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

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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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