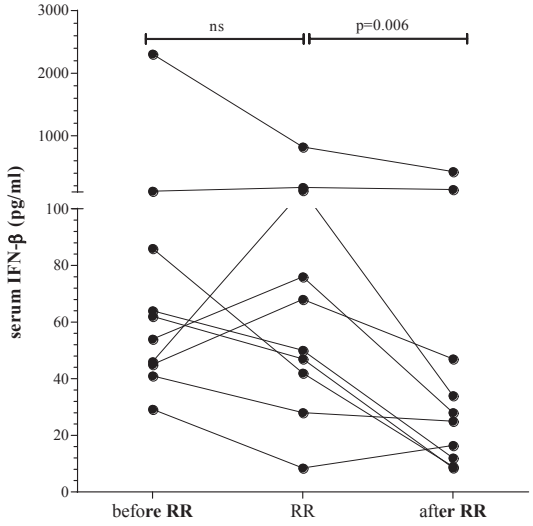


Figure S6: IFN-β in sera derived from patients developing RR in the absence of clinical signs of reactions and at least three months before reaction (**before RR**), at diagnosis of reaction before steroids (**RR**) or after MDT and RR, at least one month after end of steroids (**after RR**). For ROC values, timepoints at least three months before RR and at RR diagnosis before steroids were considered. IFN-β levels for controls were not detectable.

Chapter 5

Field evaluation of a new lateral flow assay for detection of cellular and humoral immunity against Mycobacterium leprae

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Abstract

Background: Field-applicable tests detecting asymptomatic *Mycobacterium leprae* (*M. leprae*) infection or predicting progression to leprosy are urgently required. Since the outcome of *M. leprae* infection is determined by cellular- and humoral immunity, we aim to develop diagnostic tests detecting pro-/ anti-inflammatory and regulatory cytokines as well as antibodies against *M. leprae*. Previously, we developed lateral flow assays (LFA) for detection of cytokines and anti-PGL-I antibodies. Here we evaluate progress of newly developed LFAs for applications in resource-poor settings.
Methods: The combined diagnostic value of IP-10, IL-10 and anti-PGL-I antibodies was tested using *M. leprae*-stimulated blood of leprosy patients and endemic controls (EC). For reduction of the overall test-to-result time the minimal whole blood assay time required to detect distinctive responses was investigated. To accommodate LFAs for field settings, dry-format LFAs for IP-10 and anti-PGL-I antibodies were developed allowing storage and shipment at ambient temperatures. Additionally, a multiplex LFA-format was applied for simultaneous detection of anti-PGL-I antibodies and IP-10. For improved sensitivity and quantitation upconverting phosphor (UCP) reporter technology was applied in all LFAs.
Results: Single and multiplex UCP-LFAs correlated well with ELISAs. The performance of dry reagent assays and portable, lightweight UCP-LF strip readers indicated excellent field-robustness. Notably, detection of IP-10 levels in stimulated samples allowed a reduction of the whole blood assay time from 24h to 6h. Moreover, IP-10/IL-10 ratios in unstimulated plasma differed significantly between patients and EC, indicating the feasibility to identify *M. leprae* infection in endemic areas.
Conclusions: Dry-format UCP-LFAs are low-tech, robust assays allowing detection of relevant cytokines and antibodies in response to *M. leprae* in the field. The high levels of IP-10 and the required shorter whole blood assay time, render this cytokine useful to discriminate between leprosy patients and EC.

Author Summary

Leprosy is one of the six diseases considered by WHO as a major threat in developing countries and often results in severe, life-long disabilities and deformities due to delayed diagnosis. Early detection of *Mycobacterium leprae* (*M. leprae*) infection, followed by effective interventions, is considered vital to interrupt transmission. Thus, field-friendly tests that detect asymptomatic *M. leprae* infection are urgently required.

The clinical outcome after *M. leprae* infection is determined by the balance of pro- and anti-inflammatory cytokines and antibodies in response to *M. leprae*. In this study, we developed lateral flow assays (LFA) for detection of pro-inflammatory (IP-10) vs. anti-inflammatory/ regulatory (IL-10) cellular immunity as well as antibodies against *M. leprae* and evaluated these in a field setting in Ethiopia using lightweight, portable readers.

We show that detection of IP-10 allowed a significant reduction of the overall test-to-result time from 24h to 6h. Moreover, IP-10/IL-10 ratios in unstimulated plasma differed significantly between patients and EC, which can provide means to identify *M. leprae* infection. Thus, the LFAs are low-tech, robust assays that can be applied in resource-poor settings measuring immunity to *M. leprae* and can be used as tools for early diagnosis of leprosy leading to timely treatment and reduced transmission.

Introduction

Leprosy, a curable infectious disease caused by *Mycobacterium leprae* (*M. leprae*) that affects the skin and peripheral nerves, is one of the six diseases considered by the WHO as a major threat in developing countries [1]. Despite being treatable, leprosy often results in severe, life-long disabilities and deformities [2] due to delayed- or misdiagnosis. Transmission of leprosy is clearly unabated as evidenced by the number of new cases, 10% of whom are children, that plateaued at nearly 250,000 each year since 2005 [1]. Continued transmission in endemic areas likely occurs from the large reservoir of individuals who are infected subclinically. Thus, early detection of *M. leprae* infection, followed by effective interventions, is considered vital to interrupt transmission as highlighted by the WHO 2011-2015 global strategy [3]. Despite this pressing need, field-friendly tests that detect asymptomatic *M. leprae* infection are lacking, nor are there any biomarkers known that predict progression to disease in infected individuals.

Lateral flow assays (LFAs), are simple immunochromatographic assays detecting the presence of target analytes in samples without the need for specialized and costly equipment. Combinations of LFAs with up-converting phosphor (UCP) reporter technology are useful for detection of a variety of analytes, e.g., drugs of abuse [4], protein and polysaccharide antigens from pathogens like *Schistosoma* and *Brucella* [5;6], bacterial and viral nucleic acids [7;8] and antibodies against *M. tuberculosis*, HIV, hepatitis virus and *Yersinia pestis* [9–11]. The phosphorescent reporter utilized in UCP-LFAs is excited with infrared light to generate visible light, a process called up-conversion. UCP-based assays are highly sensitive since up-conversion does not occur in nature, avoiding interference by autofluorescence of other assay components. Importantly, UCP-LF test strips can be stored as permanent records allowing re-analysis in a reference laboratory.

In leprosy, the innate and adaptive immune response to *M. leprae* matches the clinical manifestations as substantiated by the characteristic spectrum ranging from strong Th1 immunity in tuberculoid leprosy to high antibody titers to *M. leprae* with Th2 cytokine responses in lepromatous leprosy [12]. In view of this spectral character, field-applicable tests for leprosy should allow simultaneously detection of biomarkers for humoral- as well as cellular immunity.

Tests used in leprosy diagnostics include the broadly investigated serological assay detecting IgM against PGL-I [13;14]. Although this test is useful for detection of most multibacillary (MB) patients [15,16], as the antibody levels correlate well with the bacillary load, detection of anti-PGL-I Ab has limited value in identifying paucibacillary (PB) leprosy patients [17]. In areas hyperendemic for leprosy more than 50% of young schoolchildren surveyed had positive anti-PGL-I responses [18]. Still, the vast majority of individuals with a positive antibody titer will never develop leprosy [13]. With respect to cellular responses in leprosy diagnosis, studies have focussed on *M. leprae*-unique antigens which can probe T-cell *M. leprae*-specific responses resulting in the identification of *M. leprae* (-unique) antigens that specifically induced IFN-γ production in *M. leprae* infected individuals [19;20]. Combined with serology, the use of these IFN-γ release assays (IGRAs) provided significant added value since they identified the majority (71%) of PGL-I negative healthy household contacts in Brazil [21] while control individuals not exposed to *M. leprae* were IGRA-negative. Similar IGRAs allowed detection of the extent of *M. leprae* exposure along a proximity gradient in EC in one city in Brazil and in Ethiopia [22–24].

Although ELISA techniques, as used in IGRAs, are more widely applied than before, they still require laboratory facilities which are not available at all health centres in leprosy endemic areas. To accommodate ELISAs to field-applicable tests for leprosy diagnosis, we previously developed UCP-LFAs for detection of IFN-γ and IL-10 as well as antibodies against the *M. leprae*-specific phenolic glycolipid-I (PGL-I) for high-tech, laboratory-based microtiter-plate readers [25;26]. Since IFN-γ, the hallmark cytokine of Th1 cells, has generally been assessed as a biomarker to detect anti-mycobacterial immunity, we first developed a IFN-γ-UCP-LFA [25]. Recently, IFN-γ induced protein 10 (IP-10) was found useful for detection of *M. tuberculosis* infection [27] and can also be used to indicate levels of *M. leprae* exposure and thereby the risk of infection and subsequent transmission [22;23]. Furthermore, since IP-10 is produced in large quantities, facilitating the use of simplified test platforms such as LFA [28], we investigated its potential as an alternative to IFN-γ for leprosy diagnosis. Accordingly, we developed quantitative, dry reagent UCP-LFAs for field-detection of IP-10 and anti-PGL-I antibodies and evaluated these in a leprosy endemic area in Ethiopia.

Materials and Methods

Ethical statement. 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 from the National Health Research Ethical Review committee, Ethiopia (NERC # RDHE/127-83/08) and The Netherlands (MEC-2012-589). Participants were informed about the study objectives, the required amount and kind of samples and their right to refuse to take part or withdraw from the study at any time without consequences for their treatment. Written informed consent was obtained from all study participants before venipuncture.

Study participants. HIV-negative, newly diagnosed untreated leprosy patients and healthy endemic controls (EC) were recruited at the Armauer Hansen Research Institute (AHRI) in Addis Ababa, Ethiopia, The Leiden University Medical Center (LUMC) and the Erasmus Medical Center (EMC), The Netherlands from October 2011 until November 2012. Leprosy was diagnosed based on clinical, bacteriological and histological observations and classified by a skin biopsy evaluated according to the Ridley and Jopling classification [2] by qualified personnel. 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. Mantoux-negative, healthy Dutch donors recruited at the Blood Bank Sanquin, Leiden, The Netherlands were used as nonendemic controls (NEC). None of these NEC had lived in or travelled to leprosy- or TB endemic areas, and, to their knowledge, had not experienced any prior contact with TB or leprosy patients.

Recombinant proteins. *M. leprae* candidate genes were amplified by PCR from genomic *M. leprae* DNA and cloned using Gateway technology (Invitrogen, Carlsbad, CA) with pDEST17 expression vector containing an N-terminal histidine tag (Invitrogen) [29]. Purified recombinant proteins were produced as described [22;29] and contained endotoxin levels below 50 IU per mg recombinant protein as tested using a Limulus Amebocyte Lysate (LAL) assay (Cambrex, East Rutherford, NJ). 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.

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 incubation periods of 1h, 4h, 6h or 24h (as

indicated), 150 µl of supernatants were removed from each well and frozen in aliquots at –20°C until further analysis.

Synthetic PGL-I and *M. leprae* whole cell sonicate (WCS). Synthetic PGL-I (ND-O-HSA) and *M. leprae* whole cell sonicate were generated with support from the NIH/NIAID Leprosy Contract N01-AI-25469 (available through the Biodefense and Emerging Infections Research Resources Repository listed at http://www.beiresources.org/TBVTRMResearch Materials/tabid/1431/Default.aspx). Disaccharide epitope (3,6-di-O-methyl-β-D-glucopyranosyl(1→4)2,3-di-O-methylrhamnopyranoside) of *M. leprae* specific native PGL-I glycolipid was synthesized and coupled to human serum albumin (ND-O-HSA) as previously described by Cho *et al.* [30]. Inactivated (irradiated) armadillo-derived *M. leprae* whole cells were probe sonicated with a Sanyo sonicator to > 95% breakage.

PGL-I ELISA. IgM antibodies against *M. leprae* PGL-I were detected with natural disaccharide of PGL-I linked to human serum albumin (ND-O-HSA (500 ng/ well in 50 µl) provided through the NIH/NIAID Leprosy Contract N01-AI-25469) as previously described [31]. Serum dilutions (50 µl/ well; 1:800) were incubated at RT for 120 min in flat-bottomed microtiter plates (Nunc) coated with NDO-HSA. After washing diluted enzyme linked secondary antibody solution (anti-human IgG/IgM/IgA – HRP; Dako, Heverlee, Belgium; 50 µl/ well) was added to all wells and incubated at RT for 120 min. After washing diluted TMB solution (50 µl/ well) was added to all wells and incubated in the dark for 15 min at RT. The reaction was stopped by adding 50 µl/ well 0.5 N H_2SO_4. Absorbance was determined at wavelength of 450 nm. Samples with a net optical density at 450 nm (OD) above 0.149 were considered positive. The ELISA performance was monitored using a positive and negative control serum samples on each plate.

Cytokine ELISAs. For ELISAs 96 well Nunc MaxiSorp microtitre-plates were used and the presence of biotinylated antibody was detected enzymatically using streptavidin-HRP (horse-radish peroxidase): **IFN-γ** was determined using anti-IFN-γ coating Ab mAb mO-13-32-22 (U-CyTech Biosciences, Utrecht, the Netherlands) and biotinylated anti-IFN-γ pAb pB-15-43-13 (U-CyTech Biosciences) as detection Ab. Culture supernatants were diluted 1:2 in buffer (1% BSA/PBS) and 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. **IP-10** was determined using anti-IP-10 capture Ab (clone B-C50) and biotinylated anti-IP-10 detection Ab (clone B-C55; Diaclone, France) in culture supernatants diluted 1:100 with dilution buffer. The cut-off value to define positive responses was set beforehand at 2,000 pg/ml. The assay sensitivity level was 40 pg/ml. Values for unstimulated cell cultures of NEC were typically < 2,000 pg/ml. **IL-10** was determined using anti-IL-10 mAb mO-13-10-12 (U-CyTech Biosciences) as coating Ab and biotinylated anti-IL-10 pAb mB-15-10-26 (U-CyTech Biosciences) as detection Ab in culture supernatants diluted 1:2. The cut-off value to define positive responses was set beforehand at 100 pg/ml. The assay sensitivity level was 10 pg/ml. Concentration values for unstimulated whole blood were typically ≤ 10 pg/ml.

Upconverting phosphor (UCP) conjugates and LF strips. UCP conjugates specific for cytokines IP-10, IL-10, IFN-γ were prepared following earlier described protocols [26], by conjugating 5 µg anti-IP-10 (BC-50; Diaclone), 20 µg anti-IL-10 mAb (coating mAb in ELISA, mO-13-10-12; U-CyTech) or 25 µg anti-IFN-γ (BB-1; Diaclone) per 1 mg carboxylated UCP particles, respectively. Wet UCP conjugates were stored at a concentration of 1 mg/ml at 4 °C. An UCP-IP-10 dry conjugate was made by drying 100 ng in a 5% sucrose matrix overnight at 37°C in 0.65 ml U-shape polypropylene tubes (Ratiolab tubes for 96-well micro test plate, VWR International, Amsterdam, The Netherlands); dried materials were stored in aluminum foil bags (Lamigrip pouches Overtoom International, Den Dolder, The

Netherlands) with silica dry pellets at ambient temperature [6,32]. Reporter conjugates for detection of humoral immune response, an IgM- and Ig-specific UCP conjugates, were prepared as described earlier [9,26] by conjugation of 25 µg goat anti-human IgM (I0759; Sigma-Aldrich, Saint Louis, MO, USA), protein-A (Repligen Corp.) or IgG/IgM/IgA/Kappa/Lambda–HRP (Dako), respectively. Wet conjugates were stored at a concentration of 1 mg/mL at 4 °C. Freeze dried pellets, so-called lyospheres, containing 100 ng $UCP^{protein\ A}$ conjugate were produced (Biolyph LLC, Hopkins, MN, USA) and stored in vacuum-sealed glass vials as described earlier [33]. LF strips (4 mm width) for IP-10, IL-10 and IFN-γ were prepared with a test (T) line at 2.0 cm comprised of 50 ng anti-IP-10 BC-55 (Diaclone), 700 ng anti-IL-10 mAb mO-10-10-28 (U-CyTech Biosciences) or 200 ng anti-IFN-γ BG-1 (Diaclone) respectively. The antibody pairs were identical to those used for ELISA but not containing a biotin hapten. LF strips for cytokine detection were further provided with a goat anti-mouse pAb (M8642; Sigma-Aldrich) flow-control (FC) line of respectively 100 ng and 200 ng at 2.5 cm. LF strips for detection of antibodies against PGL-I were provided with 50 ng synthetic PGL-I (ND-O-HAS) on the test (T) line and 100 ng rabbit anti-goat IgG (G4018; Sigma-Aldrich) on the flow-control (FC) line. LF strips for IP-10 and PGL-I multiplex detection were prepared using the same compositions as the strips for the individual targets, but now were provided with two T- and two FC-capture lines. Capture lines were separated by 4 mm located at 1.5 (T1, IP-10), 1.9 (T2, PGL-I), 2.7 (FC1, goat anti-mouse), and 2.3 cm (FC2, rabbit anti-goat).

UCP-LFA for cytokine detection. The UCP-LFAs for cytokine detection (IFN-γ, IL-10, IP-10) comprise two phases, designated solution phase and immunochromatography phase [26]. Solution phase: 10 µl of 100-fold diluted sample (translating to 0.1 µl undiluted sample) for IP-10 and 10 µl undiluted sample for IL-10 and IFN-γ is mixed with 90 µl High Salt Lateral Flow (HSLF) buffer (100 mM Hepes pH 7.2, 270 mM NaCl, 1% BSA (w/v), 0.5% Tween-20 (v/v)) containing 100 ng specific UCP reporter conjugate and incubated for 60 min on a thermoshaker at 37 °C and 900 rpm. The immunochromatography phase: the above mixture is applied to cytokine specific LF strip and allowed to flow for at least 30 min. After immunochromatography, LF strips are scanned in a Packard FluoroCount microtiterplate reader adapted with an infrared laser. Upon IR excitation (980 nm), UCP reporter particles emit green light detectable using a 550 nm band pass filter. Results are displayed in histograms in relative fluorescence units (RFUs) measured at Test and Flow-Control lines, or as the ratio value between Test (T) and Flow-Control (FC) RFUs using Lateral Flow Studio software V 3.3.5 (QIAGEN Lake Constance GmbH). For strip analysis in Ethiopia a lightweight portable LF strip reader with UCP capability was used (UCP-Quant, an ESEQuant *LFR* reader custom adapted with IR diode; QIAGEN Lake Constance GmbH, Stockach, Germany) [6]. Best reproducibility is obtained when analyzing completely dry LF strips, whereas wet LF strips generate lower T and FC signals. Ratio values between wet- and dry-scanned strips are not significantly different when scanned with readers with sufficient sensitivity that contain a high power IR laser and an adjustable photo multiplier [34]. Since wet-format assays require a sonication step, not suitable for field applications [6], the IP-10-UCP-LFA was adapted to allow implementation of dry reagents (dry conjugate and lyophilized buffer) similar as described for Schistosomiasis [6] and RSV [33]. Next, the dry-format IP-10-UCP-LFA was transported to Ethiopia at ambient temperature and used by local staff after short instruction. In order to evaluate the field performance of these dry-format UCP-LFAs at the Ethiopian site, a lightweight dedicated UCP-LF strip analyzer was provided.

UCP-LFA for anti-PGL-I antibody detection. For detection of anti-PGL-I IgM antibodies two protocols were used: a rapid sequential flow protocol without incubation using the $UCP^{protein\text{-}A}$ or $UCP^{\alpha IgG/IgM/IgA/Kappa/Lambda}$ conjugate, or a two phase protocol similar to the above described protocol for cytokine detection only using $UCP^{\alpha IgM}$ instead of cytokine-

specific UCP conjugates. The sequential flow protocol using the $UCP^{protein\text{-}A}$ conjugate is referred to as consecutive flow (CF) as described [8;9;35]. The CF protocol comprised three sequential flow steps: first 40 µl of a diluted clinical sample (2.5% (v/v) in HSLF assay buffer), after 2 min followed by a wash step with 20 µl HSLF and a final flow after 5 min with 70 µl UCP-conjugate (100 ng in HSLF). Multiple strips can be handled simultaneously by prefilling 96 well ELISA microtitre-plates (Nunc MaxiSorp) with the appropriate three solutions and transferring LF strips from one well to the other. Immunochromatography is allowed to continue for at least 30 min before LF strips are analyzed (see above). For the dry-format UCP-LFA to detect anti-PGL-I antibodies, dry $UCP^{prot\text{-}A}$ reagent in the form of lyospheres [2] was used.

UCP-LFA for simultaneous (multiplex) cytokine and antibody. Simultaneous detection of IP-10 and anti-PGL-I IgM was performed following the two phase protocol described above for cytokine detection. The solution phase comprised the incubation (60 min; 37 °C; 900 rpm) of 10 µL 100-fold diluted sample (translating to 0.1 µL of the original undiluted clinical sample) with 90 µl HSLF buffer containing 100 ng of the $UCP^{\alpha IP\text{-}10}$conjugate (wet) and 100 ng of the $UCP^{\alpha IgM}$ conjugate. The immunochromatography phase was identical to that described for the cytokine-only testing protocol and allowed to continue for at least 30 min before analysis of LF strips (see above). Note that the above protocol may not be applicable when performing antibody detection with the $UCP^{protein\text{-}A}$ conjugate due to unwanted interaction of protein-A with the $UCP^{\alpha IP\text{-}10}$ conjugate [26].

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). For correlations R^2 was calculated with the Pearson correlation using GraphPad Prism version 5.01. The statistical significance level used was $p \leq 0.05$.

Results

Combined cytokine profiles in response to *M. leprae* antigens

M. leprae unique antigens can be used to indicate *M. leprae* exposure using IFN-γ and IP-10 as read-outs [22;23;36]. Also, IFN-γ and IP-10 are associated with Th1-mediated protection against mycobacteria, whereas the anti-inflammatory cytokine IL-10 dampens Th1 cells' responses [37–39]. In view of the high levels of IP-10 produced compared to IFN-γ [22;28] and since, in contrast to IFN-γ, IP-10 is not affected by low CD4 counts in TB patients with HIV [28], we investigated whether IP-10, as an alternative to IFN-γ, can be applied as a pro-inflammatory biomarker.

To evaluate the combined diagnostic value of IL-10, IP-10 and IFN-γ, we first determined their concentrations by ELISAs in 24h WBA of 11 Ethiopian leprosy patients (9 BL, 2 BT) and 12 EC. In addition, anti-PGL-I antibodies were determined for each individual as well (Figure 1). The IP-10 production measured in WBA displayed a pattern similar to that of IFN-γ, although the overall IP-10 concentrations were much higher: median levels of both cytokines in response to *M. leprae* and ML2478 in patients' WBA were not significantly different from those for EC in this leprosy endemic area. These data are consistent with our previous findings, leading to the use of IFN-γ/ IP-10 production in response to ML2478 to determine the level of exposure to *M. leprae* irrespective of infection [22].

In contrast, IL-10 concentrations in response to ML2478 were significantly lower for EC (Figure 1C). Since the balance of pro- and anti-inflammatory cytokines in response to *M. leprae* regulates the clinical outcome after infection, diagnostic tests for leprosy measuring both types

of responses will be helpful in the decision on which individuals need (preventive) treatment. IP-10/IL-10 ratios for stimulated and unstimulated WBA samples demonstrated significantly different values between patients and EC, in particular for unstimulated samples (Figure 1D). Finally, detection of a biomarker for humoral immunity, anti-PGL-I antibody levels, demonstrated significantly higher titers for leprosy patients, further contributing to a discriminating profile between leprosy patients and EC in leprosy endemic areas (Figure 1E).

Kinetics of cytokine production in WBA

Since short overall test-to-result times are preferred for diagnostic assays, the supernatants of WBA of Ethiopian leprosy patients and EC were analyzed for the presence of IFN-γ, IL-10 and IP-10 after 1h, 4h, 6h and 24h stimulation. For IFN-γ and IL-10, levels that varied significantly from unstimulated samples were only detected after 24h (data not shown). For IP-10, however, already after 6h significant production was observed in antigen stimulated samples (Figure 2). Important to note is that after 6h, IP-10 levels in ML2478-stimulated samples were significantly higher ($p = 0.02$) in patients compared to EC (Figure 2B), whereas no distinctive responses were observed for IFN-γ at that time point. PHA-induced IP-10 levels were high for all individuals after 6h and substantial IP-10 levels were only detectable in *M. leprae*-stimulated samples after 24h. Thus, besides the higher levels of IP-10, also the shorter whole blood assay time required render IP-10 combined with ML2478 or as ratio with IL-10 directly in serum, a preferred pro-inflammatory biomarker to discriminate between leprosy patients and EC.

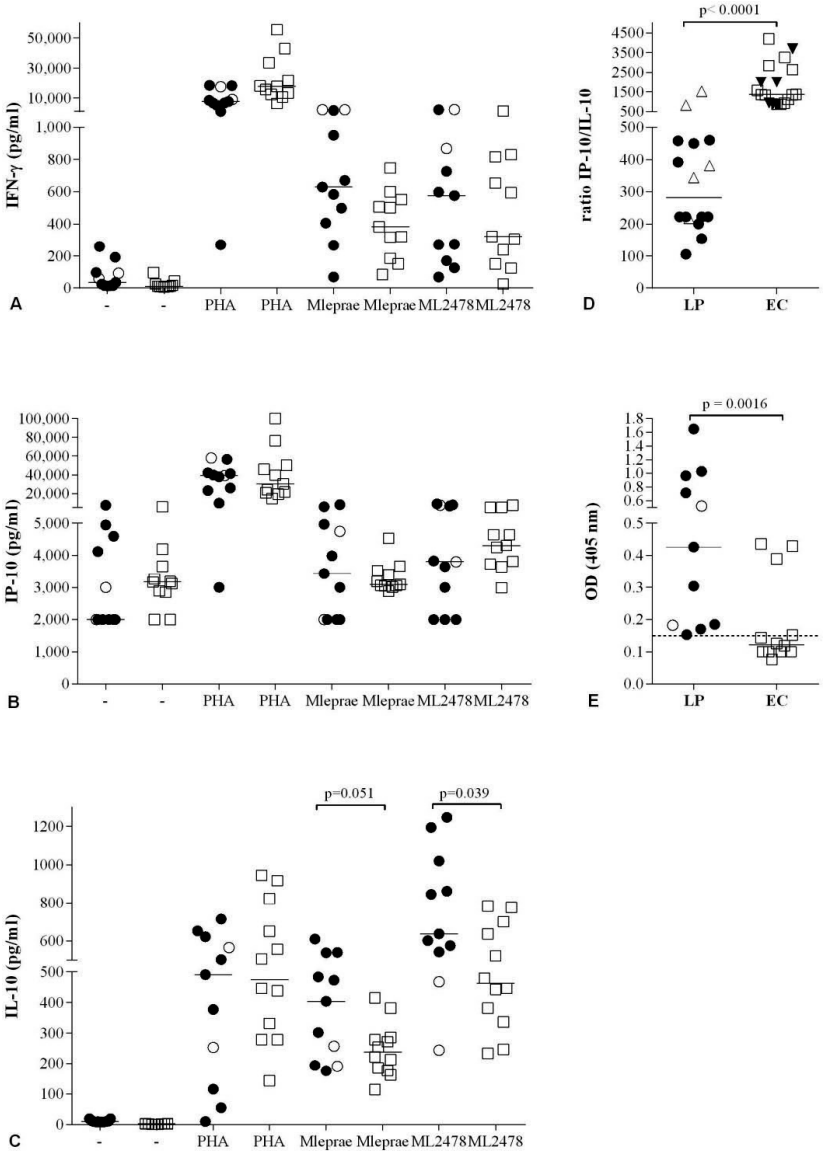

Figure 1. Combined cytokine profiles in response to *M. leprae*. Production of IFN-γ (A), IP-10 (B) and IL-10 (C) determined by ELISA, in response to medium (-), PHA, M. leprae WCS or the M. leprae-unique protein ML2478 in 24 h WBA for Ethiopian leprosy patients (n = 11: 2 BT (○) and 9BL(●), and healthy endemic controls (EC; n = 12; □). For comparison between BT and BL, significant differences were found for *M. leprae* WCS (Mlep) induced IFN-c responses (p = 0.036) and ML2478 induced IL-10 responses (p = 0.035). D): IP-10/IL-10 ratios are depicted for unstimulated samples after 24 h {LP (●) and EC (□)} or after 1 h WBA {LP (Δ) and EC (▼)}. (E): Anti-PGL-I antibodies for BL (○) and BT (•) patients were detected by ELISA using natural disaccharide of PGL-I linked to HSA [31] (ND-O-HSA). Optical density (OD_{450}) readings were performed using 1:800 serum dilutions. Median values per group are indicated by horizontal lines. The cut-off for positivity is indicated by the dashed horizontal line.

Development and evaluation of UCP-LFAs

For detection of IFN-γ, IL-10 as well as antibodies against *M. leprae* PGL-I, we previously developed up-converting phosphor lateral flow assays (UCP-LFAs) [25;26]. Because of the potential of IP-10 to identify *M. leprae* infection in a shorter test-to-result time as well as the value of IP-10/IL-10 ratios, we now selected IP-10 for UCP-LFA development, using the wet-format for IL-10 described previously [26]. Validation of these IL-10 and IP-10 UCP-LFA by comparison to ELISAs utilizing the same antibody pairs and antigen-stimulated WBA samples of non-endemic controls (NEC), demonstrated good correlations between UCP-LFAs and ELISAs for IP-10 and IL-10 (R^2 0,854 and R^2 0,816, respectively; Figure 3).

In view of the greater stability in the field, dry assay format IP-10-UCP-LFA were produced and evaluated in Ethiopia as well: IP-10 values obtained in both wet and dry assays showed a good correlation (R^2 0,790; Figure 4A) indicating the value for field application of the dry-format IP-10-UCP-LFA. Similarly, the unstimulated WBA samples were locally (in Ethiopia) tested for the presence of antibodies against PGL-I as well. Quantitive analysis of the UCP^{prot-A} ratios and ELISA OD values correlated well (R^2 0.689; Figure 4B) indicating 100% agreement in respect to serological status of the samples (qualitative analysis).

To further evaluate UCP-LF applications with this Ethiopian sample set, IL-10 levels of 84 samples (21 patients, 3 stimuli and medium) were also tested, using the available wet-format IL-10-UCP-LFA in parallel with ELISA. Since the IL-10-UCP-LFA was used with 100-fold larger sample input than the IP-10 assay, some of the discrepancies observed for IL-10 between ELISA and UCP-LF assay were probably due to particulate material present in WBA samples. Despite these differences, IL-10-UCP-LFA and ELISA correlated well (R^2 0,735; Figure 4C).

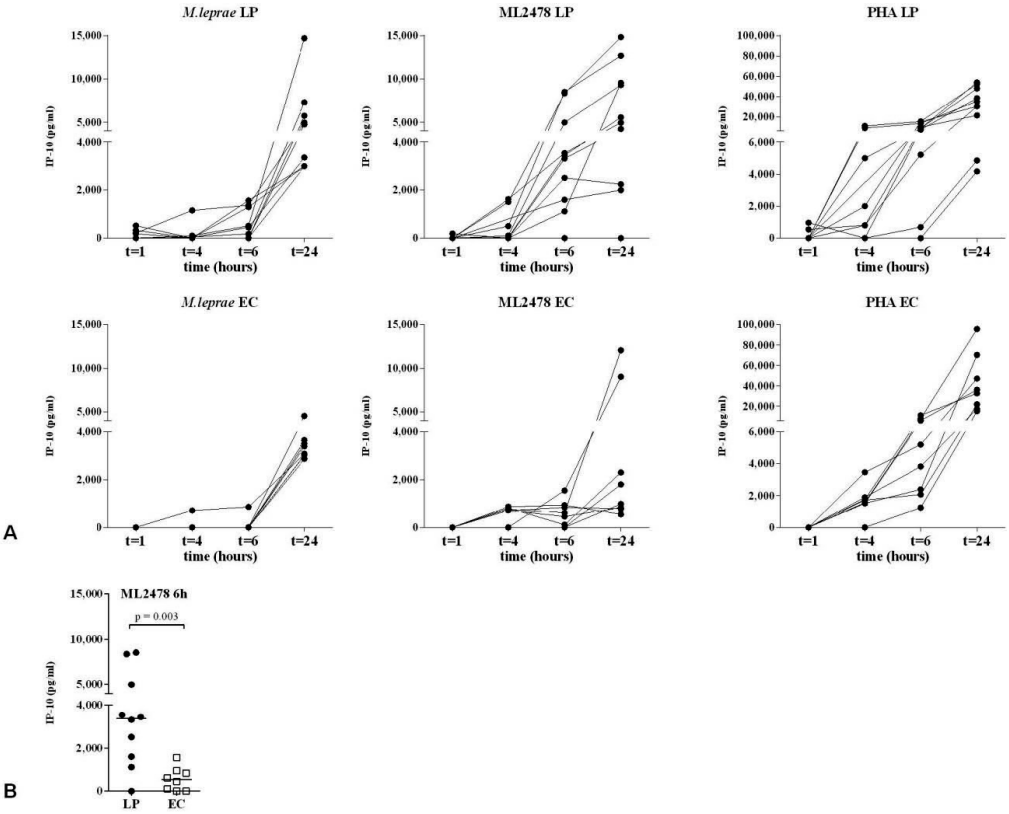

Figure 2. Kinetics of IP-10 production in WBA. (A): IP-10 concentrations produced in stimulated whole blood cultures of leprosy patients (upper panel; LP; n = 10: 5 BL (Ethiopia); 2 BT (Ethiopia); 3 BT (The Netherlands) and healthy endemic controls (lower panel; EC, n = 8) in response to M. leprae WCS (left panel; 10 mg/ml), M. leprae unique protein ML2478 (middle panel; 10 mg/ml) and PHA (right panel; 1 mg/ml). IP-10 concentrations were determined by ELISA after 1 h, 4 h, 6 h and 24 h antigen stimulation. Values on the y-axis are concentrations corrected for background values. (B): Comparison of IP-10 concentrations determined by ELISA after 6 h stimulation with ML2478 (10 mg/ml) of whole blood samples.

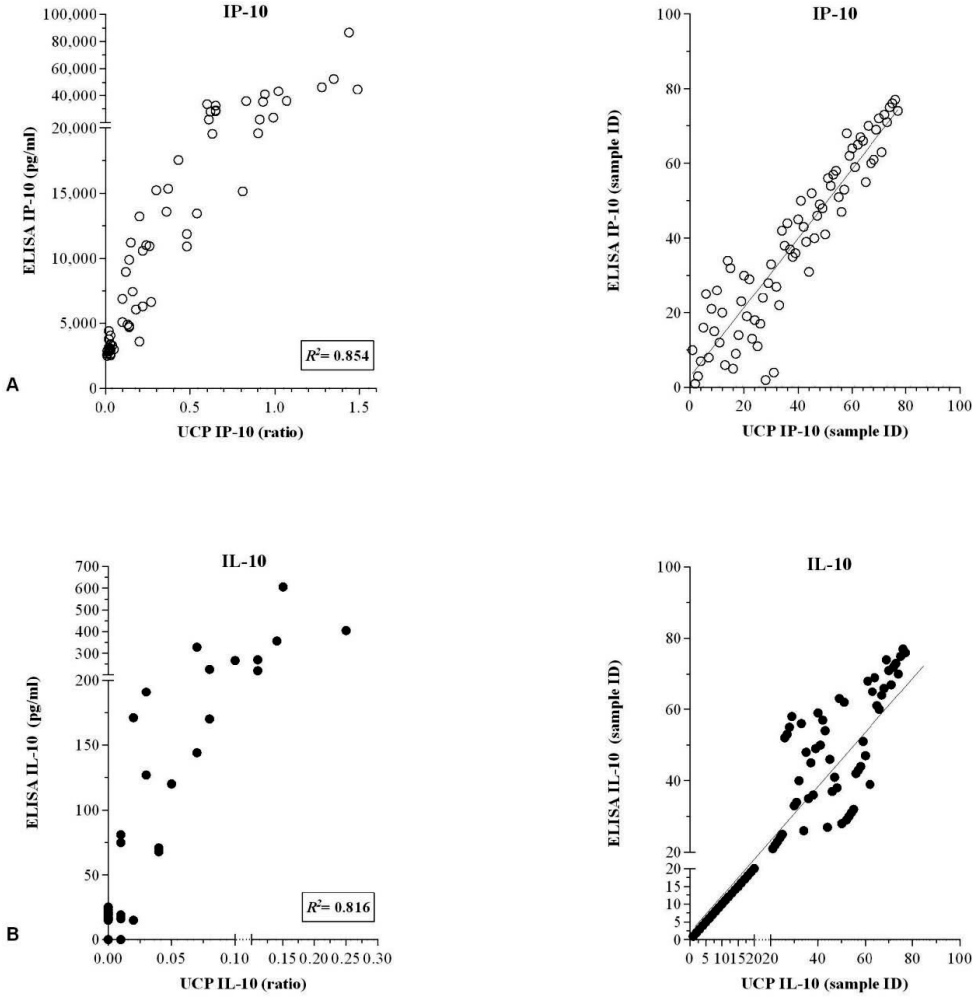

Figure 3. Correlation between ELISAs and UCP-LFAs. Levels of IP-10 (A) and IL-10 (B) in 24 h whole blood samples of 77 M. leprae (antigen), LPS and PHA stimulated WBA samples of Dutch healthy controls were simultaneously determined by ELISAs and wet-format UCP-LFAs. Left panels: results for ELISAs are indicated in pg/ml (ELISA) or as the ratio of the relative fluorescence units (RFUs) measured at Test and Flow-Control lines (UCPLFA). R^2 equals the square of the Pearson correlation coefficient. Right panels: Spearman ranking.

For direct comparison of single UCP-LFAs performance in a field- versus laboratory setting, the UCP-LF strips for IP-10 and anti-PGL-I antibodies analyzed in Ethiopia were sent to The Netherlands and re-analysed using a dedicated, high-tech UCP scanner, a Packard FluoroCount microtiter-plate reader adapted with an infrared laser (980 nm) capable to scan 20 strips simultaneously. Comparison of ratios obtained in both tests showed an excellent correlations between both scanners (IP-10: R^2 0,960 and PGL-I: R^2 0,901; Figure 5), demonstrating that the UCP-LF strips can be stored as permanent record allowing re-analysis in a reference laboratory. Since leprosy endemic areas are often short of sophisticated laboratories, these results indicate that UCP-LFAs represent robust test suitable for resource-poor settings.

Multiplex UCP-LFA for detection of IP-10 and anti-PGL-I antibodies

IP-10 levels as well as anti-PGL-I antibody concentrations were present in high concentrations allowing reliable detection even with small amounts of serum thereby improving the robustness in field assays. To further simplify the use of the UCP-LFA for leprosy diagnostics in a field setting, we next developed a multiplex UCP-LFA for simultaneous detection of anti-PGL-I antibodies and IP-10 in whole blood samples, analogous to the earlier described anti-PGL-I/ IL-10 multiplex UCP-LFA [26]. The advantage of this specific chemokine/ antibody combination is that similarly diluted serum samples can be used, facilitating multiplex analysis of cellular and humoral immunity. For extensive comparison of single and multiplex UCP-LFAs Dutch leprosy patients' WBA samples were used as well to accommodate for more samples. Multiplex UCP-LFA and the single UCP-LFA for IP-10 and anti-PGL-I antibodies showed good correlations (R^2 0,961 and 0, 897; Figure 6) demonstrating the applicability of this multiplex UCP-LFA.

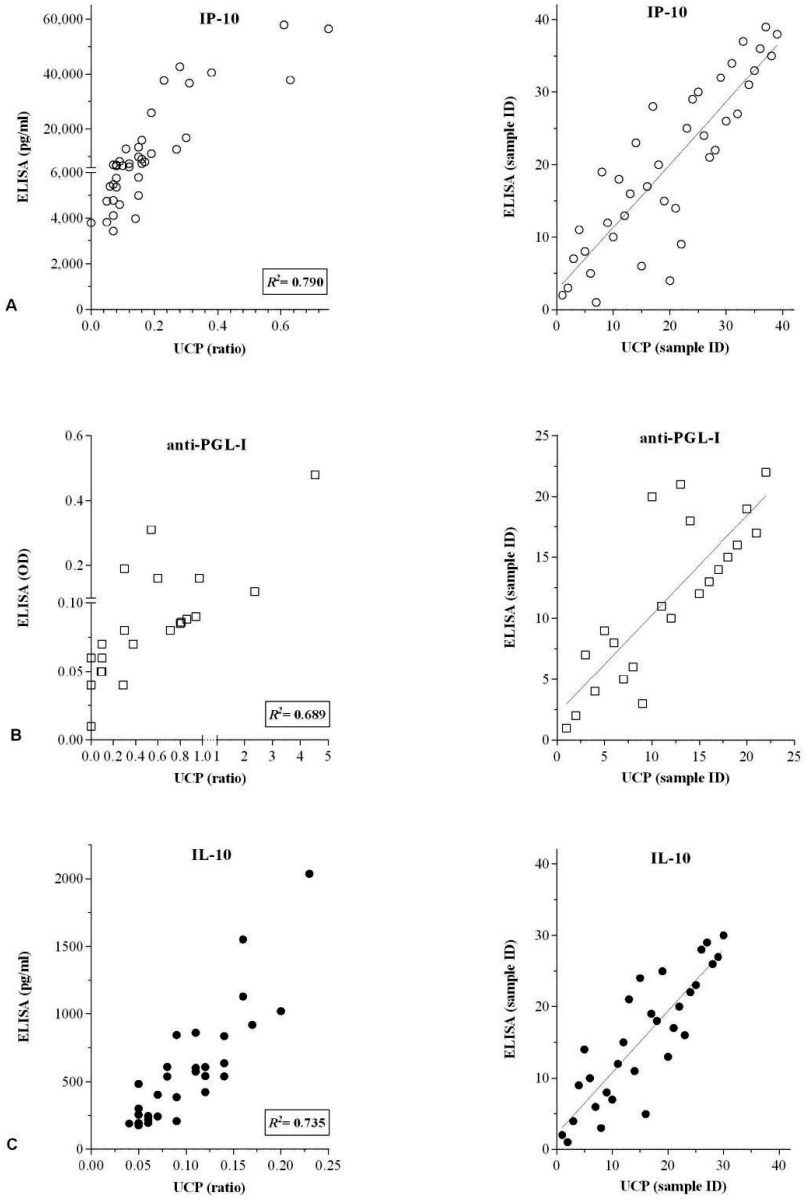

Figure 4. Correlation between ELISAs and UCP-LFAs. Levels of IP-10 (A; n = 40), anti-PGL-I antibodies (B; n =22) or IL-10 (C; n =40) in WBA samples were simultaneously determined by ELISAs and UCP-LFAs in Ethiopia using dry-format (A, B) or wet format (C) UCP-LFAs. For cytokine analysis (A and C), samples of Ethiopian leprosy patients (2 BT and 8 BL) that were unstimulated or stimulated with M. leprae WCS, ML2478 or PHA were used. For anti-PGL-I antibodies (B), samples of Ethiopian leprosy patients (2 BT and 8 BL) and healthy endemic controls (n = 12) were used. Left panels: results for ELISA are indicated in pg/ml (A, C)orOD450 (B) or as the ratio of the relative fluorescence units (RFUs) measured at Test and Flow-Control lines (UCP-LFA). R^2 equals the square of the Pearson correlation coefficient. Correlation was calculated for samples with ELISA values higher than the cut-off threshold. Right panels: Spearman ranking.

Discussion

Effective diagnostics are essential tools for the control, elimination and eradication of neglected diseases such as leprosy. Since leprosy endemic areas are often short of sophisticated laboratories, it is imperative to develop diagnostic tests for early detection of *M. leprae* infection that are suitable for field settings. The main requisite for such diagnostic tests is the selection of suitable biomarkers. WBA using *M. leprae* (-specific) antigens induce a 'fingerprint' of (the ratio of) pro- and anti-inflammatory cytokines that, combined with detection of anti-PGL-I antibodies, can be used as a biomarker profile for *M. leprae* infection.

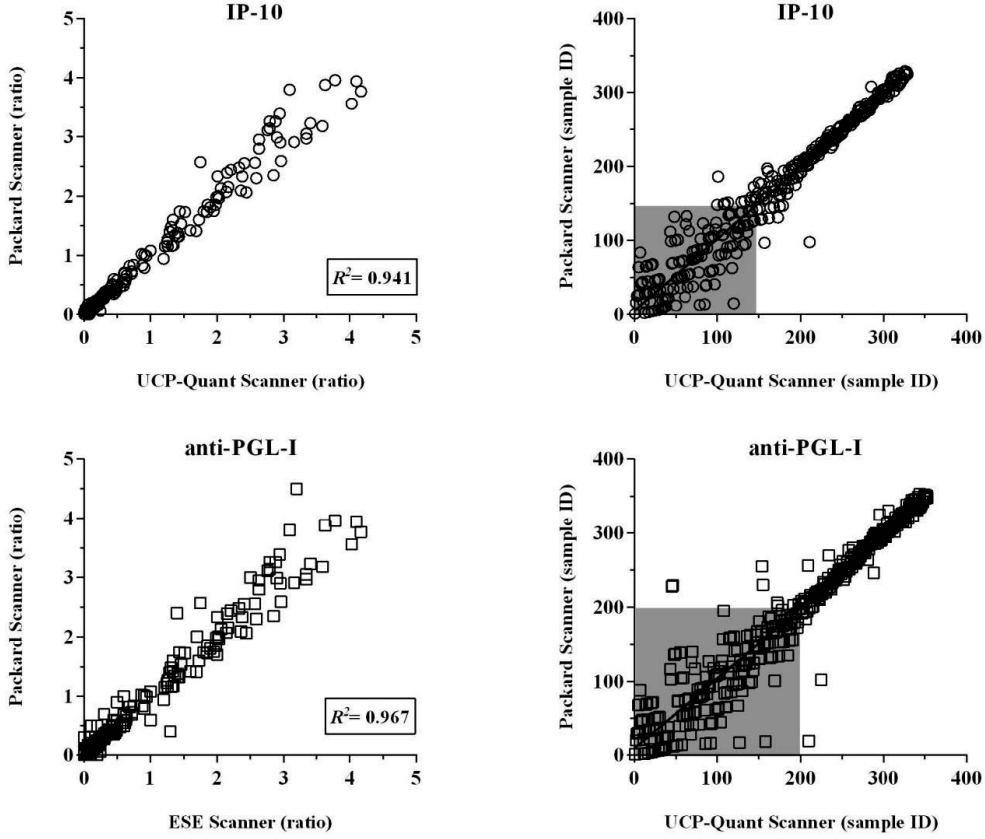

Figure 5. Performance of the portable lightweight UCP-Quant LF strip reader. Dry-format UCP-LFAs were performed for single detection of IP-10 and anti-PGL-I antibodies in an Ethiopian field setting (Figure 3). LF strips were analyzed using a portable reader (UCP-Quant). Subsequently, LF strips were shipped to The Netherlands and re-analysed using a dedicated lab-based FluoroCount microtiterplate reader (Packard) adapted for reading UCP-LF strips. Left panel: results are indicated as the ratio of the relative fluorescence units (RFUs) measured at Test and Flow-Control lines. R2 equals the square the Pearson correlation coefficient. Right panel: Spearman ranking. The grey box indicates samples scoring values below the specificity threshold.

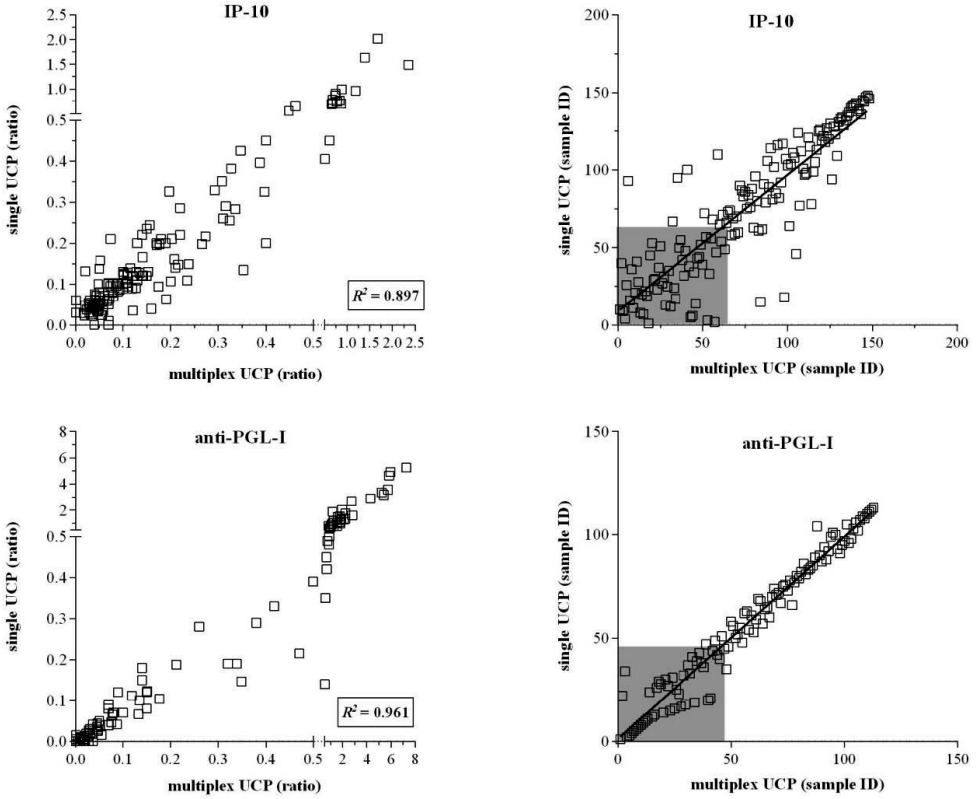

Figure 6. Comparison between single and multiplex UCP-LFAs. UCP-LFAs were performed for single or multiplex detection of IP-10 (upper panel; n = 149 samples) and anti-PGL-I (lower panel; n = 115 samples) using M. leprae antigen-stimulated WBA samples of Dutch and Ethiopian leprosy patients. Simultaneous detection of IP-10 and anti-PGL-I IgM was performed following the two phase protocol using the UCP $^{\alpha IP-10}$conjugate and the UCP $^{\alpha IgM}$ conjugate. Left panel: Results for UCP-LFAs are displayed as the ratio of the relative fluorescence units (RFUs) measured at Test and conjugate. Left panel: Results for UCP-LFAs are displayed as the ratio of the relative fluorescence units (RFUs) measured at Test and Flow-Control lines. R^2 equals the square of the Pearson correlation coefficient. Right panel: Spearman ranking. The grey box indicates samples scoring values below the specificity

Notwithstanding the frequent use of IFN-γ, IP-10 represents an equally valid biomarker for pro-inflammatory responses to mycobacteria [22;23;27;36;40;41]. This chemokine is produced by various cell types, including monocytes/macrophages, and is involved in recruitment of lymphocytes and neutrophils to sites of inflammation. IP-10 can be used to differentiate between high and low *M. leprae* exposure levels [22] and it also provides a biomarker associated with type 1 reaction (T1R) in leprosy patients [42;43]. Moreover, IP-10, is much less influenced by CD4 cell count and, in contrast to IFN-γ, can be used in HIV^+ individuals [28]. Considering the similarities in IP-10 responses of *M. leprae*- and *M. tuberculosis* infected individuals, and the high concentrations in which it is produced, we developed a UCP-LFA for IP-10 and investigated its diagnostic potential for leprosy (this study) and TB in Africa (Corstjens *et al*., in preparation). Although most IGRAs require an antigen stimulation time of at least 24h, we here demonstrate that IP-10, in contrast to IFN-γ, already showed a significant divergence between Ethiopian leprosy patients and EC after 6h

stimulation with the *M. leprae*-unique protein ML2478. This considerably reduces the overall assay time and could conveniently provide a sample-to-result on the same day.

Since host immunity and immuno-pathogenicity in response to *M. leprae* comprises multifaceted interactions between a diversity of cells secreting different molecules, it is rather unlikely that only a single compound is linearly correlated to protection or to disease progression [44]. Diagnostic tests that determine ratios of different types of cytokines will therefore be informative regarding disease development after *M. leprae* infection [19;45] as was previously illustrated by IFN-γ/ IL-10 and IFN-γ/ IL-17 ratios in *Mtb* infected individuals [46;47], but also for the development of T1R [42]. Relatedly, another valuable observation made here was the significant difference in IP-10/IL-10 ratios in sera of leprosy patients and EC, even without antigen stimulation. These data illustrate that the proportion of pro- to anti-inflammatory cytokines is consistent with clinical outcome after infection. Consequently, over time changes in the IP-10/IL-10 ratio for one individual will provide relevant clinical information with respect to the outcome of infection.

Dry-format UCP-LFAs are ideally suited for performance in the field and can be shipped and stored conveniently at ambient temperature and have prolonged shelf life of more than two years in African settings [6]. In this study we selected IP-10 and anti-PGL-I antibodies for field-evaluation of the dry-format UCP-LFA and development of dry-format UCP-LFA for more analytes is in progress. This evaluation showed that both dry-format UCP-LFAs were equally sensitive as ELISAs and could be applied in the concentration range of 100 to >100,000 pg/ml. Also, the availability of affordable and portable UCP-LF strip readers showed suitability of the assay in field settings where ELISA equipment is not available or is more challenging to use. The LF strips were read with an easy to operate, portable reader that allows full instrument-assisted assay analyses avoiding operator bias. Due to the chemical stability of the assay components, the strips can be kept in patients' files and read again after long periods of time.

Besides the speed and ease of performance, another advantage of the UCP-LFA is that multiple analytes can be detected on the same LF-strip. Feasibility of multiplexed analysis was demonstrated previously for IL-10 and anti-PGL-I antibodies in spiked sera [26]. In this study multiplexing was successfully shown for IP-10 and anti-PGL-I antibodies in whole blood samples. Although the current UCP-LFA conditions for IL-10 quantitation demand a 100-fold larger sample input than the IP-10 assay, a single strip allowing quantitative detection of IP-10, IL-10 as well as anti-PGL-I antibody detection is feasible. Revision of the position (distance from the sample pad) and antibody load of the test lines, would allow the use of 1 μL samples instead of the currently applied 0.1 and 10 μL for IP-10 and IL-10 respectively. Moreover, multiplexing can be achieved by running two or more LF strips from a single sample in parallel as was for instance described for a simple multiple channel device running ten UCP-LF strips from a single sample [11].

This study describes the first steps towards development of a UCP-LFA as a field test measuring pro- and anti-inflammatory cellular- as well as humoral immunity to *M. leprae*, thereby including read-outs for multiple classifications of the leprosy spectrum. Such tests can be useful tools in leprosy control programs for classification of leprosy and allow early diagnosis of leprosy or leprosy reactions, leading to timely treatment and reduced transmission.

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Chapter 6

T-cell regulation in lepromatous leprosy

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Abstract

Regulatory T (T_{reg}) cells are known for their role in maintaining self-tolerance and balancing immune reactions in autoimmune diseases and chronic infections. However, regulatory mechanisms can also lead to prolonged survival of pathogens in chronic infections like leprosy and tuberculosis (TB). Despite high humoral responses against *Mycobacterium leprae* (*M. leprae*), lepromatous leprosy (LL) patients have the characteristic inability to generate T helper 1 (Th1) responses against the bacterium.

In this study, we investigated the unresponsiveness to *M. leprae* in peripheral blood mononuclear cells (PBMC) of LL patients by analysis of IFN-γ responses to *M. leprae* before and after depletion of $CD25^+$ cells, by cell subsets analysis of PBMC and by immunohistochemistry of patients' skin lesions.

Depletion of $CD25^+$ cells from total PBMC identified two groups of LL patients: 7/18 (38.8%) gained *in vitro* responsiveness towards *M. leprae* after depletion of $CD25^+$ cells, which was reversed to *M. leprae*-specific T-cell unresponsiveness by addition of autologous $CD25^+$ cells. In contrast, 11/18 (61.1%) remained anergic in the absence of $CD25^+$ T-cells. For both groups mitogen-induced IFN-γ was, however, not affected by depletion of $CD25^+$ cells. In *M. leprae* responding healthy controls, treated lepromatous leprosy (LL) and borderline tuberculoid leprosy (BT) patients, depletion of $CD25^+$ cells only slightly increased the IFN-γ response.

Furthermore, cell subset analysis showed significantly higher ($p = 0.02$) numbers of $FoxP3^+$ $CD8^+CD25^+$ T-cells in LL compared to BT patients, whereas confocal microscopy of skin biopsies revealed increased numbers of $CD68^+CD163^+$ as well as $FoxP3^+$ cells in lesions of LL compared to tuberculoid and borderline tuberculoid leprosy (TT/BT) lesions. Thus, these data show that $CD25^+$ T_{reg} cells play a role in *M. leprae*-Th1 unresponsiveness in LL.

Author summary

Leprosy is a curable infectious disease caused by *Mycobacterium leprae* (*M. leprae*) that affects the skin and peripheral nerves. It is manifested in different forms ranging from self-healing, tuberculoid leprosy (TT) with low bacillary load and high cellular immunity against *M. leprae*, to lepromatous leprosy (LL) with high bacillary load and high antibody titers to *M. leprae* antigens. However, LL patients have poor cell mediated response against *M. leprae* leading to delayed clearance of the bacilli. A possible explanation for this bacterial persistence could lie in the presence of more regulatory cells at infection sites and in peripheral blood. This study shows the recovery of the cell mediated response by depletion of $CD25^+$ cells in a subset of LL patients, while another patient subset was not affected similarly. Moreover, an increased frequency of $FoxP3^+$ T cells together with anti-inflammatory macrophages was observed in LL patients' skin biopsies. Thus, these data show that $CD25^+$ T_{reg} cells play a role in *M. leprae*-unresponsiveness in leprosy patients.

Introduction

The human immune system strives to maintain the delicate balance between preventing host susceptibility to various pathogens and limiting immunopathology due to an exacerbated immune response to infections. Sub-populations of T-cells previously identified as suppressor T-cells and later as T_{reg} cells are the major players in the regulatory network of the immune system [1,2]. Although the idea of suppressor T-cells was a key topic of research already in the 70's and 80's it was not successfully established because of poor cellular characterization, and it took until mid-1990's before T_{reg} cells were recognized as a different lineage [1]. More recently, studies clearly demonstrated the suppressive ability of this sub-population contributing to the re-acceptance of suppressor T-cell as a different T-cell lineage [3,4].

Characterization of this T-cell sub-population has continued and currently the thymus-derived T_{reg} cells (tT_{reg} cells) and peripherally derived T_{reg} cells (pT_{reg} cells) [5] are the two widely accepted categories of T_{reg} cells [1,6,7]. Both T-cell subtypes play a role in limiting immune reactions in autoimmune diseases and chronic infections [8–11]. In addition, $CD39^+$ T_{reg} cells have also been reported as a subset of the $CD4^+$ $CD25^{high}FoxP3^+$ T_{reg} cells in association with chronic infections like tuberculosis (TB) [12], hepatitis B (HBV) and in graft rejections [13,14] and the ability of $CD8^+$ $CD39^+$ T_{reg} cells to suppress antigen specific $CD4^+$ proliferation clearly demonstrated the importance of this sub-population [15].

Leprosy is a chronic infectious disease leading to more than 200,000 new cases every year [16]. The remarkable inter-individual variability in clinical manifestations of leprosy closely parallels the hosts' abilities to mount effective immune responses to *M. leprae*. This is clear from the well-known immunological and clinical spectrum in those who progress to disease ranging from polar T helper 1 (Th1) to Th2 responses. TT and BT show more dominant Th1 responses which limit *M. leprae* growth resulting in clinical paucibacillary (PB) leprosy whereas, BL/LL patients demonstrate dominant Th2 responses as well as more permissive growth of *M. leprae* resulting in clinical multibacillary (MB) leprosy. TT/BT patients in general show high cellular responses and low antibody titers to *M. leprae* antigens, and develop localized granuloma with often no detectable bacilli in their lesions. At the opposite pole, LL/BL patients are incapable to generate *M. leprae* specific Th1 cell responses, show high antibody titers to *M. leprae* antigens including PGL-I, with numerous bacilli and disorganized granuloma in their lesions. The borderline states of leprosy are immunologically unstable. The different outcomes of infection in leprosy are most likely caused by host defense mechanisms [17]. However, the mechanism underlying the *M. leprae*-specific T-cell anergy in LL patients is still not completely understood.

In chronic bacterial or viral infections, evidence exists that T_{reg} cells suppress effector T-cells (T_{eff} cells) in order to limit damage to the host caused by the immune responses against pathogens [18]. In this situation, the regulatory activity of T_{reg} cells may lead to prolonged survival of pathogens in the host [9,19]. As evidenced in a previous study, higher levels of $CD4^+CD25^+FoxP3^+$ T_{reg} cells were observed in active TB patients in the periphery compared to latently infected individuals and healthy controls [20,21]. Also, an increased number of T_{reg} cells expressing FoxP3, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced tumour-necrosis-factor-receptor-related protein (GITR) were reported in lymph nodes from children with tuberculosis lymphadenitis [22]. Similarly, in leprosy, higher numbers of T_{reg} cells in PBMC from BL and LL patients stimulated with *M. leprae* cell wall antigen (MLCWA) were observed compared to TT/BT forms, indicating the possibility that T_{reg} cells may have a role in persistence of *M. leprae* bacteria as well as unresponsiveness of

Th1 cells in BL/LL patients [23]. Recently, the mechanism of action of FoxP3 in $CD4^+CD25^+$ T cells derived from BL/LL leprosy patients was shown to result from increased molecular interactions of FoxP3 with Histone deacetylases (HDAC7/9) in the nucleus of $CD4^+CD25^+$ T cells derived from BL/LL patients [24].

In the presence of pathogens, T_{reg} cells can also be induced by certain macrophages as evidenced by the anti-inflammatory, $CD163^+$ macrophages, known as type 2 macrophages (mφ2), that exert a suppressive effect on Th1 responses [25,26]. On the other hand, IL-10 induced phagocytosis of *M. leprae* by mφ2 without induction of microbicidal activity in LL lesions has been described [27] indicating the role of IL-10 producing T_{reg} cells in the persistence of the pathogen within the host. Similarly, the presence of higher IL-10 expression correlated with increased CD163 and indoleamine 2,3-dioxygenase (IDO) proteins in tissues and sera of LL patients further evidenced their potential [28].

In this study, we have investigated the functional role of $CD25^+$ T_{reg} cells in *M. leprae* unresponsiveness of LL patients as well as the frequency of $CD25^+$ and $FoxP3^+$ cells in the PBMC of leprosy patients. Additionally, lesions of LL and TT/BT patients were assessed for the presence of $FoxP3^+$ cells and $CD163^+$ macrophages (mφ2).

Materials and Methods

Ethical statement. Ethical approval of the study protocol was obtained from the National Health Research Ethical Review committee, Ethiopia (NERC # RDHE/127-83/08) and the Nepal Health Research Council (NHRC #751). Participants were informed about the study objectives, the required amount and kind of samples and their right to refuse to take part or withdraw from the study at anytime without consequences for their treatment. Written and Informed consent was obtained from study participants before enrollment.

Study participants. The following HIV-negative individuals were recruited on a voluntary basis: newly diagnosed, non reactional leprosy patients from Ethiopia (ALERT hospital, Addis Ababa, Ethiopia) classified as LL (n=40) and TT/BT (n=16) and healthy endemic controls from health centers in Addis Ababa (EC; n=5); Treated, non reactional LL (n=6) and TT/BT (n=9) patients and EC (n=10) from Anandaban Hospital, (Kathmandu, Nepal); and non-endemic Dutch healthy controls (NEC; n=13). Leprosy was diagnosed based on clinical, bacteriological and histological observations and classified by a skin biopsy evaluated according to the Ridley and Jopling classification [17] by qualified microbiologists and pathologists. All patients were enrolled before treatment was initiated. EC were assessed for the absence of clinical signs and symptoms of tuberculosis and leprosy. Individuals working in health facilities were excluded as EC.

PBMC Isolation, freezing and thawing. PBMC were isolated by Ficoll-Hypaque density gradient method, cells were washed and suspended in 20% fetal calf serum (FCS) in AIM-V (Invitrogen, Carlsbad, CA) and kept cool on ice, counted and frozen using a cold freshly prepared freezing medium composed of 20% FCS, 20% dimethyl sulphoxide (DMSO) in AIM-V. Cells were kept at -80 °C for 2-3 days and transferred to liquid nitrogen until use. During thawing, cells were transported in liquid nitrogen to a water bath (37^oC) for 30 to 40 seconds until thawed half way and resuspended in 10% FCS in AIM-V (37^oC) containing 1/10,000 benzonase until completely thawed, washed 2 times (5-7 minutes each) and counted. The percentage viability obtained was > 75% and cells were incubated with anti-CD25 magnetic beads or used for FACS analysis.

CD25 $^+$cell separation. Frozen PBMC were thawed, washed and incubated with 20µl of the CD25 micro beads II, human (Miteny Biotec, Bergisch Gladbach, Germany) in 80µl MACS

buffer (Phosphate-buffered saline (PBS) with 0.5% Bovine serum albumin (BSA) and 2mM EDTA) for 20 minutes at 4°C. Cells were washed and added to MS column attached to Magnetic Cell Sorter (MACS) (Milteny Biotec) where $CD25^-$ cells were collected as flow through and the $CD25^+$ population was collected by detaching the column from the magnetic cell sorter. Cells were washed with MACS buffer and resuspended in AIM-V medium. The purity of the $CD25^-$ and $CD25^+$cell populations was > 80% (supplementary figure S2A and S2B).

Lymphocyte stimulation tests (LST). Total PBMC (150,000 cells/well), $CD25^-$ cells (150,000 cells/ well) or $CD25^-$ cells with proportionally added $CD25^+$ cells (10,000 and/or 25,000) were added in triplicate into 96 well U bottom tissue culture plates and cultured with *M. leprae* whole cell sonicate (WCS; 10 μg/ml), phytohaemagglutinin (PHA; 1 μg/ml) or AIM-V medium at 37°C with 5% CO_2 and 70% humidity. After 6 days, supernatants were collected and kept frozen until used in ELISA.

***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 kindly provided by Dr. J.S. Spencer 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/TBVTRMResearch Materials/tabid/1431/Default.aspx).

IFN-γ ELISA. IFN-γ levels were determined by ELISA (U-CyTech, Utrecht, The Netherlands) [29]. The cut-off value to define positive responses was set beforehand at100 pg/ml. The assay sensitivity level was 40 pg/ml. Values for unstimulated cell cultures were typically < 40 pg/ml.

Flow cytometry. After depletion, the total PBMC, $CD25^-$ or $CD25^+$ populations (25,000 to 200,000 cells) were stained for CD3 (clone SK7, PerCP; Becton, Dickinson and Company, New Jersey, USA), CD4 (clone SK3, FITC; BD) and CD25 (PE; MACS) to check the purity. Frozen PBMC of patients and healthy controls (2 x 10^6 cells/ml) were thawed, washed and treated with benzonase (10 U/ml, Novagen, Merck4Biosciences, Merck KGaA, Darmstadt, Germany) for 2 hours prior to *in vitro* stimulation with PMA (20ng/ml)/ionomycine (500 ng/ml) in the presence of 1 μg/ml anti CD28 (Sanquin, the Netherlands) and 1 μg/ml anti CD49d (BD Biosciences, Eerbodegem, Belgium). After 4 hours, Brefeldin A (Sigma Aldrich) was added at 3 μg/ml and cells were left for an additional 16 hours in the incubator at 37°C with 5% CO_2 and 70% humidity. After live/dead staining with Vivid (Invitrogen, Life technologies, Merelbeke, Belgium), surface staining was performed for 30 minutes at 4°C with the labeled antibodies directed against: CD14-Pacific Blue, CD19-Pacific Blue (eBioscience), CD3-PE-TexasRed (Invitrogen, Life technologies), CD8-Horizon V500, CD4-Pe-Cy7, CD25-APC-H7 (all BD Biosciences, CD39-PE (Biolegend, ITK Diagnostics, Uithoorn, The Netherlands). Samples were washed, fixed and intracellular staining was performed using the intrastain kit (Dako Diagnostics, Glostrup, Denmark) with IFN-γ -Alexa700 (BD Biosciences), IL-10 APC (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and FoxP3 PE-Cy5 (eBioscience, Hatfield, UK) labeled antibodies. Cells were acquired on a FACS LSR Fortessa with Diva software (BD Biosciences, The Netherlands) and analyzed with FlowJo version 9.4.1 (Tree Star, Ashland, OR, USA). The full gating strategy for live $CD4^+$ $CD3^+$ cells or $CD8^+$ $CD3^+$ cells (supplementary Figure S1A and S1B) was performed in compliance with the most recent MIATA [30] guidelines according to the following procedure: events were first gated using a forward scatter area (FSC-A) versus height (FSC-H) plot to remove doublets. Subsequently, the events were subjected to a lymphocyte gate using a side scatter (SSC) followed by a live/dead gating. Then, live $CD3^+$ cells were gated and $CD14^+$ and $CD19^+$ events were excluded from analysis using a dump channel. Finally, CD3 live cells were separated in to $CD4^+$ and $CD8^+$. After the gates for each function were created, we used the

Boolean gate platform to identify all functions within each cell preparation using the full array of possible combinations.

Immunohistochemistry and confocal microscopy. Skin biopsies taken from leprosy lesions of LL (n=10) and TT/BT (n=4) patients were fixed in formalin and embedded in paraffin. Tissue sections with 4 μm thickness were prepared using a microtome (LEICA RM 2165). The prepared tissues sections were stained for hematoxylin and eosine (H & E; images are shown in supplementary figure S3) and also used as previously described [31] for immunofluorescence staining. Tissue sections were deparaffinised and rehydrated using graded concentrations of ethanol to distilled water. Antigen retrieval was performed in boiling Tris-EDTA buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0) for 12 minutes. After two hours of cooling at room temperature in antigen retrieval buffer, slides were washed twice in distilled water and twice in PBS, blocked for 15 min with 5% goat serum in PBS, washed again with PBS and stained with primary antibodies for FoxP3 (1:100, mouse anti-human IgG1 Abcam; Cambridge, UK), CD8 (1:100 mouse anti-human IgG2b, Abcam), CD68 (mouse anti-human IgG2a AbD serotec/Bio-Rad; Veenendaal, The Netherlands), CD163 (1:400, mouse anti-human IgG1, Leica; Rijswijk, The Netherlands) and CD39 (1:100, mouse anti-human IgG2a, Abcam). Two antibodies were used per tissue section: FoxP3 with CD68, CD163, CD39 or CD8; CD68 with CD163 and CD39 with CD163. After overnight incubation at room temperature in the dark, sections were washed and incubated for 1 hour in the dark with secondary antibodies; goat-anti-mouse IgG1 coupled with Alexa 488 (1:200) (Invitrogen, Bleiswijk The Netherlands), goat-anti-mouse IgG2a or goat-anti-mouse IgG2b with Alexa 546 (1:200) (Invitrogen). Tissue sections were then washed three times with PBS and mounted with Vectashield (DAPI, 4', 6-diamidino-2-phenylindole; Vector Laboratories, Brussels, Belgium). Immunofluorescence of skin sections was examined and images were taken from 5 different fields per section using a Leica-TCS-SP5 confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany). Nucleated cells that positively stained for the specific marker were counted from five different fields per section by two laboratory persons independently. Average counts for each marker per section were compared for all samples.

Statistical analysis. Differences in cytokine concentrations were analyzed with the two-tailed Mann-Whitney U test or Wilcoxon signed rank 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

Depletion of $CD25^+$ cells enhanced pro-inflammatory response in LL patients

To analyse the role of $CD25^+$ cells in the production of IFN-γ, PBMC from Ethiopian LL patients (n=17) and Dutch healthy controls (n=12) were depleted of $CD25^+$ cells and cell subsets with and without re-added $CD25^+$ cells were stimulated with *M. leprae* WCS in 6 days culture.

PBMC from treated Nepali LL (n=6), BT (n=9) patients and EC (n=10) were depleted for $CD25^+$ cells but only the total PBMC and $CD25^-$cell subset were stimulated with *M. leprae* WCS. When compared according to clinical classification, there was a trend of higher IFN-γ production in PB compared to MB samples. IFN-γ production of total PBMC (undepleted fraction) from LL patients in response to *M. leprae* (WCS) was significantly lower ($p = 0.001$) compared to responses by PBMC from TT/BT patients, whereas IFN-γ responses to PHA

were high in both groups (Fig 1). These data further confirm the *M. leprae*-specific lack of cell mediated immunity (CMI) in LL patients.

Analysis of IFN-γ production in response to *M. leprae* (WCS) by $CD25^-$ cells alone or $CD25^-$ cells (150,000 cells per well) supplemented with the $CD25^+$ fraction (10,000 or 25,000 cells/well) discriminated two groups of LL patients: those that produced IFN-γ in response to *M. leprae* after $CD25^+$ cell depletion and those that did not (Fig 2A, 2B and 2E). Among the 18 LL Ethiopian patients, 7 (38%) responded to *M. leprae* WCS after depletion of $CD25^+$ cells whereas they lacked any response in total PBMC. IFN-γ production in response to PHA in both groups was not affected by the depletion of or enrichment with $CD25^+$ cells.

In the LL patient group, in which recovery of IFN-γ responses was observed to *M. leprae* WCS after depletion of $CD25^+$ cells, this could be reversed proportionally by the addition of $CD25^+$ cells (Fig 2A). In the patient group in which $CD25^+$ cell depletion did not reverse anergy to *M. leprae*, there was no effect observed by addition of $CD25^+$ cells to the depleted fraction (Fig 2B).

In similar analysis of treated leprosy patients (LL and BT) and endemic controls from a Nepali population, PBMC responded to *M. leprae* WCS in the presence of $CD25^+$ cells and a slight increase in IFN- γ levels after $CD25^+$ cell depletion was also observed (Fig 2C). Similarly, healthy Dutch controls (n=8) responding to *M. leprae* WCS before depletion of $CD25^+$ cell showed a slight increase after depletion (Fig 2D left panel) as well, while other NEC (n=5) remained unresponsive after $CD25^+$ cell depletion (Fig 2D right panel).

FoxP3 expressing $CD8^+$ $CD25^+$ T-cell are more abundant in PBMC of LL

For cell subset analysis, PBMC from Ethiopian LL (n=13), TT/BT (n=5) and EC (n=7) and Dutch healthy controls (NEC; n=4) were stained for surface and intra-cellular markers. The frequency of $FoxP3^+$ $CD8^+CD25^+$ cells was significantly higher in PBMC of LL patients compared to TT/BT patients ($p = 0.02$) (Fig. 3). Although not statistically significant ($p= 0.05$), we also observed a higher frequencies of $FoxP3^+$ $CD4^+$ $CD25^+$ T-cell in the LL group compared to the TT/BT patients (Fig. 3). In contrast, analysis of the frequency of IL-10 producing $CD4^+$ $CD25^+$ or $CD8^+CD25^+$ T-cell showed no significant differences between patients and healthy controls. The frequency of IL-10 production in $CD4^+$ $CD25^+$ or $CD8^+CD25^+$ T-cell in general was very low in all groups.

Mφ2 ($CD68^+$ $CD163^+$) and $FoxP3^+$ cells are more frequent in skin lesions of LL patients

Confocal analysis of two-colour immunofluorescence was used to localize specific cell markers in skin biopsies of Ethiopian LL (n=10) and TT/BT (n=4) leprosy patients. Higher number of $CD68^+$ cells in LL lesions ($p = 0.02$) (Table 1, Fig.4A, 5A and B) indicated the presence of more infiltrating macrophages compared to TT/BT (Fig.5C and D). In addition, $CD68^+$ $CD163^+$ cells (mφ2) and $FoxP3^+$ cells were present to a larger extent in LL patients' lesions ($p = 0.02$) compared to TT/BT (Table 1 and Fig. 4B, 4C, 5C and 5D). With respect to the numbers of $CD68^+$ $CD163^+$ cells (mφ2) and $FoxP3^+$ cells, differences were observed among the LL patients which could be explained by variations in the time elapsed since skin lesions were noticeable or by influence of other host factors. Although we found significantly higher frequency of $CD8^+FoxP3^+$ in PBMC, we could not clearly detect $CD8^+FoxP3^+$ in skin lesions indicating $CD4^+FoxP3^+$ cells could play a regulatory role in these tissues. In addition, skin lesions were stained with CD39 combined with FoxP3 to localize $CD39^+FoxP3^+$ regulatory T-cells. However, in most skin tissues, $CD39^+$ cells were not detected except for two LL skin tissues in which CD39 and FoxP3 positivity was observed simultaneously in

macrophage-like shaped cells (Fig 4E). Thus, these results indicate the induction of more FoxP3^{+} but not CD39^{+} Treg cells in LL patients' skin lesions probably by the presence of type 2 macrophages.

Discussion

Decreased *M. leprae*-specific T-cell mediated immunity is the hall mark of lepromatous multibacillary leprosy and can be assessed by *in vitro* unresponsiveness to *M. leprae* (antigens) or clonal anergy [2,23,32]. In this study, we confirm the *M. leprae*-specific unresponsiveness by the absence of IFN-γ responses to *M. leprae* WCS.

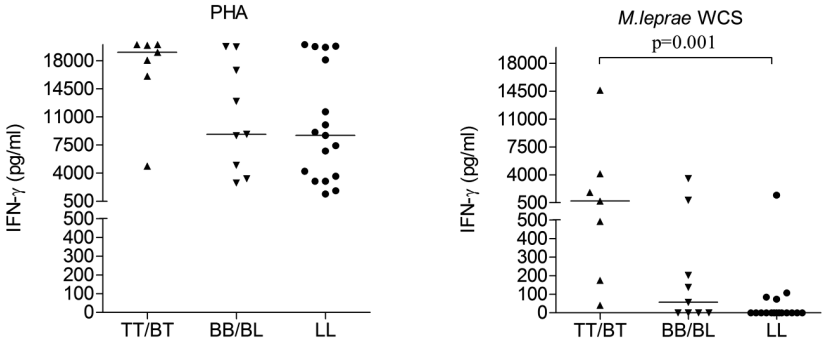

Figure 1. IFN-γ responses to PHA and *M. leprae* whole cell sonicate (WCS) by PBMC of TT/BT (n=7), BB/BL (n=9) and LL (n=16) patients. Median values for each group are indicated by horizontal lines.

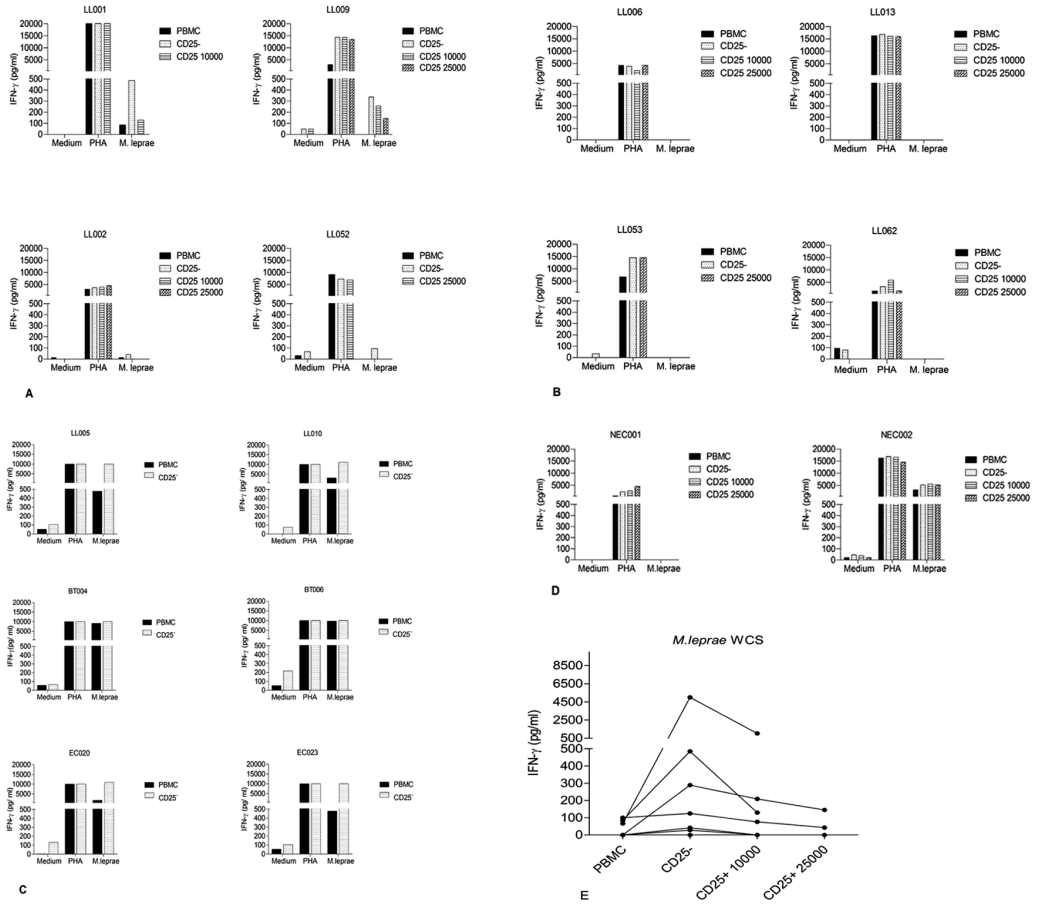

Figure 2. IFN-γ responses of total PBMC, $CD25^-$ cells and $CD25^-$ cells supplemented with $CD25^+$ cells from LL patients. (**A**) representatives for the group responding to *M. leprae* after depletion of $CD25^+$ cells (n = 7); (**B**) representatives for the group not responding to *M. leprae* after depletion of $CD25^+$ cells (n = 11); (**C**) LL005 and LL010 representatives for Nepali treated LL patients (n=10), BT004 and BT006 representatives for Nepali treated BT patients (n=7) and EC020 and EC023 representatives for Nepali EC (n=10) before and after depletion of $CD25^+$ cells; (**D**) NEC001 and NEC002 representatives for healthy Dutch controls (n=10) after depletion of $CD25^+$ cells with and without response to *M. leprae* WCS; (**E**) Dot plot graph showing IFN-γ responses of both groups of Ethiopian LL patients in dot-plot graph. Medium indicates AIM-V medium used in the assays as negative control. In 2A and 2B: for LL001, CD25-25000 and for LL052 and LL053, CD25-10000 were not done.

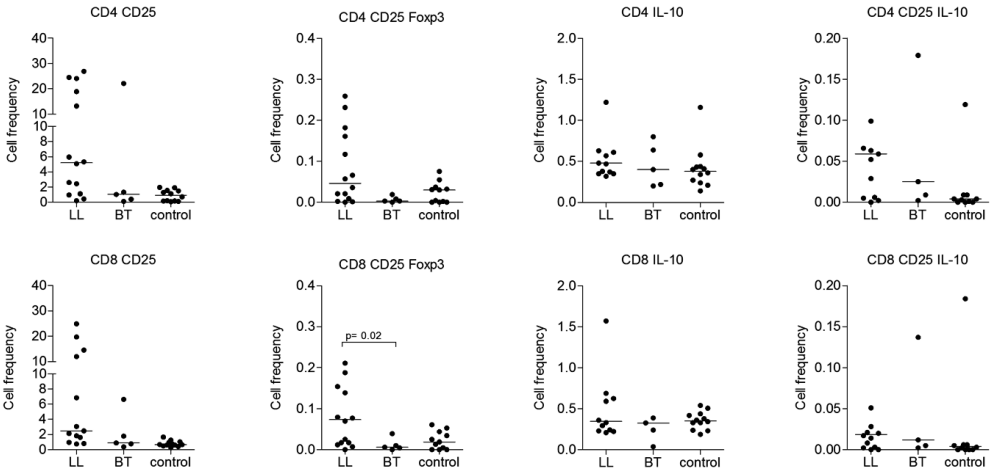

Figure 3. T-cell subset analysis of PBMC from LL, TT/BT and the control group consisting of EC and NEC showing the frequencies of FoxP3 expressing T-cells and IL-10 producing FoxP3$^+$ T-cells.

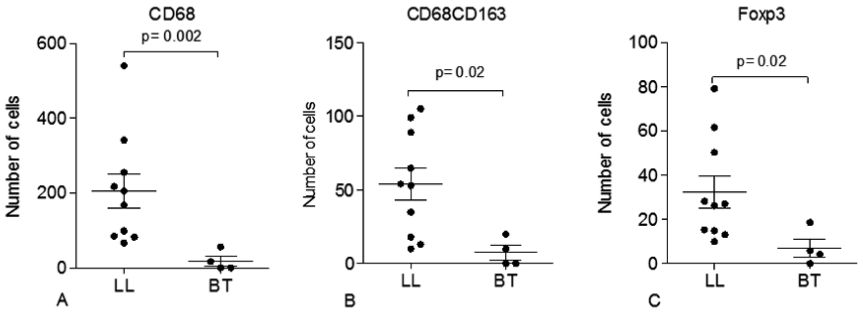

Figure 4. Immunohistochemical analysis of skin lesions of LL (n=10) and BT patients (n=4) showing the number of (**A**) CD68$^+$ cells (**B**) CD68$^+$ CD163$^+$ cells and (**C**) FoxP3$^+$ cells.

Several studies have investigated the possible causes leading to hyporesponsiveness in LL patients such as formation of foamy macrophages in presence of IL-10 [27], cholesterol dependent dismantling of HLA-DR raft in macrophages of BL/LL [33] and other factors, including T_{reg} cells. Some of these studies on T_{reg} cells have shown their presence and role either in the periphery or in skin lesions through measuring T_{reg} associated markers, mainly CD25, TGF-β, CTLA4, IL-10, and FoxP3 [23,24,34,35, 45]. Recently, Teles *et al.* showed higher expression of IFN-γ and the downstream vitamin D-dependent antimicrobial pathway related genes including CYP27B1 and VDR (Vitamin D receptor) in TT/BT as well as an increased IL-10 expression induced by IFN-β in LL lesions [36]. Some reports have revealed the limitations of the available T_{reg} markers due to their lack of specificity [37–39]: CD25, for example, is expressed on activated T and B cells and is not exclusively found on T_{reg} cells. However, noting that CD25 is still a crucial marker for T_{reg} cells in the unstimulated situation, we performed depletion of CD25$^+$ cells from unstimulated PBMC to isolate the T_{reg} cells and demonstrated their involvement in *M. leprae*-specific unresponsiveness in LL patients.

The BL/LL patients are known for their poor CMI and this is commonly assessed by measuring IFN-γ responses to *M. leprae* WCS. The total PBMC of the LL patients were analysed along with the $CD25^+$ depleted and enriched fraction for their IFN-γ responses to *M. leprae* WCS and was negative. However, the depletion of $CD25^+$ cells from total PBMC of LL patients showed an enhanced pro-inflammatory response as measured by the level of IFN-γ in response to *M. leprae* WCS in some but not all patients. Two distinct groups of LL patients were identified after depletion of $CD25^+$ cells; 38% (7/18) of the LL patients showed enhanced IFN-γ responses in the $CD25^-$ population while the remaining 62% of the LL patients did not respond to *M. leprae* WCS at all. The recovered IFN-γ production in the first group was reversed by addition of $CD25^+$ cells, clearly indicating that this $CD25^+$ cell population conferred the unresponsiveness in these LL patients. However, we did not stain the $CD25^+$ cell populations with FoxP3 which could have allowed more detailed characterization as $CD25^{high}$ FoxP3 or $CD25^{low}$ FoxP3 sub-populations which might have explained differences between the responders and non-responders. Nonetheless, the presence of non-responding LL patients after depletion of $CD25^+$ cells indicates that $CD25^+$ T_{reg} cells do not represent the sole factor responsible for T-cell anergy in LL leprosy. As the Th1 arm is responsible for killing and clearing bacilli, there could have been enormous damage to tissues in BL/LL patients where high load of bacilli and antigens are available. However, the presence of T_{reg} in these patients represents one important factor that can avoid tissue damage but, on the other hand, creates a convenient environment for bacilli to survive through suppression of Th1 response. In addition, the significant IFN-γ production observed in treated LL patients in our study before depletion of $CD25^+$ T cells showed how treatment and thereby the level of bacillary load can influence the Th1 response and T_{reg}. Similar findings were reported for TB patients with recovered IFN-γ production and reduced number of T_{reg} cells after treatment [21,40]. The slight increases observed in IFN-γ production after depletion of $CD25^+$ T cells in treated LL and BT patients and in EC tested in the depletion experiments could also indicate the regular presence of T_{reg} cells to maintain homeostasis in the host. However, the overall ratio of $CD25^+$ T_{reg} cells to effector T cells will be crucial in determining the outcome of *M. leprae* infection in the host.

Previous studies which aimed at identifying potential factors for *M. leprae*-specific unresponsiveness in LL used the addition of IL-2 [2,41–43] or anti-DQ monoclonal antibodies [44] or offered isolated antigenic fractions of *M. leprae*. Interestingly, each of the studies similarly identified two groups of LL patients, in one of which *M. leprae* unresponsiveness could be reversed. This indicated that the unresponsive phenotype in LL patients is likely mediated through the collective effects of various molecules. The more recent observation of cholesterol-dependent dismantling of HLA-DR raft and an increased membrane fluidity in BL/LL patients which causes a major defect in antigen presentation provides additional evidence for the presence of multiple different factors leading to T-cell anergy [33]. Thus, *M. leprae* specific unresponsiveness/anergy in LL patients very likely is a complex phenomenon mediated by multiple host and pathogen associated factors, one of which is represented by T_{reg} cells.

Several studies have reported on the *ex vivo* frequency of T_{reg} cells in peripheral blood of LL and TT/BT patients in unstimulated or *M. leprae* antigens stimulated PBMC [23,35]. Attia *et al*. showed, elevated frequencies of circulating T_{reg} cells ($CD4^+CD25^{high}FoxP3^+$) in TT patients [35] whereas Palermo *et al.,* showed that PBMC stimulated with *M. leprae* antigen for 6 days in culture had significantly higher number of T_{reg} cells ($CD4^+$ $CD25^+FoxP3^+$) in LL patients [23]. Recently, Saini *et al.,* further confirmed the importance of Tregs in LL non-

responsiveness by measuring TGF-β producing $CD4^+$ $CD25^+FoxP3^+$ cells in stimulated PBMC culture [45]. In this study, we analysed the frequency of T_{reg} cells in PBMC briefly activated with PMA/ionomycin. The frequency of $CD4^+$ $CD25^+FoxP3^+$ cells was higher in LL compared to BT but not statistically significant (Fig 3). However, with the visible difference observed between LL and BT and with the evidences from previous studies, their presence and role in BL/LL patients cannot be denied. For example, the recent molecular analysis of FoxP3 in $CD4^+CD25^+$ T cells nuclei has revealed that the FoxP3 interaction with histone deacetylases drives the immune suppression by $CD4^+$ $CD25^+$ T_{regs} in BL/LL unlike in other forms of leprosy [24].

On the other hand, the frequency of $CD8^+$ $CD25^+FoxP3^+$ cells found in this study was significantly higher in LL (Fig 3). This suggests that $FoxP3^+$ $CD8^+$ $CD25^+$ T_{reg} cells may also play a role in unresponsiveness in LL although not specifically analyzed for their functional role in our depletion experiments. Although lower in frequency compared to the $CD4^+$ $CD25^+FoxP3^+$, Saini *et al.,* also reported higher numbers of $CD8^+$ $CD25^+FoxP3^+$ in LL compared to BT but without induction of TGF-β [45]. Most studies focused on $CD4^+$ $CD25^+FoxP3^+$ in leprosy [23,35]. In contrast one study on LL lesions showed the presence of increased numbers of $CD8^+$ T cells with suppressive type in LL indicating the importance of $CD8^+$ T_{reg} cells in leprosy [46]. In addition few other studies identified $CD8^+$ Treg as a potential suppressive sub-population [47,48]. Recent evidence from an *in vitro* study also revealed $CD8^+$ T_{reg} cells ($CD8^+$ $LAG\text{-}3^+$ $FoxP3^+CTLA\text{-}4^+$) induced by matured plasmacytoid dendritic cells (pDC) with suppression activity on allo-reactive T memory cells [49]. In our opinion, the $CD8^+$ Treg population is not sufficiently studied in leprosy and we believe further analysis of this population in all forms of leprosy in periphery and lesionary tissues will be vital.

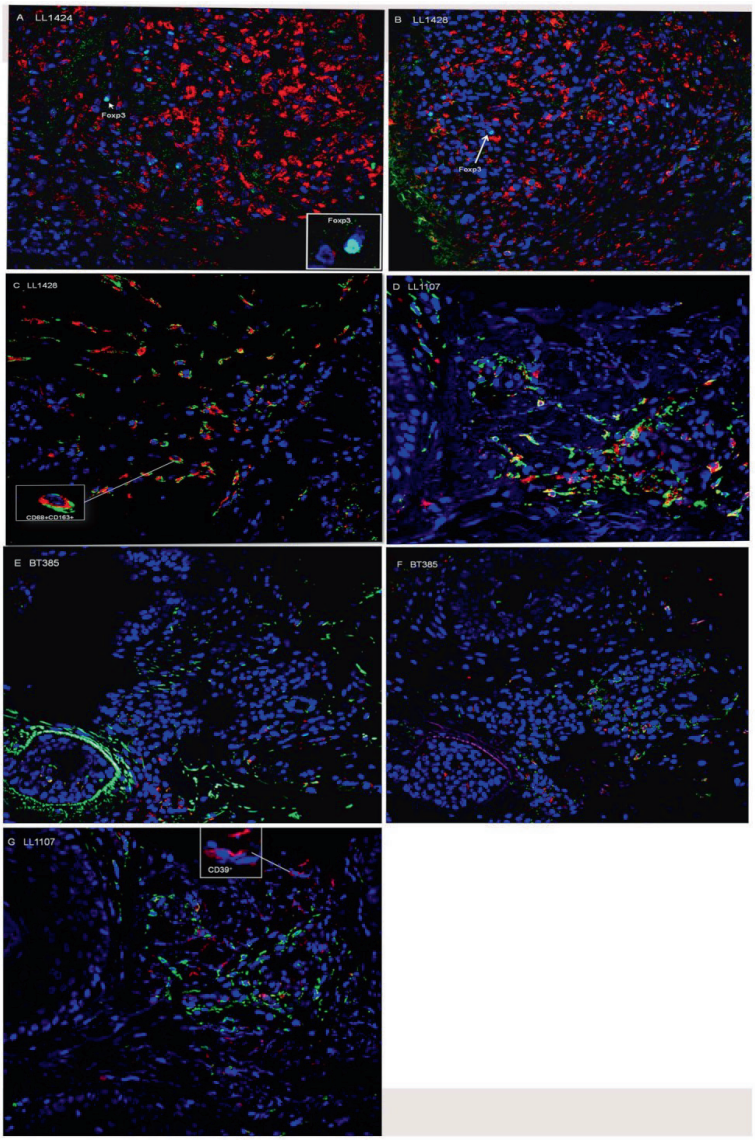

Figure 5. Immunohistochemical analysis (Original magnification, 400x; image size 359μm x 359 μm) of skin lesions. Sequential skin sections from LL (n = 10) and BT (n = 4) patients were stained with mAb specific for CD68 (red) and FoxP3 (green) [**A**, **B, E, F**], for CD68 (red) and CD163 (green) **[C**, **D]** and CD39 (red) [**G**]. Representatives LL [**A, B, C, D, and G**] and BT [**E, F,**] patients are shown.
Insets represent 1500x magnification of FoxP3^{+} cells [**A**, **B**]; 800x magnification of CD68^{+} CD163^{+} [**C**, **D]**; 1000x magnification of CD39^{+} cells [**G**].

The low IL-10 frequency measured by FACS analysis in all groups did not allow detection of significant differences among groups as expected in view of the crucial role of IL-10 as an anti-inflammatory cytokine in the unresponsiveness in LL patients [27,36]. This could be due to the short PMA/ionomycin stimulation inherent to the procedure for *ex vivo* determination

of the frequency of $CD25^+$ cells. However, 6 days stimulation of PBMC from BL patients with *M. leprae* induced high levels of IL-10 [50].

Although, it will not be easy to generalize or conclude on frequencies and numbers of $CD4^+$ $CD25^+FoxP3^+$ T_{reg} cells in different forms of leprosy since the experimental procedures used in each study vary, most of the studies including ours, point to the presence of increased numbers of T_{reg} cells in LL patients either in periphery as well as lesions. Detailed characterization of T_{reg} cell subsets in large cohorts of leprosy patients as well as the ratio to effector T cells may provide additional insights in this area.

The dominant presence of $CD163^+$ macrophages in LL lesions [27,28] and the significantly higher expression of IL-10 and CTLA4 in LL tissues have been reported previously [25]. The role of T_{reg} cells ($FoxP3^+$ $GITR^+$ $CD25^+$) and their induction by $CD163^+$ anti-inflammatory human macrophages was demonstrated *in vitro* since $CD4^+$ T-cells gained a potent regulatory/suppressor phenotype and functions after activation by mφ2 [25]. In the current study, we show the presence of significantly higher number of $CD68^+$ $CD163^+$cells (mφ2) in the vicinity of $FoxP3^+$ cells in LL lesions compared to TT/BT lesions. These findings support the involvement of both cell types in the induction and/or maintenance of *M. leprae* directed T_{reg} cells in LL lesions.

Since a suppressive effect of $CD4^+CD39^+FoxP3^+$ T_{reg} cells was described in TB patients [12], we also analysed the frequency of $CD39^+FoxP3^+$ cells in PBMC but observed no differences between LL and TT/BT patients except for few LL skin lesions, in which macrophage-shaped $CD39^+$ cells were observed. A recent study has shown that CD39 expression on macrophages has an important role in self-regulation mechanism during inflammation [51]. These cells may also play a similar role in LL patients but this has to be further analysed.

In summary, this study clearly show that $CD25^+$ T_{reg} cells play a role in unresponsiveness in LL, and that there are two subtypes of *M. leprae* unresponsive LL patients. Furthermore, the co-existence of T_{reg} cells with mφ2 in LL lesions further supports the potential role of these regulatory cell subsets at the site of infection.

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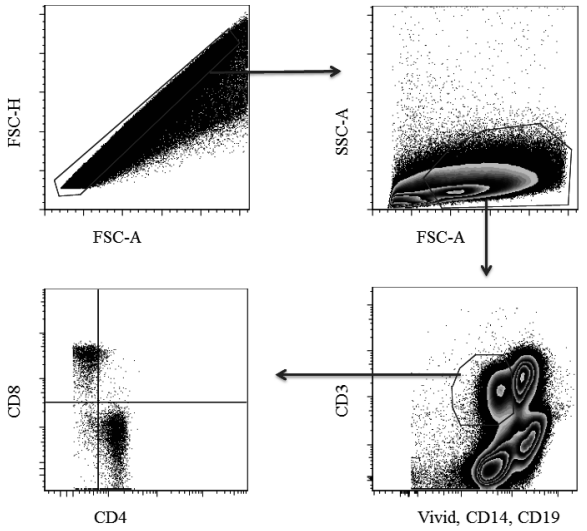
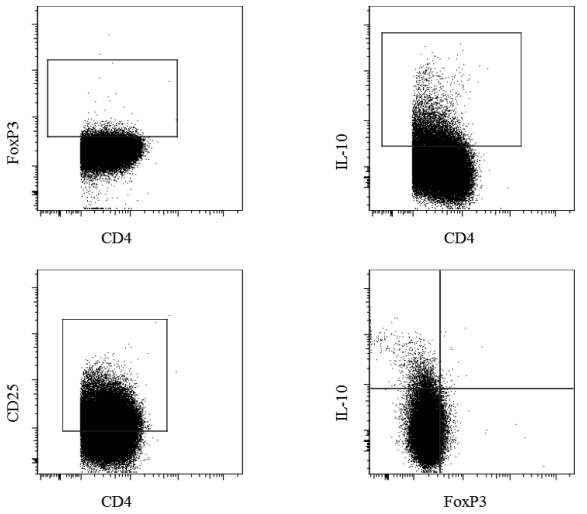
Supplementary File 1A. Gating strategy for live $CD4^+CD3^+$ cells or $CD8^+CD3^+$ cells in PBMC



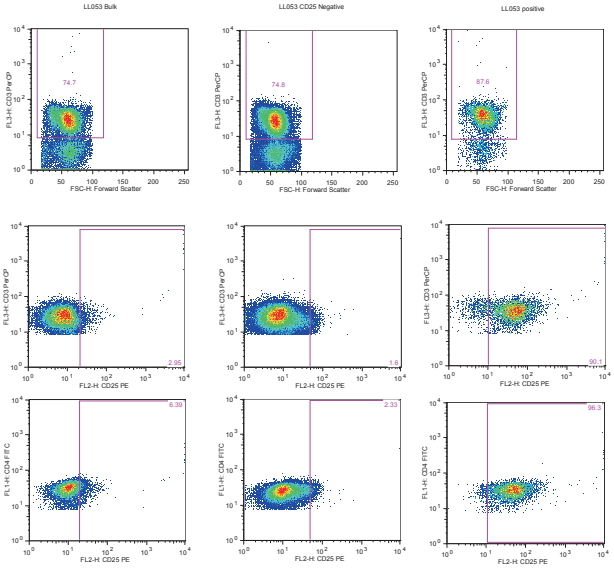
Sup. Fig **S1A.** Ungated events were first gated using a forward scatter area (FSC-A) versus height (FSC-H) plot to remove doublets. Subsequently, the events were subjected to a lymphocyte gate by gated through a side scatter (SSC). Subsequently, live $CD3^+$cells were gated by live/dead staining using Vivid (Invitrogen, Life technologies) as a marker for viability and $CD14^+$ or $CD19^+$ events were excluded from analysis using a dump channel. Finally, $CD3^+$ live cells were separated into $CD4^+$ and $CD8^+$.

Supplementary File 1B. Gating strategy for IL-10 and FoxP3 expression in $CD4^+CD3^+$ cells or $CD8^+CD3^+$ cells



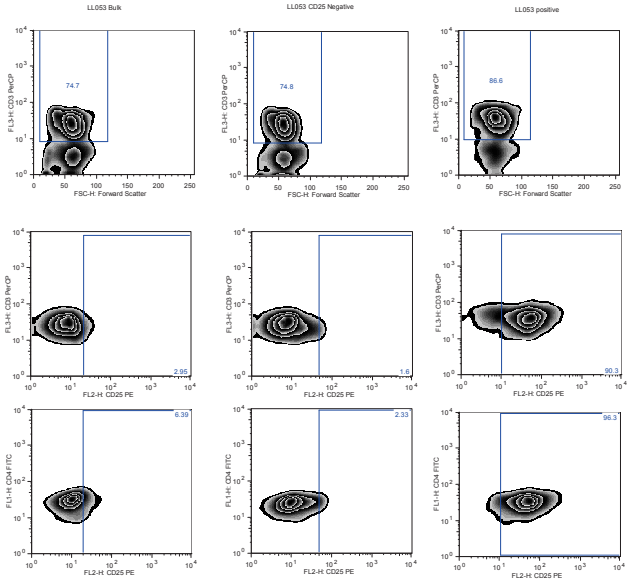
Supp. Fig. S1B After the gates for each function were created, we used the Boolean gate platform to identify all functions within each cell population using the full array of possible combinations FACS LSR Fortessa as shown here for IL-10 and FoxP3 expression in $CD4^+$ T cells.

Supplementary File 2A. Dot plot analysis of bulk (total) PBMC, CD25 depleted and CD25 positive population of a representative LL patient (LL053).



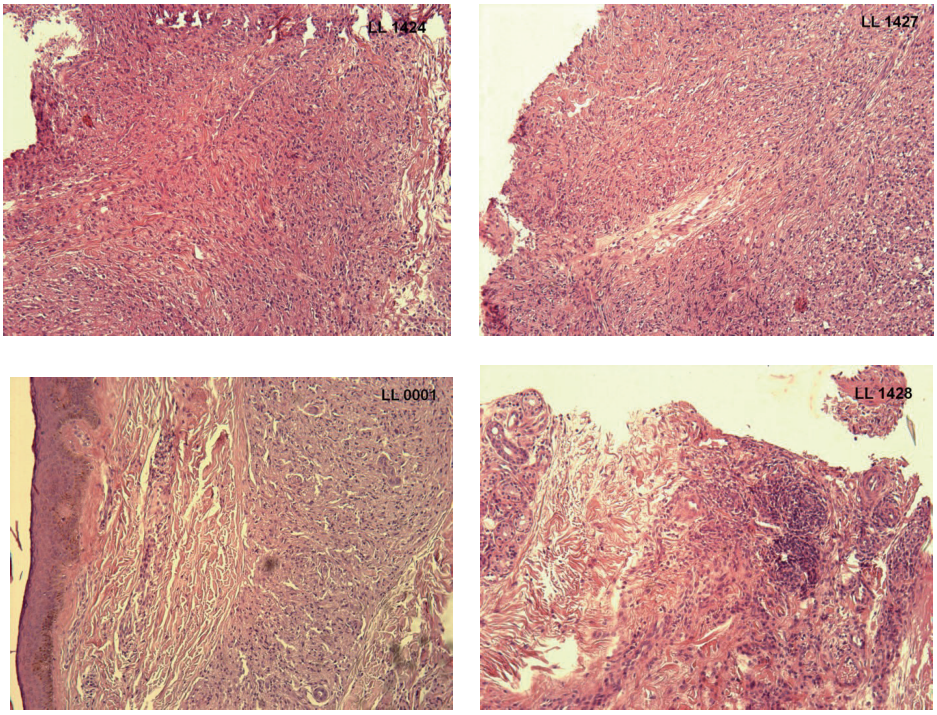
Supp. Fig S2A.After separating the CD25 negative and CD25 positive cell population using Magnetic cell sorter, fractions of each cell population including the bulk (total) PBMC were analysed for their expression of CD3, CD4 and CD25. Here the data are presented in dot plots.

Supplementary File 2B. Zebra plots of bulk (total) PBMC, CD25 depleted and CD25 positive population of a representative LL patient (LL053).



Supp. Fig. S2B. After separating the CD25 negative and CD25 positive cell population using Magnetic cell sorter, fractions of each cell population including the bulk (total) PBMC were analysed for their expression of CD3, CD4 and CD25. Here the data are presented in zebra plots.

Supplementary File 3. Hematoxylin and Eosin staining of four representative LL patients (original magnification X100).



Supp. Fig. S3. Tissue sections from paraffin embedded biopsy samples of leprosy patients were stained for H&E. Here images of H&E staining of four representative LL patients are presented.

Chapter 7

Immune responses in Ethiopian leprosy patients coinfected with HIV or helminthes

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Abstract

The host immune response against *Mycobacterium leprae* (*M. leprae*) determines the type and severity of the disease. Early detection of leprosy and asymptomatic *M. leprae* infection is key to reducing transmission. We have developed diagnostic tools based on cellular immune responses to *M. leprae* antigens. However, coinfections with HIV or helminths may reduce the host immune response to *M. leprae* thereby possibly hampering diagnosis of infected cases in tests based on anti-mycobacterial cellular immunity. This study characterized the immune profiles of HIV- or helminth coinfected leprosy patients in order to estimate the effect of coinfection in immunodiagnostic tools. Production of selected host biomarkers from peripheral blood mononuclear cells (PBMC) stimulated with *M. leprae* whole cell sonicate (WCS) and mRNA expression level of 76 genes was measured.
Similar T helper 1 (Th1) and Th2 responses were measured in WCS stimulated PBMC of leprosy patients with HIV coinfection and matched leprosy patients without HIV infection. In non-reaction BL/LL patients with and without helminth infection, the IFN-γ production was similar in both groups but the reductive effect of helminth coinfection in patients with T1R suggests the interference of helminth driven Th2 responses.
mRNA expressions of IL15 (p=0.0001), CTLA4 (p=0.003) and TLR10 (CD290) (p=0.0001) were significantly higher in HIV coinfected patients than in non-HIV patients, whilst ZNF532 expression was significantly lower (p =0.002). In BL/LL patients with and without helminth coinfection, similar mRNA expressions were observed for all 76 genes tested.
In summary, the similarity in immune responses in leprosy patients with and without HIV and helminth coinfections allows the use of identified immune- and transcriptomics biomarkers in diagnostic tests irrespective of patients' coinfection status. However, in patients with reactions, helminth infections may mask the severity of the reaction in Th1-based diagnostic tests, which warrants further investigations in larger/ longitudinal cohorts.

Author Summary

Early detection of leprosy is the main strategy to reduce transmission. Recently, we have developed diagnostic tools based on cellular immune responses of the host to *M. leprae* antigens. HIV or helminth coinfections in leprosy patients may interfere and hamper the diagnostic potential of these new tools. Therefore, in this study, the immune profiles of coinfected leprosy patients were characterized and compared with non coinfected leprosy patients to estimate the effect of coinfection in immunodiagnostic tools.

Similar host immune responses were measured in both HIV co-infected and non-coinfected leprosy patients. The IFN-γ production in helminth co-infected patients without T1R (type 1 reaction) was similar with that of non coinfected patients. However, a reduced IFN-γ production was measured in helminth coinfected patients with T1R.

mRNA expressions for majority of the genes were similar except for a few genes namely IL15 CTLA4 and TLR10 (CD290) which were found significantly higher in HIV coinfected patients and ZNF532 expression was significantly lower. In BL/LL patients with and without helminth coinfection, similar mRNA expressions were observed for all genes tested.

In summary, the similar immune responses shown in this study for both groups allows the use of identified host biomarkers in diagnostic tests irrespective of patients' coinfection status. However, further investigations in larger/ longitudinal cohorts are warranted in helminth coinfected patients with reaction.

Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*) and about 200,000 new cases of leprosy are reported by the WHO every year [1]. The disease manifestation has a wide spectrum which ranges from the tuberculoid form with high cell mediated immunity (CMI) and low number of bacilli to the lepromatous form with poor CMI and high bacillary load [2]. Host immunity determines the clinical manifestation after infection with *M. leprae*. However, coinfections may interfere with host immunity, thereby determining clinical manifestations either by up regulating or down regulating the different arms of immunity [3;4].

Over the last three decades, HIV has killed millions through increasing susceptibility of infected people to many opportunistic infections. Mycobacterial infections are among these opportunistic threats, and tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*) is the leading cause of death among HIV infected individuals [5;6]. After initiation of anti retroviral therapy (ART), diseases like TB may manifest as a result of immune reconstitution inflammatory syndrome (IRIS) which is an exaggerated immune reaction against re-current or unrecognized sub-clinical infections [7].

Similarly, increased manifestation of leprosy, especially of the lepromatous form, was predicted in HIV infected patients taking ART [8;9]. In contrast to the expectations, the impact of HIV has not worsened the leprosy situation, although some patients on ART are being diagnosed as new leprosy cases [10]. Few studies have analysed some clinical, immunological and pathological parameters [11-14]. Most of these features in *M. leprae*-HIV coinfected patients were reported to be similar compared to patients with one infection, indicating that each disease progresses independently [15].

The occurrence of type 1 leprosy reactions (T1R) in association with anti-retroviral therapy (ART) initiation was reported and is often considered as immune reconstitution inflammatory syndrome (IRIS) in *M. leprae*-HIV coinfected patients on ART [11;14;16-20]. In a non-HIV leprosy patient with T1R, our group has previously shown increased expression of cytotoxicity-associated genes granzyme A (GZMA), granzyme B (GZMB) and perforin 1 (PRF1) [21]. In *M. leprae*-HIV coinfected patients, increased frequency of $CD8^+$ T cells was also reported as a potential triggering factor for the occurrence of T1R along with increased production of PRF and GZMB compared to non-HIV leprosy patients [22]. Therefore, in this study, we compared the mRNA expressions of these and other related genes in both groups of patients.

Coinfection with intestinal parasites, mainly helminthic infections are known to elicit immune modulation characterized by up-regulating Th2 responses in the infected host [23-28]. In mycobacterial infections like TB, studies have shown the effect of helminth infestation through weakening Th1 immunity [29]. Moreover, poor immunogenicity induced by BCG vaccination was also observed in helminth infested groups compared to de-wormed groups in an Ethiopian cohort [30;31]. There are also evidences for Omega-1 (with glycosylation and ribonuclease activity) secreted by *Schistosoma mansoni* eggs in conditioning dendritic cells in priming Th2 responses [32;33]. In addition, helminthic infections are common in HIV infected people [34]. The presence of intestinal helminths in leprosy patients may potentially facilitate the progression of *M. leprae* infection to more severe forms of leprosy [35]. Significant association of lepromatous leprosy (LL) with

helminths and higher production of Th2 type cytokines such as IL-4 and IL-10, were reported in coinfected patients [35;36].

Our group has selected specific *M. leprae* proteins and identified host biomarkers such as IFN-γ, IP-10, MIP-1β, MCP-1, IL-1β, IL-6 for detection of *M. leprae* infection and/ or exposure in different groups including leprosy patients, household contacts and endemic controls [37-40]. These biomarkers are currently applied in development of field friendly rapid diagnostic tests. Assessing the effect of coinfections on these biomarkers is essential for the interpretation of these tests in leprosy patients with coinfections.

In this study, we analysed immune responses to *M. leprae* antigens in HIV or helminth coinfected Ethiopian leprosy patients to assess the effect of these coinfections on host immune biomarkers.

Materials and Methods

Ethical statement. This study was performed according to the Helsinki Declaration (2008 revision). Ethical approval of the study protocol was obtained from the National Health Research Ethical Review committee, Ethiopia (NERC # RDHE/127-83/08). Participants were informed about the study objectives, the required amount and kind of samples and their right to refuse to take part or withdraw from the study at anytime without consequences to their treatment. Written and informed consent was obtained from study participants before enrollment. Pre- and post counseling for HIV testing was performed by the recruiting nurse and patients identified as HIV-positive were referred to ALERT ART clinic.
Study participants. During 2009 and 2012 *M. leprae*-HIV coinfected patients (n=21) and leprosy patients without HIV (n=256) were enrolled in this study. Leprosy was diagnosed based on clinical, bacteriological and histological observations and classified by a skin biopsy evaluated according to the Ridley and Jopling classification [2] by qualified microbiologists and pathologists. All patients were enrolled before initiation of MDT antibiotic treatment for leprosy infection or steroid treatment for leprosy associated inflammation or reactions. or steroid. The HIV test was done using KHB (Shanghai kehua Bioengineering CO-Ltd, Shanghai, China) and if positive a second test was performed using STAT-PAK™ (Chembio HIV1/2, Medford, New York, USA). Stool samples were prepared using the direct stool smear (wet smear) protocol and examined under microscope within 30 min for the presence of ova, cysts and parasites. Patients with positive microscopic result were further categorized into patients with helminth and protozoan infection.
PBMC isolation, freezing and thawing. PBMC were isolated by density gradient method using Ficoll-paque, cells were washed and suspended in 20% fetal calf serum (FCS) in AIM-V (Invitrogen, Carlsbad, CA) and kept cool on ice, counted and frozen using a cold freshly prepared freezing medium composed of 20% FCS, 20% dimethyl sulphoxide (DMSO) in AIM-V. Cells were kept at -80 °C for 2-3 days and transferred to liquid nitrogen until use. During thawing, cells were transported in liquid nitrogen to a water bath (37^{o}C) incubated for 30 to 40 seconds until thawed half way and resuspended in 10% FCS in AIM-V (37^{o}C) containing 1/10,000 benzonase until completely thawed, washed twice (5-7 minutes each) and counted.
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 kindly provided by Dr. J.S. Spencer 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/TBVTRMResearch Materials/tabid/1431/Default.aspx).

Lymphocyte stimulation tests (LST). PBMC (200,000 cells/well) were added in triplicate into 96 well U bottom tissue culture plates and cultured with *M. leprae* whole cell sonicate (WCS; 10 μg/ml), phytohaemagglutinin (PHA; 1 μg/ml) or AIM-V medium at 37°C with 5% CO_2 and 70% humidity. After 6 days, supernatants were collected and kept frozen until used in ELISA.

IFN-γ ELISA. IFN-γ levels were determined by ELISA (U-CyTech, Utrecht, The Netherlands) [41]. The cut-off value to define positive responses was set beforehand at100 pg/ml. The assay sensitivity level was 40 pg/ml. Values for unstimulated cell cultures were typically < 40 pg/ml.

Multiple cytokine and chemokine assays. The concentrations of 12 analytes (IL-1β, IL-10, IL-12p70, IL-17, IFN-γ, IP-10 (CXCL10), G-CSF, GM-CSF, MCP-1 (CCL2), MIP-1β (CCL4), VEGF and TNF) in supernatants from 6 days LST 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). The assay protocol described in Bobosha, K et al., 2012 was followed [42].

PGL-I ELISA. IgM antibodies against *M. leprae* PGL-I were detected with natural disaccharide of PGL-I linked to BSA (ND-O-BSA (0.01 ng/ well) provided through the NIH/NIAID Leprosy Contract N01-AI-25469) as previously described [43]. Serum dilutions (100 μl/ well; 1:300) were incubated at 37°C for 90 min in flat-bottomed microtiter plates (Nunc) coated with NDO-BSA. After washing diluted enzyme linked secondary antibody solution (100 μl/ well) was added to all wells and incubated at 37°C for 30 min. After washing diluted TMB solution (100 μl/ well) was added to all wells and incubated in the dark for 15 min at RT. The reaction was stopped by adding 100 μl/ well 0.5 N H_2SO_4. Absorbance was determined at wavelength of 450 nm. Samples with a net optical density at 450 nm (OD) above 0.199 were considered positive. The ELISA performance was monitored using a positive and negative control serum sample on each plate.

PAXgene whole blood RNA isolation. PAXgene tubes were stored at -80°C and shipped to LUMC. Total RNA from venepuncture PAXgene blood collection tubes was extracted and purified using the PAXgene Blood RNA kit (BD Biosciences) including on-column DNase digestion according to the manufacturers' protocol. The RNA yield from 2.5 ml of whole blood was determined by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and ranged from 4.2 to 8.5 μg of total RNA (average 6.02 ± 1.5 μg) with an average $OD_{260/280}$ ratio of 2.0 ± 0.04.

Dual color Reverse Transcription Multiplex Ligation-dependent Probe Amplification (dcRT-MLPA) assays. dcRT-MLPA assay was performed as described previously [44]. Briefly, for each target-specific sequence, a specific RT primer was designed that is complementary to the RNA sequence and located immediately downstream of the probe target sequence. Half-probes consisted of chemically synthesized oligonucleotides and right hand half-probes were 5' phosphorylated to facilitate ligation. As a positive control, chemically synthesized oligonucleotides were used that were complementary to the RNA sequence and encompassed the combined target-specific sequences of the left and right hand half-probes. Primers and probes were purchased from Sigma-Aldrig Chemie (Zwijndrecht, The Netherlands) and MLPA reagents from MRC Holland (Amsterdam, The Netherlands). To avoid detection of contaminating DNA fragments, all target sequences have an exon boundary near the probe ligation site. Also, splice variants and SNPs present in the mRNA were taken into account. Trace data were analyzed using GeneMapper software package (Applied Biosystems, Warrington, UK). The areas of each assigned peak (in arbitrary units)

were exported for further analysis in Microsoft Excel spreadsheet software. Signals below the threshold value for noise cut-off in GeneMapper (log2 transformed peak area ≤ 7.64) were assigned the threshold value for noise cut-off. Results from target genes were calculated relative to the average signal of the reference gene, *GAPDH*, present within the gene sets. Following normalization of the data, signals below the threshold value for noise cut-off (peak area ≤ 7.64) were again assigned the threshold value for noise cut-off. To monitor assay performance, a negative control (without RNA), a positive control (using synthetic template oligonucleotides as hybridization templates) and a commercial Human Universal Reference RNA (Clontech, Palo Alto, CA, USA) were included on each 96-well plate. dcRT-MLPA experiments for RNA samples of all time points were performed simultaneously.

Statistical analysis. Data were analyzed 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). The statistical significance level used was $p<0.05$.

Results

T1R in *M. leprae*-HIV coinfected patients

to assess the effect of HIV coinfection, we recruited, 10 male and 10 female HIV coinfected leprosy patients with age range of 18 to 50 including 16 BB/BL/LL patients and 4 BT. The BI ranged from 0 to +4 (Table 1).

Table 1. *M. leprae*-HIV coinfected patients' demographic and clinical status

	Lep-HIV Coinfected	Sex	Age	Rxn	Status	BI	PGL-I (OD_{450})	ART status*	CD4 count
1	Lep HIV 002	M	39	T1R	BT	0	0.02	ART	242
2	Lep HIV 011	F	23	T1R	BT	0	0.018	ART	90
3	Lep HIV 008	F	18	T1R	BT	0	0.103	Not received	581
4	Lep HIV 001	M	40	T1R	BB	0	0.726	ART	NA
5	Lep HIV 006	F	35	T1R	BB	0	0.2	ART	21
6	Lep HIV 018	F	50	T1R	BB	0	0.027	Not received	247
7	Lep HIV 004	M	25	T1R	BL	0	0.096	ART/IRIS	206
8	Lep HIV 009	M	36	T1R	BL	0	0.068	ART	86
9	Lep HIV 012	F	30	T1R	BL	0	0.599	ART	332
10	Lep HIV 014	M	38	T1R	BL	0	0.981	Not received	359
11	Lep HIV 016	M	28	T1R	BL	0	0.08	Not received	216
1	Lep HIV 017	F	39	T1R	BL	0	0.042	Not received	218
13	Lep HIV 019	F	26	T1R	BL	2	2.183	ART	216
14	Lep HIV 020	M	30	T1R	BL	0	0	ART	144
15	Lep HIV 007	F	30	T2R	BL	0	0.612	ART	109
16	Lep HIV 003	M	30	T2R	LL	3	0.091	Not received	238
17	Lep HIV 025	M	38	T2R	LL	2	1.375	Not received	375
18	Lep HIV 015	M	34	No rxn	BT	0	0.026	Not received	565
19	Lep HIV 021	F	40	No rxn	LL	4	ND	Not received	59
20	Lep HIV 005	F	24	No rxn	LL	3.3	1.375	ART	425

Rxn: leprosy reaction; T1R: type 1 reaction; T2R: type 2 reaction

*: at diagnosis of leprosy

The patients who were referred from the ART clinic at ALERT were all on ART (11/20), whereas the other patients who were first diagnosed for leprosy had not received ART (9/20). T1R was diagnosed in 14 out of 20 (70 %) and T2R in 3 out of 20 (15 %) (LL= 2 and BL= 1). Only 3 patients did not have reactions (LL=2 and BT=1) at the time of leprosy diagnosis. Among the HIV coinfected patients on ART; 9/11 (69.2%), and among those naive to ART 5/9 (30.7%) had a clinical T1R. The CD4 count ranges from 21 to 425 cells/µl in patients on ART and ranges from 59-581 cells/ul in patients naïve to ART.

Similar cytokine responses in non-HIV and HIV coinfected leprosy patients

To assess the influence of HIV infection on recently identified potential biomarkers for early diagnosis, cytokine/chemokine responses against *M. leprae* WCS in both HIV uninfected and HIV coinfected leprosy patients were analysed. As shown in Figure 1, the IFN-γ responses to *M. leprae* WCS measured by ELISA in both groups were found similar.

The IFN-γ responses to PHA in both groups were higher in general compared to responses to *M. leprae* WCS. Also, the IFN-γ response in non-HIV leprosy patients to PHA was significantly, though slightly, higher (p =0.045) compared to that in those infected with HIV (Figure 1).

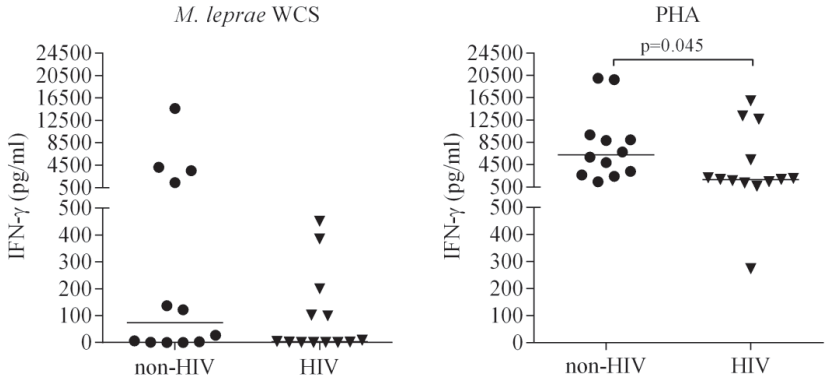


Fig 1: IFN-γ responses of PBMC from HIV coinfected (n=13) and non-coinfected leprosy patients (n=13) stimulated with *M. leprae* Whole Cell Sonicate (WCS) and PHA positive control in 6 days culture.

Similarly, the 11 analytes (IL-1β, TNF, IL-12p70, IL-17, IL-10, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1α, G-CSF, GM-CSF and VEGF) measured in supernatants of 6 days PBMC culture were also found similar in both groups (Figure 2) affirming the possibility to use the previously identified host biomarkers as diagnostic markers in non-HIV as well as HIV coinfected individuals.

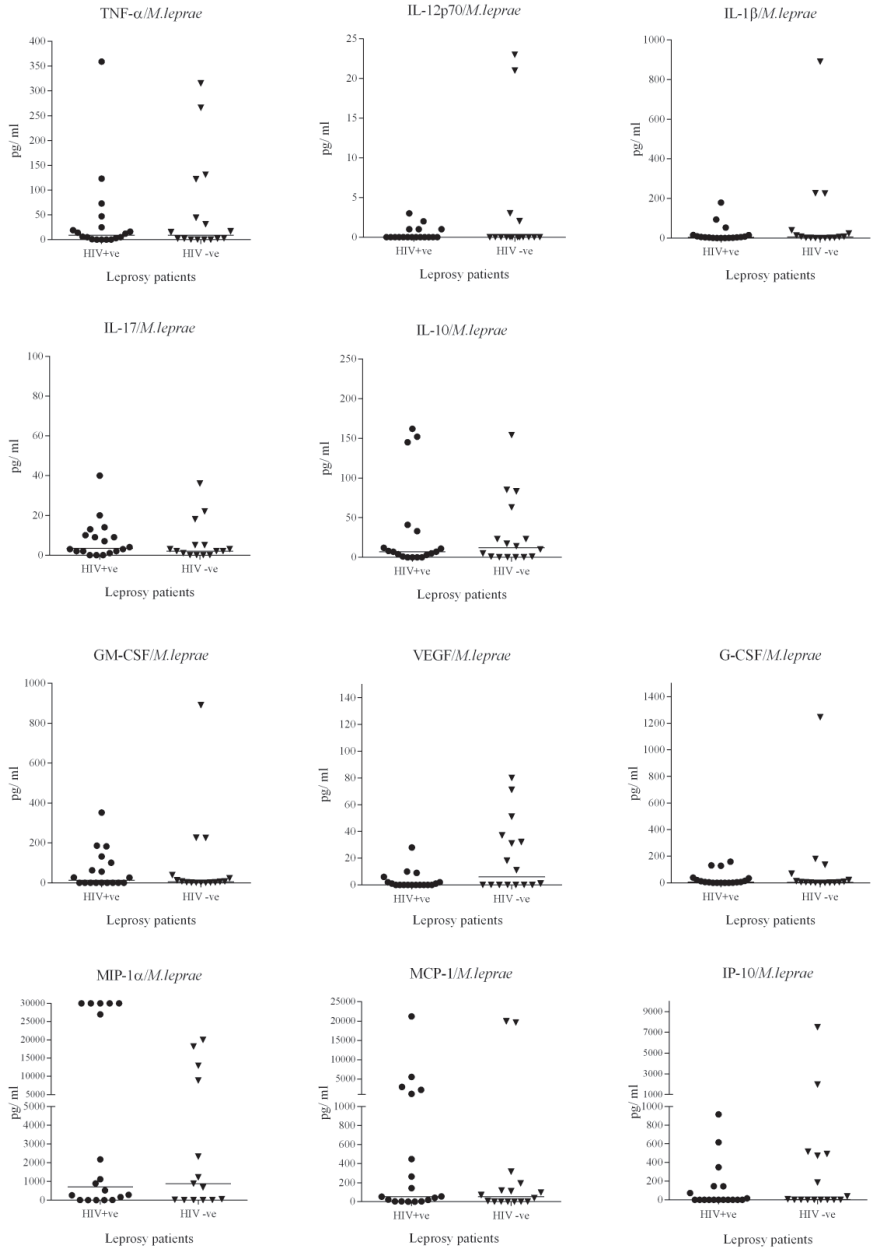


Fig 2: Multiple cytokine and chemokine responses of PBMC of HIV coinfected (n=18) and non-coinfected leprosy patients (n=15) stimulated with *M. leprae* Whole Cell Sonicate (WCS).

Increased mRNA expression of IL15 and CTLA4 in HIV coinfected leprosy patients

mRNA expression of 76 target genes involved in innate and adaptive immunity or associated with leprosy [21] were analysed in both HIV-coinfected (n=20) and HIV-uninfected leprosy patients (n=20). Only a few genes showed significant differences between both groups of

leprosy patients. Significantly higher CD4 (p <0.0001) and CD8 (p <0.002) mRNA expression levels were measured in non-HIV and HIV coinfected respectively (Figure 3). The expressions of IL15 (p =0.0001), CTLA4 (p =0.003) and TLR10 (CD290) (p =0.0001) were found significantly higher in HIV coinfected leprosy patients (Figure 3). In contrast, ZNF532 mRNA expression was found significantly higher (p =0.002) in non-HIV patients. The expression of all other tested genes including IL1β, CCL4, CCR7 and VDR did not show significant differences between the two groups.

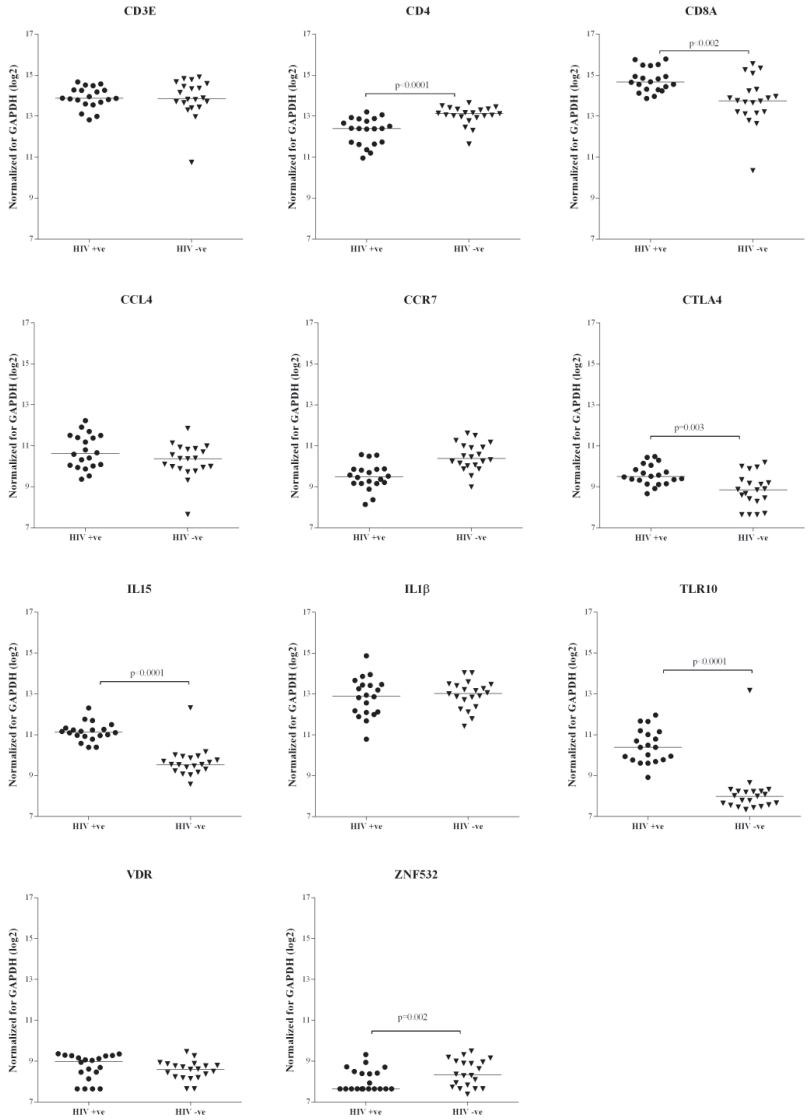


Fig 3: mRNA expression of 11 target genes in peripheral blood of HIV coinfected (n=20) and non-coinfected leprosy patients (n=20).

Comparable frequency of helminth coinfection in MB and PB leprosy patients

Leprosy patients were recruited consecutively at ALERT hospital and tested for helminth infestation where 218 BL/LL and 38 BT patients were included from 2009 – 2013. Among these patients, 54 out of 218 BL/LL patients (24.7%) and 6 out of 38 (15.7%) BT patients were found coinfected with parasites; helminths (51.6%) and protozoans (48.3%) (Table 2).

Table 2. Type and distribution of parasites in leprosy patients

Stool result	# of patients	%	Sex ratio (M/F)	Age range (yrs)	Type of leprosy	Reaction (No/Yes)
Cyst of *Giardia lamblia*	8	3.13	5/3	18-55	BL/LL	4/4
Cyst of *Giardia lamblia* and *Entamoeba histolytica*	1	0.39	1/0	33	BL	1/0
Cyst of *Entamoeba histolytica*	9	3.50	6/3	24-46	BL/LL	5/4
Trophozoites of *Giardia lamblia*	7	2.72	6/1	20-55	BL/LL (5) BT (2)	4/3
Trophozoites of *Giardia lamblia* and *Entamoeba histolytica*	1	0.39	1/0	18	LL	1/0
Trophozoites of *Entamoeba histolytica*	3	1.17	2/1	29-40	BL/LL	1/2
Larvae of *Strongyloides stercolaris*	4	1.56	4/0	22-28	BL/LL	1/3
Ova of *Ascaris lumbricoides*	10	3.89	7/3	18-47	BL/LL	7/3
Ova of *Enterobius vermicularis*	1	0.39	1/0	31	BL	1/0
Ova of hookworm	10	3.89	8/2	18-46	BL/LL (6) BT (4)	6/4
Ova of *Hymenolepis nana*	1	0.39	0/1	21	BL	1/0
Ova of *Taenia Spp*	2	0.78	1/1	25-29	BL/LL	0/2
Ova of *Trichuris trichiura*	1	0.39	0/1	18	LL	1/0
Ova of *Trichuris trichiura* and *Ascaris lumbricoides*	2	0.78	2/0	25	LL	2/0
No parasites	196	76.26	133/63	18-65	BL/LL (164) BT (32)	97/89
Total	256					

Protozoans	29	48.3
Helminths	31	51.6

Parasites	60	23.35
No parasites	196	76.26
	256	100

M/F: Male –Female ratio; BT: Borderline tuberculoid; BL: Borderline lepromatous

The most frequent protozoans were *Giardia lamblia* and *Entamoeba histolytica* and the most frequent helminths were *Ascaris lumbricoides* and hookworm. Helminth infected BL/LL patients were 27 out of 218 (12.4%) and BT were 4 out of 38 (10.5%).

Helminth coinfection suppresses the Th1 response in leprosy patients with T1R

As shown in Figure 4, the IFN-γ (hallmark cytokine for Th1 immunity) production in non-reactional BL/LL patients with and without helminths in response to *M. leprae* WCS were low (less than 100 pg/ml) and no difference was observed between the BL/LL patients with and without helminths. However, when PBMC of BL/LL patients (n=4) with T1R not treated with steroids were stimulated with *M. leprae* WCS, patients free of helminth infestation showed significantly higher (p=0.028) IFN-γ responses than those coinfected with helminths (n=4).

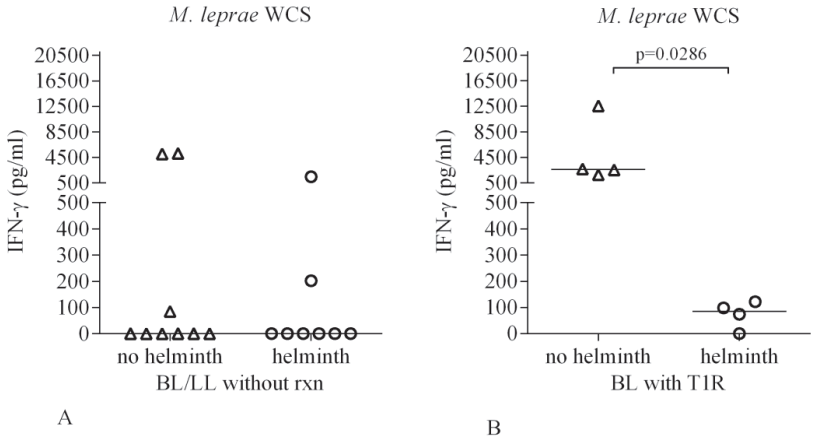
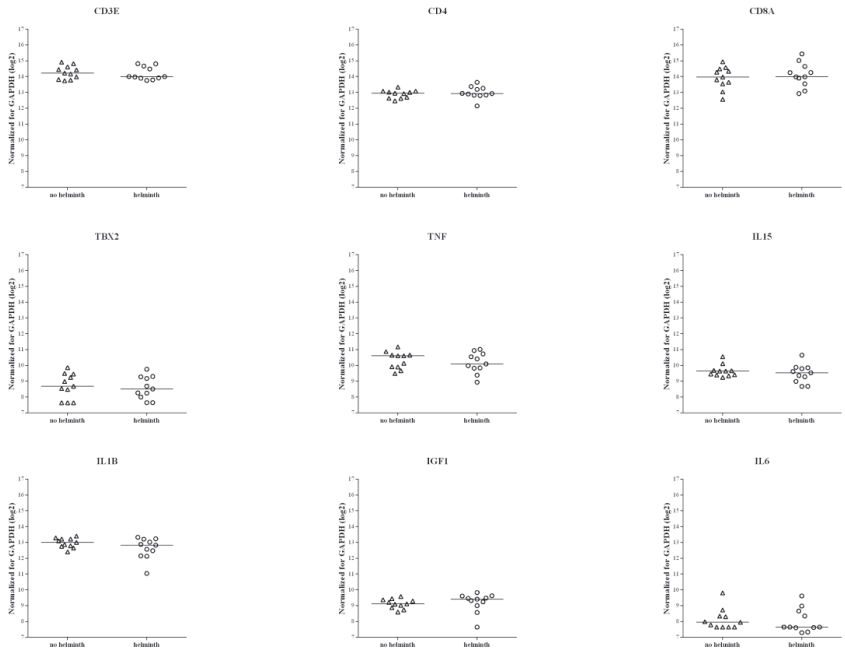


Fig 4: IFN-γ responses of PBMC of helminth coinfected and non-coinfected leprosy. Patients stimulated with *M. leprae* Whole Cell Sonicate (WCS) in 6 days culture A. patients without reaction: no helminth n=9 and helminth infested n=8 B. patients with T1R: no helminth n=4 and helminth infested n=4

Similar mRNA expressions of leprosy associated genes in patients with and without helminth

The mRNA expression of 76 genes associated with innate and adaptive immunity were assessed using dcRT-MLPA [41] in whole venous blood of BL/LL patients without helminth (n=11) and coinfected with helminth (n=11). The mRNA expressions in both groups were found similar. In figure 5, the mRNA expression levels for a selection of 27 genes are shown.



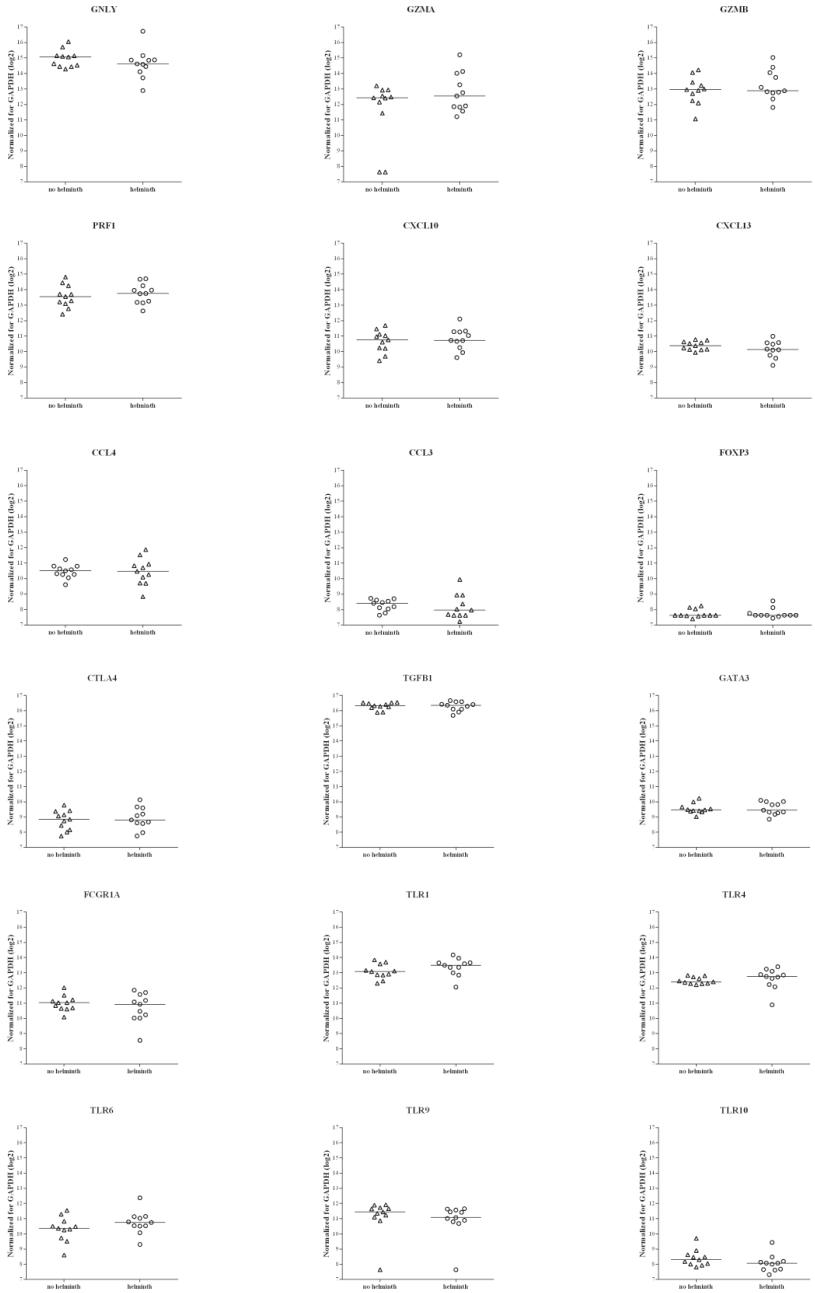


Fig 5: mRNA expression of 27 target genes in peripheral blood of BL/LL patients without helminths (n=11) and with helminths (n=11).

Discussion

Coinfections in leprosy have not been studied intensively although some reports have shown coinfections as risk factors for leprosy reactions (both type 1 and 2) [45;46]. Reports on the occurrence of TB in HIV patients who are naïve to ART as a result of diminished $CD4^+$ T cells [47;48] and in those who are on ART as IRIS [7] prompted the leprosy research community to evaluate HIV-infection in leprosy patients [13;17;18;49-51]. HIV coinfection was reported previously to occur in any form of leprosy [11] and a strong association of ART with the occurrence of T1R was shown in several studies [11;12;17;22] including this study. ART is known to restore $CD4^+$ T cell numbers but also drives an excessive and tissue destructive inflammation in some individuals [52]. This inflammatory environment may facilitate the occurrence of T1R in *M. leprae*-HIV coinfected patients. However, taking into consideration that T1R represents a common phenomenon that occurs in 30-50% of leprosy patients at any time before, during or after MDT, the interference or influence of ART needs to be investigated. In addition, studies on the interaction of various factors including ART, MDT, steroids, reactions, leprosy forms and various immune cells including T cells, dendritic cells and macrophages will certainly generate valuable information which could be used in management of coinfected patients in general and specifically those with T1R.

The clinical and histopathologcial features of leprosy in HIV coinfected patients did not differ from non-HIV leprosy patients in our study, as also evidenced by previous studies [15;49] except the above discussed ART and T1R association. No differences were observed in immune responses against *M. leprae* WCS as measured by different cytokines/chemokines suggesting the feasibility of the use of the newly identified diagnostic biomarkers in coinfected patients as well. Still, helminths-induced Th2 response may suppress stronger Th1, which could become evident upon treatment with anti-helminth prophylaxis. Therefore, further longitudinal studies are required to monitor the effect on anti-*M.leprae* responses effected by treatment.

Lower CD4:CD8 ratio [14] and an increased $CD8^+$ memory T cells in HIV coinfected patients with T1R [22] were reported previously. In our study, mRNA expression levels in whole blood of CD4 was significantly lower ($p<0.0001$), whereas CD8 expression was significantly higher ($p<0.002$) in HIV coinfected leprosy patients. In addition, IL15 and TLR10 were higher expressed in coinfected and ZNF532 was higher expressed in leprosy patients without HIV. IL-15 is known as a pleiotropic cytokine dominant in lesions of TT/BT patients [53;54]. Increased IL15 mRNA expression in coinfected patients may be associated with the Th1 dominated immune response as a result of ART and/or T1R. Toll like receptors (TLR) are important in triggering inflammatory and adaptive immune responses in a host invaded by pathogens. A recent study revealed that TLR10 shares microbial derived agonists of TLR1 and also requires TLR2 for innate immune recognition [55]. Although not particularly demonstrated for TLR10, there is evidence that HIV infected individuals in general show pro-inflammatory responsiveness to TLR agonists [56]. Therefore, the significantly increased TLR10 mRNA expression ($p =0.0001$) in the leprosy HIV coinfected patients demonstrated in this study, indicates its potential as a biomarker for HIV infection in leprosy patients. Zinc fingers in general are interaction modules that bind to different compounds including nucleic acids, proteins and small molecules [57]. There is insufficient information particularly on Zinc Finger 532 (ZNF532) function and the significantly higher mRNA expression ($p =0.002$) shown in non-HIV patients in this study calls for further investigation in this domain. There are few recent reports on Zinc finger nucleases, which are generated by fusing Zinc finger DNA-binding domain to a DNA cleavage as therapy for HIV.

Therefore, our finding on ZNF532 may have relevance in future diagnostic or therapeutic research in HIV.

On the other hand, in non-helminth and helminth coinfected leprosy patients, the comparable mRNA expressions may implicate the minimal interference of helminth but assessing the protein expression levels is warranted. Decreased Th1 responses in presence of helminths in leprosy patients was reported previously [35]. In this study, the majority of helminth coinfected and non-helminth leprosy patients were BL/LL patients and showed comparable Th1 responses because the cellular immune responses in both groups were low, in line with the general phenomenon in BL/LL patients. Importantly, however, we were able to show the effect of helminths in leprosy patients with T1R as their IFN-γ responses were significantly lower compared to non-helminthic leprosy patients with T1R. This suggested interference of helminth infestation and domination of helminth driven Th2 type response in patients with reaction as T1R generally induces a dominant Th1 type responses. Furthermore, since delay in clearance of bacilli is a major issue in BL/LL patients, the risk of helminth infection in further delaying the bacterial clearance and whether de-worming puts patients at risk of developing T1R have to be investigated in longitudinal studies to develop proper management algorithms of coinfected patients and potentially adapt diagnostic tests.

In general, characterization of host immune profiles in coinfected patients is relevant for the validation of newly developed diagnostic tools. In this study, the previously identified biomarkers for early diagnosis such as IP-10, CCL4 and CCL2 [58;59] were evaluated and comparable responses were observed in mono- and coinfected groups indicating the potential use of these biomarkers in both groups. Therefore, it is essential for future biomarker screenings or diagnostic tool development for leprosy to consistently include patients who are infected with other microbes besides *M. leprae* and thereby account for the interference of coinfections.

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Chapter 8

Summary and General Discussions

Summary

Globally more than 200,000 people develop leprosy every year and 2-3 million people live with leprosy associated disabilities. Despite the availability of efficient multi drug therapy, leprosy has continued affecting many individuals, including children because of the uninterrupted transmission in the population. Untreated MB cases as well as non-symptomatic *M. leprae* infected individuals in the population are believed to be the major sources of *M. leprae* infection and transmission. The currently available clinical and laboratory diagnostics methods have limitations for detection of PB patients and asymptomatic, *M. leprae* infected individuals at high risk of developing the disease. In addition, leprosy reactions are the major causes of disabilities and occur as a result of host immunological responses against whole bacilli and/or its antigens before, during or after treatment. However, no tools are available to predict leprosy reactions.

The availability of the whole genome sequence of *M. leprae* has opened the opportunity to understand the pathogen and the disease more than ever. *In silico* identification of unique *M. leprae* genes and production of the encoded recombinant proteins have broadened the possibilities to develop diagnostic tools, in particular for early detection of infection which eventually helps to reduce transmission.

In vitro assessment of recombinant *M. leprae* proteins and synthetic peptides for their immunogenicity and specificity in populations with different genetic backgrounds by measuring cell mediated immunity has shown the presence of potential antigens. Further in depth analysis of the host immune responses against these unique antigens in leprosy patients, their close household contacts and healthy endemic controls is of immense importance in development of new diagnostic tools. Therefore, field friendly tests for early detection are currently developed at the LUMC using through identification of *M. leprae* antigens and host biomarkers with diagnostic potential.

Thus, this thesis focuses on the selection and evaluation of immunogenic *M. leprae*unique proteins and peptides thereof as well as identification of potential host biomarkers for detection of *M. leprae* infection and early diagnosis of leprosy reactions.

General Discussion

In search of immunogenic, *M. leprae* unique antigens

The leprosy elimination goal will only be achieved if the ongoing transmission within the endemic populations is abrogated. Untreated MB cases as well as certain *M. leprae* infected individuals in the population are believed to be the major sources of *M. leprae* infection and transmission. Early detection of leprosy still remains the core strategy of WHO [1], leprosy control programs and researchers in the field. In addition, various strategies are designed to block transmission by identification of *M. leprae* infected individuals. In order to develop tests that allow detection of infection, about 200 candidate *M. leprae* antigens were screened for induction of cellular or humoral responses in leprosy endemic populations. These candidate antigens were selected based on analysis of the whole genome sequence of *M. leprae* [24] . The host immune response to *M. leprae,* characterized by both cell mediated immunity (CMI) and humoral mediated immunity (HMI) is the main factor that determines disease outcome in leprosy. PB patients mostly develop CMI whereas MB patients develop predominantly HMI. Therefore, both types of immune responses need to be determined infield applicable tests.

CMI based antigen selection

The studies described in this thesis aimed at the identification of specific and immunogenic *M. leprae* antigens (proteins and peptides) for eventual application as diagnostic tools. This was accomplished by performing extensive screening of antigens in leprosy endemic populations with different genetic backgrounds from Asia, Africa and Latin America as well as from very low and non-endemic populations in South Korea and The Netherlands, respectively [27]. Being a stable cytokine, it is used as read out in T cell *in vitro* assays as well as in commercialized diagnostic kits like QuantiFERON TB Gold and T Spot TB tests. The level of IFN-γ secreted by immune cells in response to the *M. leprae* proteins and synthetic peptides was used as readout for preliminary selection of immunogenicity of *M. leprae*-specific candidate antigens [8;26;27] (chapter 2). Subsequently, promising candidate diagnostic antigens were analysed in leprosy patients, their household contacts and endemic controls for their potential to induce additional biomarkers besides IFN-γ (chapter 3 and chapter 4). Subsequently, the application of such biomarkers in field-friendly diagnostic tests was investigated (chapter 5).

Among several antigens tested, the “hypothetical proteins” ML2478 and ML0840 were able to discriminate between the likely levels of infection as judged by their ability to induce high IFN-γ responses in TT/BT, HHC and EC living in high endemic areas as compared to healthy controls from non-endemic area [25]. Similarly, responses to ML1601, a group IV hypothetical protein with less than 30% identity in most mycobacterial species discriminated groups according to their level of *M. leprae* exposure [7] (Chapter 3). Few other research groups have explored such potential *M. leprae* antigens based on CMI [19;20;38;48;53]. So far, ML2478 and ML1601were reported as promising and we have studied these further for the development of diagnostic tools.

Considering the potential of peptides to induce more specific T cell responses by avoiding T cell cross reactivity to conserved stretches of amino acids in the protein, several *M. leprae* peptides and pools thereof were tested for their ability to induce recall CMI [17;18;28;53]. Due to the inherent highly polymorphic HLA-restriction of antigen presentation to T cells, multiple peptides would be required in order to cover populations with different genetic

backgrounds. Most of the *M. leprae* peptides and peptide pools induced very low IFN-γ (in the range of 50 to 100 pg/ml concentration) in all study groups [8;27] as described in this thesis (chapter 2). Especially in whole blood assays (WBA), the level of IFN-γ barely exceeded the background levels, thus rendering these peptides not useful for application in diagnostic tools. Previously, addition of co-stimulants including cytokines (IL-2, IL-7, IL-12, IL-18 and IL-23) and antibodies (anti-IL-10, anti CD49d, anti CD28 and anti-CD40) were tested by our group to enhance peptide-induced IFN-γ responses. Mannosylation of peptides was also investigated. IL-12 was the only co-stimulus which enhanced *M. leprae* specific IFN-γ response in WBA [29]. However, further studies using IL-12 at a low concentration in an endemic area in Ethiopia showed that this cytokine also induced some individual-specific background responses when used in combination with *M. leprae* unique peptides in WBA, thereby excluding this strategy as a dependable assay for selection of immunogenic peptides (Bobosha *et al.*, unpublished data). Aabye *et al.* investigated a simple strategy to enhance CMI by incubating cell cultures at temperatures ranging from 38 - 41°C mimicking the fever temperature known for increased inflammatory responses *in vivo*. Those cell cultures incubated at 39°C showed enhanced immune responses to *Mtb* antigens (peptides of TB10.4 and peptides in the QuantiFERON-TB Gold test) and mitogen (PHA), especially in individuals with low responses at base line [2]. Thus, evaluation of such simple boosting techniques may also enhance the weak *in vitro* responses against *M. leprae* specific peptides.

The other approach described in chapter 3 of this thesis is *in silico* discovery of promiscuously binding HLA class I and class II epitopes, which should highly enrich for relevant peptides [38]. Among the 29 *in silico* selected HLA class I (n=21) and class II (n=8) peptides derived from virulent proteins group IV.A (sanger.ac.uk/Projects/M_leprae/Ml_gene_list_hierarchical.shtml), we identified two peptides of ML2055 (p35: IPASVSAPA and p42: LAIAVVASA), by measuring IFN-γ, that were able to differentiate endemic controls living in areas with high versus low leprosy endemicity [5]. Similarly, a study conducted in Brazil identified 58 additional peptides following a similar *in silico* prediction approach. IFN-γ responses to these selected peptides and their pools showed interesting differences as to their level of exposure to *M. leprae* and/or bacillary load among healthy controls from hyper endemic areas, close HHC of MB patients and HHC of PB patients which is also supported by the anti-PGL-I IgM values of each group [38].

HMI based antigen selection

High humoral and poor cell mediated immunity is a typical feature of MB patients and is an indication of the failure to contain *M. leprae* infection. Clinically, it is relatively simple to diagnose MB patients without performing tests such as the anti-PGL-I IgM assays which are mainly used in epidemiological studies [49]. As mentioned in chapter 1 of this thesis, humoral responses against most *M. leprae* antigens studied so far are more potent in detecting MB patients. However, the potential of humoral responses in monitoring treatment outcome and in assessing possible incipient disease in close household contacts [54] requires further exploration. In this thesis, we showed that HMI as estimated by IgM responses against PGL-I can be combined in a field friendly assay with CMI responses as described in chapter 5 indicating the applicability of combined approach in diagnostic test development.

Potential host biomarkers relevant in leprosy diagnosis and prediction of reactions

In leprosy, the cytokine- and chemokine environment at the sites of infection is a major determinant influencing outcome of the disease. For instance, higher IL-15 production in leprosy lesions drives the differentiation of monocytes to pro-inflammatory macrophages (Mφ1) which is typical feature of TT/BT patients. Instead, higher IL-4 and IL-10 leads to

differentiation of monocytes towards anti-inflammatory macrophages (Mφ2) in MB patients [41;42]. IFN-γ, the hallmark cytokine produced by Th1 cells, induces the differentiation of Mφ1 and expression of microbicidal pathways.

Several host chemokines and cytokines other than IFN-γ were also analyzed in other populations, using multiplex assays [5;25;45]. IP-10, IL-1β, IL-6, TNF-α and MCP-1 were able to differentiate healthy controls residing in relatively high (EC_{high}) and low (EC_{low}) leprosy endemic areas (Chapter 3), suggesting an ability to detect differences in the levels of *M. leprae* exposure. Of interest, a heterogeneous IL-1β response was found within the household contacts group [5;25]; this might suggest that some individuals in this group may induce protective versus pathogenic immune responses to *M. leprae*. Similarly in TB, IP-10, MCP-1 and IL-4 were among the potential biomarkers reported for detection of latent or active TB, disease progression or protection. Such markers could be important in treatment monitoring or in vaccine development [3;11;31;33;40;46]. Therefore, investments in larger-scale longitudinal follow-up studies [44], allowing intra-individual comparison of immune profiles of healthy controls as well as household contacts from leprosy-endemic areas worldwide, will be essential to evaluate which biomarkers correlate with true progression to disease and thus can be used as predictive tools. Some of these biomarkers (such as IP-10 and MIP-1β) are abundantly produced and can be measured easily from small amounts of samples or from shortly stimulated WBA. This makes them attractive candidates for development of simple and rapid field friendly diagnostic tests.

Tools for early detection or prediction of leprosy reactions are highly relevant and a key goal in leprosy research, since these could be instrumental in reducing severe complications and disabilities in leprosy patients. In a recent longitudinal study by our group, an increased production of cytokines: IFN-γ, IP-10, CCL9, IL-17A and VEGF (vascular endothelial growth factor) and a decrease in IL-10 and GCSF was reported in patients with active T1R in response to *M. leprae* (whole cell sonicate) as compared to time points prior to the onset of the reaction. Upregulated mRNA expressions of VEGF and cytolytic proteins like GRMA, GRMB and PRF1 in T1R were also detected [30]. In other multicenter longitudinal studies by our group, the ratios of cytokines such as IFN-γ/IL-10 and IL-17/IL-10 appeared as potential tools for predicting T1R in leprosy patients [34] (Chapter 4). In line with this, high expression of TNF-α mRNA and protein in nerves and lesions [35] and over expression of VEGF and its receptor KDR in lesions [23] of patients with T1R were reported previously. Elevated IL-13, IL-6 and IL-10 [4] in lesions of T1R and decreased IL-13, IL-10 and sIL-6R in patients on steroid [32] and also increased IP-10 in plasma of T1R patients [50;55] showed the importance of these host biomarkers in leprosy reactions, suggesting utility in diagnostic platforms and possibly also in monitoring the efficacy of clinical reaction management. Further intensive longitudinal assessment of patients at multiple time points before, during and after reactions will reveal more biomarkers for prediction of reactions.

The biomarkers discussed in this thesis (IFN-γ, IP-10, MCP-1, MIP-1β, IL-1β and others) are expressed by either innate cells (monocytes, macrophages, DCs, NK cells, ILCs) or adaptive immune (T) cells. A recent study in mice demonstrated that during re-exposures or re-infections, memory T cells secrete IFN-γ which induces a cascade of innate cells to produce different cytokines and chemokines to control the infection [52]. This example illustrates the synergy of innate and adaptive immunity in controlling infections. Future in depth analysis of which cells produce the biomarkers discovered in our work will provide new insights into the cellular networks and mechanisms involved. These can be further studied to obtain

relevant information on these and new biomarkers that could in turn be analysed for improved diagnosis of *M. leprae* infection, leprosy reactions, and predictive capacity.

Application of up-converting technology in development of leprosy diagnostic tools
The development of rapid diagnostic tests that detect *M. leprae* infection is an urgent topic. As a study in India reported, inadequate monitoring of a policy of 'new case validation' in which treatment was not initiated until the primary diagnosis had been verified by a leprosy expert, may have led to approximately 26% of suspect cases awaiting confirmation of diagnosis 1–8 months after their initial primary health care visit [51]. This clearly shows the need for rapid leprosy diagnostic kits applicable in field settings where there is scarcity of leprosy experts.

In recent studies, up-converting phosphor technology (UPT) has been applied in diagnostics via detection of various analytes derived from the host or pathogen. Similarly, the application of UCP-LFA for T-cell based responses or in combination with humoral responses was previously optimized for leprosy to measure IFN-γ, IL-10 and anti PGL-IgM [12] and more recently also IP-10 [6] (Chapter 5). The abundant IP-10 response against *M. leprae* specific antigen ML2478, which allows differentiating highly exposed individuals from those with low exposure, provided a rationale for optimizing the UCP-LFA for IP-10. Along with this, the possibility of detecting IP-10 already 6 hrs after stimulating with ML2478, the reproducibility of readings in dry and wet UCP-LF format using portable and bench readers makes it a highly promising candidate for POC test development [6]. The possibility of measuring both humoral and cell mediated responses against *M. leprae* on the same UCP-LF strip is an additional advantage that may simplify diagnosis of leprosy. Currently, the IP-10, IFN-γ and anti-PGL-I IgM based UCP-LFA is being field-tested in several endemic areas in Asia and Africa in combination with clinical follow-up of leprosy patients and their contacts. In addition, several other cytokines and chemokines are under investigation for application in the UCP-LFA platform in order to allow multiplex formats of different T cell subset-related cytokines as well as antibodies. A similar UCP-LFA test was also field-tested for its utility in TB diagnosis in five African countries [13], which further corroborated the value and robustness of this assay.

Regulatory T cells contribute to non-responsiveness in Lepromatous patients
The T cell non responsiveness in LL patients is mediated by multiple host and pathogen factors. We have shown regulatory T cells as a major factor for the non-responsiveness in at least one third of LL patients using a functional assay [9] (Chapter 6). In addition, the co-presence of significantly higher number of FoxP3 positive cells with $CD163^+$ Mφ2 in LL lesions shown in this thesis [9], and the higher number of $CD163^+$ [41] and increased IL-10 and CTLA-4 in lesions of LL reported previously [43] further strengthens the role of regulatory T cells in leprosy. The presence of high frequency of $CD8^+$ T regs in peripheries of LL patients as shown in ours and in another recent study [47] certainly indicates the need for functional characterization of this T reg population at infection sites and in the systemic circulation. In TB, the suppressive role of $CD8^+CD39^+$ T reg cells has been shown previously [10]. Increasing basic understanding of pathogenic mechanisms in leprosy will facilitate the design of treatments that can boost the CMI and down regulate the regulatory function to re-establish normal function of macrophages and T cells in MB patients which eventually facilitate bacterial clearing from the host.

Co-infections in leprosy

The impact of HIV has not worsened the leprosy situation as predicted [36;37]. However, manifestation of sub-clinical leprosy infection in some patients on anti-retroviral therapy (ART) was observed [37] although it is difficult to clearly show the onset of the infections, whether the HIV infection precedes leprosy or the other way round. The number of co-infected patients in our study was small but among them the majority (66.6%) were patients with T1R and a strong association of ART and T1R was observed as described in chapter 7 and was found similar with previous reports [15;39]. However, this association has to be further investigated in larger groups of patients on ART or naïve to ART in longitudinal studies.

Characterization of immunological profiles in co-infected individuals is also important to generate information for the development of new diagnostic tools that can be used in both groups, in addition to what it might add in better understanding the co-existence of the two infections. In this thesis, significantly higher mRNA expression of $CD8^+$ T cells in co-infected patients is reported (chapter 7) and in previous study, in addition to the higher frequency of $CD8^+$ T cells, their role in triggering T1R was reported in co-infected patients [14]. Therefore, further detailed investigations of immune cells derived from co-infected and leprosy patients without HIV are required for better understanding of the influence of one infection on the other and to generate information useful for management of co-infected patients.

Another common co-infection is infection with helminthes. In TB, helminth infestation upregulates Th2 responses and weakens Th1 immunity induced e.g. by BCG vaccination [21;22] which may play a role in delayed clearance of the bacilli. In our study, the majority of patients were BL/LL patients (n=218). However, comparable percentages of BL/LL (12.4%) and BT (10.5%) patients tested positive for helminthes, unlike a previous study that showed higher (22.8%) helminth infection in MB compared than PB (6.8%) [16]. Although our findings require further analyses in a larger sample size, high IFN-γ responses against *M. leprae* WCS in helminth-free leprosy patients with T1R were measured as compared to helminth co-infected BL/LL patients with T1R. This indicates a skewing of helminth driven Th2 responses over Th1 also during T1R. We hypothesize that this Th2 biased immune response may further delay the clearance of the bacilli in these patients. De-worming could be an option in regaining the Th1 type response in these patients to facilitate the bacterial clearance but may aggravate on the other hand the severity of the T1R. These issues need to be investigated urgently before they can be applied in patient management.

Conclusions and recommendations

The achievements in leprosy control in the last three decades are remarkable, especially the replacement of life-long treatment dapsone with MDT and the global decline of leprosy prevalence. However, the incidence of leprosy registered every year in some countries such as India, Bangladesh, Brazil and Ethiopia has become stable because of the ongoing transmission within the endemic population.

The major known sources of *M. leprae* infection are untreated MB patients and the non-symptomatic sub-clinically infected individuals, although the nine-banded armadillos has also been reported as a source of infection in places where these animals are living close to humans [56]. The existence of some environmental reservoirs such as soil and water is also not resolved yet [57]. The established clinical investigation and the lab assessments including

AFB staining, the PGL-I based ML-Flow and LID-1 (See page 7) based rapid tests are more appropriate in identifying MB patients. However, developing diagnostic tests capable of identifying patients with few clinical signs and sub-clinically infected non-symptomatic individuals is by far more important in reducing leprosy transmission.

Measuring IP-10 in whole blood assays briefly stimulated with the immunogenic and specific protein ML2478 and peptides of ML1601 has been found relevant in differentiating groups of individuals by level of their *M. leprae* exposure. IP-10 in UCP-LF assays is reproducible in different settings and preparations in dry or wet format and can be measured in combination with the humoral anti-PGL-I IgM in the same assay. Other biomarkers including MCP-1, MIP-1β and IL-1β were also found to have potential in differentiating groups by level of exposure or infection. In addition, the ratios of IFN-γ/IL-10 and IL-17/IL-10 were found relevant in predicting reactions. The identified antigens and biomarkers here in this thesis have to be further validated in larger sample sizes focusing on higher numbers of HHC. Studies are ongoing by our group in Asian and African countries to facilitate the application of biomarker tests in active case detection and in contact tracing in control programs. Furthermore, detailed analysis of the source and role of the identified cytokines and chemokines in the immunopathogenesis of leprosy will be relevant and will include study of the interactions of host immune cells with *M. leprae* in diverse host genetic backgrounds. It is also obvious that co-infections in HIV-positive patients are high and helminthes are common in people with low economic or living status. Therefore, including leprosy patients with co-infections as study populations in every step in the diagnostic tool development process is important to ensure the applicability of the diagnostic tools in these groups of people.

Our studies started with the screening of hypothetical (unknown functions) but unique *M. leprae* candidate antigens, assessing their immunogenicity in populations in different continents that covered large host background genetic and environmental diversity. We next used the most promising antigens to develop a simple and rapid diagnostic test format for early detection of infection, and of disease onset, including type-1 leprosy reactions. However, our studies also led to major questions on exposure vs infection: does a high pro-inflammatory response in highly exposed individuals represent a signature of protective immunity, or a risk of developing disease? Such questions can only be answered by longitudinal assessment of well defined *M. leprae* exposed cohorts at different endemic sites.

Finally, as all research activities in leprosy involve vulnerable groups, it is crucial that a strong public awareness program is installed to avoid stigma, to facilitate research and develop reliable tools relevant for leprosy control.

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Nederlandse Samenvatting

Lepra is een chronische infectieziekte veroorzaakt door *Mycobacterium leprae*. Wereldwijd leven er 2-3 miljoen mensen met lepra en ervaren de permanente, bijbehorende handicaps. Daarnaast ontwikkelen er jaarlijks meer dan 200.000 mensen deze ziekte. Ondanks de beschikbaarheid van effectieve antibiotica (*multidrug treatment*), worden als gevolg van de aanhoudende transmissie van de leprabacterie, veel mensen, waar onder kinderen, getroffen door lepra. Aangenomen wordt dat onbehandelde multibacillairepatiënten als ook *M. leprae* geïnfecteerde individuen zonder klinisch-zichtbare symptomen de belangrijkste bronnen van besmetting zijn. De momenteel beschikbare klinische- en serologische laboratoriummethoden zijn niet geschikt voor detectie van paucibacillaire patiënten en (nog) asymptomatische, *M. leprae* geïnfecteerde individuen die een hoog risico lopen op het ontwikkelen van de ziekte. Ook bestaan er geen diagnostische testen om leprareacties, de belangrijkste oorzaak van zenuwschade bij lepra, te voorspellen.

In silico identificatie van genen die uniek zijn voor*M. leprae* en productie van de bijbehorende eiwitten heeft de mogelijkheden om diagnostische testen te ontwikkelenaanzienlijk uitgebreid, in het bijzonder voorde vroegdiagnostiek van infectie. Dergelijke testen kunnen uiteindelijk aanzienlijk bijdragen aan vermindering vantransmissie.
Door middel van *in vitro*screening van de specifieke cellulaire immuniteit van recombinante eiwitten en synthetischepeptiden van *M.leprae*in populaties met verschillende genetische achtergronden, zijn diverse antigenen geïdentificeerd met diagnostisch potentieel. Uitgebreid onderzoek naar de cellulaire immuunresponsetegen deze unieke antigenen bij leprapatiënten, hun huisgenoten en gezonde personen uit hetzelfde, lepra-endemische gebiedzijn van immens belang bij de ontwikkeling van nieuwe diagnostische testen.
Dit proefschrift bespreekt de selectie en evaluatie van immunogene,*M. leprae* unieke eiwitten en peptiden, alsmede identificatie van gastheer biomarkers met als doel de detectie van *M. leprae* infectie en vroegdiagnostiek van lepra en leprareacties.

Het meten van de hoeveelheid van het chemokine IP-10 in bloed,na 24 uur stimulatie met de specifieke *M.lepraee*iwitten ML2478 en ML1601 (of hun bijbehorende peptiden), kan worden gebruikt om de mate van blootstelling aan *M. leprae*, en hiermee het risico op infectie, te bepalen in verschillende populaties.
Aangezien klinieken in gebieden waar lepra voorkomt veelal niet beschikken over high-tech apparatuur, moeten diagnostische testen voor detectie van leprareacties gebruiksvriendelijk zijn. Om een gebruiksvriendelijke test voor het meten van IP-10 in veld situaties te ontwikkelen, werden er UCP-LF assays ontwikkeld en getest in diverse, verschillende populaties in lepra endemische gebieden. Deze testen bleken robusten reproduceerbaar voor gelijktijdige bepaling van zowel de hoeveelheid IP-10 (cellulaire immuniteit) als het aantal antilichamen (humorale immuniteit) tegen *M. leprae* PGL-I
Daarnaast werden andere biomarkers, waaronder MCP-1, MIP-1β en IL-1β,gevonden die gebruikt kunnen worden om de mate van infectie te helpen vaststellen.
Een belangrijk aspect bij het bepalen van biomarkers is dat niet de absolute hoeveelheid maar de verhoudingen van verschillende markers correspondeert methet optreden van ziekte. In dit proefschrift wordt dit beschreven voor de ratio IFN-γ/IL-10 en IL-17/IL-10 bij type 1 lepra reacties.

Aangezien bij het ontstaan van lepra meerdere factoren een rol spelen, is het duidelijk dat niet één maar meerdere biomarkers moeten worden toegepast in nieuwe diagnostische testen voor lepra en *M. leprae* infectie. Daarom zullen de in dit proefschrift beschreven biomarkers

verder moeten worden gevalideerd in grote aantallen contacten van leprapatienten. Hierbij is het vooral van belang dat de bepaling van de markers longitudinaal (op meerdere tijdstippen) plaats vindt omdat de intra-individuele verandering in biomarker ratios van belang is bij de diagnostiek van lepra.

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Curriculum Vitae

Kidist Bobosha Aboma was born in Addis Ababa, Ethiopia on October 12, 1972. She got her BSc. degree in Biology in July 1992 and her MSc. degree in Applied Microbiology in July 2003 from Addis Ababa University. She worked for Awassa Agricultural Research Center as a researcher in the plant pathology field from 1994 to 2000. Later after she got her MSc, in September 2003, she joined the Armauer Hansen Research Institute and started working on infectious diseases research, mainly in leprosy and tuberculosis. In 2009, she got a PhD opportunity at Leiden University Medical Center (LUMC) in the Department of Infectious Diseases to work on a project proposed to develop novel tools for early detection of *M.leprae* infection under close supervision of her promotors: Prof. dr. A. Geluk and Prof. dr. T.H.M. Ottenhoff. Since January 2013, she returned to her regular research at her home institute, AHRI, and is engaged in leprosy- and other research projects, supports MSc and PhD students and is also actively involved in various administrative responsibilities.

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List of Abbreviations

AFB	acid fast bacilli
ART	anti retroviral treatment
BB	borderline borderline leprosy
BI	bacterial Index
BL	borderline lepromatous leprosy
BT	borderline tuberculoid leprosy
CMI	cell mediated immunity
DALY	disability adjusted life years
DAWLY	disability adjusted working life years
DC	dendritic cell
EC_{high}	endemic controls living in relatively high leprosy endemic area
EC_{low}	endemic controls living in relatively low leprosy endemic area
ELISA	enzyme linked immunosorbent assay
ENL/T2R	erythema nodosum leprosum/Type 2 reaction
GCF	growth colony factor
GNLY	granulysin
GZMA	granzyme A
GZMB	granzyme B
HAART	highly active antiretroviral treatment
HIV	human immune deficiency virus
IFN-γ	interferon gamma
IL	interleukin
IP-10	interferon gamma induced protein 10
IRD	immune reconstitution syndrome
KAP	knowledge Attitude Practice
LL	lepromatous leprosy
M. leprae	*Mycobaterium leprae*
MB	multi bacillary
MDT	multi-drug therapy
MF1 (Mϕ1)	pro-Inflammatory macrophages
MF2 (Mϕ2)	anti-inflammatory macrophages
MHC	major histocompatibility complex
MLPA	multiple ligation probe amplification
Mtb	*Mycobacterium tuberculosis*
ND-O-HSA	natural disaccharide octyl human serum albumin
NOD2	nucleotide-binding oligomerization domain
PAMP	pathogen associated molecular patterns
PB	pauci bacillary
PGL-I	phenolic glycolipid-I
POC	point of care
PRR	pathogen recognition receptor
RR/T1R	reversal reaction/Type 1 reaction
SC	schwann cell
SNP	single nucleotide polymorphism
SSS	skin slit smear
TB	tuberculosis
Th	T helper cell

TLR	toll like receptor
TT	tuberculoid leprosy
UCP	up-converting phosphor technology
LF	lateral flow
VDR	vitamin D receptor
WHO	world health organization