Immunopathology of leprosy: towards the search for diagnostic and prognostic biomarkers to elucidate pathobiology and their utility in patient care

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Towards the search for diagnostic and prognostic biomarkers in elucidating pathobiology and their utility in patient care

Anand Mahadevan Iyer
Immunopathology of leprosy

Towards the search for diagnostic and prognostic biomarkers to elucidate pathobiology and their utility in patient care
Text: A passage from Sushruta Samhita (about 600 B.C.) describes Tuvaraka, identified as chaulmoogra or marotti, as one of the prominent plant oils used in Leprosy. Translated as: The regular use of Tuvaraka seeds cleanses the system of the patient and is a potent remedy against Kushtha (leprosy) and Meha (probably gonorrhoea).
Source: www.theacworthleprosymuseum.org/
Immunopathology of Leprosy

Towards the search for diagnostic and prognostic biomarkers in elucidating pathobiology and their utility in patient care

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties
ingestelde commissie
in het openbaar te verdedigen in de Agnietenkapel
op vrijdag 30 januari 2009, te 14:00 uur

door

Anand Mahadevan Iyer

geboren te Palghat, India
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### Abbreviations

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<tbody>
<tr>
<td>ADCC</td>
<td>antibody dependant cell cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cells</td>
</tr>
<tr>
<td>BB</td>
<td>mid-borderline leprosy</td>
</tr>
<tr>
<td>BCG</td>
<td><em>Mycobacterium bovis</em> Bacille Calmette-Guerin</td>
</tr>
<tr>
<td>BI</td>
<td>bacterial index</td>
</tr>
<tr>
<td>BL</td>
<td>borderline lepromatous leprosy</td>
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<tr>
<td>BT</td>
<td>borderline tuberculoid leprosy</td>
</tr>
<tr>
<td>CMI</td>
<td>cell mediated immunity</td>
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<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DC-SIGN</td>
<td>dendritic cell specific intercellular adhesion molecule 3 grabbing non-integrin</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
</tr>
<tr>
<td>ENL</td>
<td>erythema nodosum leprosum</td>
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<tr>
<td>FDT</td>
<td>fixed duration therapy</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HI</td>
<td>humoral immunity</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>I</td>
<td>indeterminate leprosy</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>LAM</td>
<td>lipoarabinomannan</td>
</tr>
<tr>
<td>LL</td>
<td>lepromatous leprosy</td>
</tr>
<tr>
<td>MΦ</td>
<td>macrophage</td>
</tr>
<tr>
<td>M. leprae</td>
<td><em>Mycobacterium leprae</em></td>
</tr>
<tr>
<td>MB</td>
<td>multibacillary</td>
</tr>
<tr>
<td>MBL</td>
<td>mannan binding lectin</td>
</tr>
<tr>
<td>MDT</td>
<td>multi-drug therapy</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MLS</td>
<td><em>M. leprae</em> whole sonicate</td>
</tr>
<tr>
<td>MR</td>
<td>mannose receptor</td>
</tr>
<tr>
<td>NCDR</td>
<td>new case detection rate</td>
</tr>
<tr>
<td>NE</td>
<td>non-ENL LL and BL</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NK</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>NRB</td>
<td>non-reactional borderline (BL, BB, BT)</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>PB</td>
<td>paucibacillary</td>
</tr>
<tr>
<td>PGE2</td>
<td>prostaglandin E2</td>
</tr>
<tr>
<td>PGL-I</td>
<td>phenolic glycolipid-I</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptors</td>
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<tr>
<td>R-J</td>
<td>Ridley-Jopling classification</td>
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<tr>
<td>RR</td>
<td>reversal reaction</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>TCC</td>
<td>T-cell clones</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>Th</td>
<td>T-helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T-regulatory cell</td>
</tr>
<tr>
<td>Ts</td>
<td>T-suppressor cell</td>
</tr>
<tr>
<td>TT</td>
<td>polar tuberculoid leprosy</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>ZNCF</td>
<td>Ziehl-Neelsen’s Carbol Fuchsine</td>
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</tbody>
</table>
Introduction and outline of the thesis
*Immunopathology of leprosy*

Leprosy is widely regarded as one of the oldest diseases known to man. References to leprosy have been found in the Bible, although Cochrane [1] argued that the biblical usage of the word probably indicated a defiling or cursed disease rather than leprosy *per se*. Leprosy was prevalent in ancient Egypt, with indisputable evidence for leprosy in a mummy dating from the 2nd century BCE [2; 3]. In ancient India, leprosy was a common disease and was mentioned in the Vedas [4]. The Sushruta Samahita provides a detailed account of the various clinical features and treatment of leprosy [5]. Inscriptions from ancient China dating from around 600 BCE also reveal the prevalence of the disease there [6]. In ancient Greece, clinical descriptions of what was probably the lepromatous form of leprosy was included under the name ‘Elephantiasis graecorum’ [7; 8]. The disease is thought to be introduced to Europe by the soldiers of Alexander the Great returning from the Indian campaign [7; 8] or Pompey’s soldiers returning from Egypt [3]. Leprosy was introduced in the Americas largely as a result of colonization by Europeans and to some extent by the slave trade from West Africa in the 18th century [9].

Much has improved in our understanding of leprosy since the causative bacterium, *Mycobacterium leprae*, was discovered by Armauer Hansen in 1874 [1]. However, despite this fact, leprosy is one of the few diseases where Koch’s postulates have not yet been fulfilled due to a lack of a suitable in vitro culture medium and the absence of good animal models. Leprosy is a chronic infectious disease primarily affecting the skin and the peripheral nerves. *Mycobacterium leprae* is primarily an intracellular rod-shaped bacterium, which occurs in groups resembling cigar bundles called globi in highly bacilliferous patients. The presence of high molecular weight mycolic acids in the cell renders the bacillus resistant to decolourization by acid-alcohol using the Ziehl-Neelsen Carbol Fuchsin method and hence its acid-fast nature. It is on basis of this acid-fast nature that the Hansen’s bacillus was included in the genus Mycobacterium, together with other closely related species like *Mycobacterium tuberculosis* and *Mycobacterium kansasii* [10]. The 8th edition of the Bergey’s Manual classifies the organism as: Class II – Schizomycetes; Order VI – Actinomycetales; Family II – Mycobacteriaceae; Genus – Mycobacterium; Species – M.leprae.

**Magnitude of the leprosy problem**

Leprosy is still far from being a disease of the past, although recent data indicate stabilization and even a steady declining trend in the yearly new case detection rate (NCDR) [11]. The globally registered prevalence of leprosy at the beginning of 2007 was 224,717 cases, with 259,017 new cases detected in 2006, a decline of about 13.4% compared to 2005 [11]. This is a decline of almost 98%, from roughly 12 million cases in 1985 [12; 13]. With this declining trend, the goal of eliminating leprosy as a public health problem (defined as
having a prevalence rate of < 1 per 10,000 population) has been reportedly achieved in most
countries except Brazil, the Democratic Republic of Congo, Mozambique and Nepal [11].
This is largely attributed to the introduction of multi drug therapy (MDT) in 1982 [14; 15] and
the WHO-led campaign for elimination of leprosy as a public health problem [16; 17]. On the
other hand, the high number of new cases still being detected may indicate the presence of a
hidden reservoir involved in the continued transmission of leprosy. What remains alarming is
the high number of multibacillary leprosy cases, ranging from 27-91% in different regions,
amongst the new cases detected which is a potential source for the continued transmission
of the bacterium [11]. The WHO global strategy for leprosy 2006-2010 aims for a further
reduction in global leprosy numbers, for improved diagnosis, management and information
systems with leprosy services integrated into the general health care system and provision of
adequate tools for disability management and rehabilitation [18; 19]. However, despite the
undoubted success of the WHO global strategy in the control of leprosy, the progress towards
leprosy “elimination” needs to be reviewed cautiously. This is especially important since
doubts exist about the reliability of statistics from some of the highly endemic countries such
as India and Madagascar which have registered a decline of almost 30% between 2003 and
2006 [11]. This is despite no significant changes in the policies and anti-leprosy activities in
these countries compared to previous years and in the face of a much slower decline globally.
Hence, in order to consolidate the successes of global strategy, the focus of future efforts
needs to be on accurate and reliable assessment of the global prevalence and incidence to
pursue the goal of leprosy control further.

Pathogenesis of leprosy
Sources and routes of infection
Although *M. leprae*-like, non-cultivable, acid-fast organisms have been found
in Mangabey monkeys [20], wild armadillos from the southern United States [21] and
sphagnum moss from coastal Norway [22; 23; 24], the only known sources of infection are
human beings disseminating viable bacilli in nasal secretions [25], sputum [26] or from skin
[27]. *M. leprae* is known to remain viable for several days in droplets from the infected upper
respiratory tract, in dried discharges from ulcers or on fomites such as bedding or clothing.
Although several routes of infection have been proposed, the skin and respiratory tract
appear to be the most likely routes of entry. Studies in mice demonstrating the development
of disseminated infection in mice exposed to either aerosols [28] or droplet instillation of
*M. leprae* suspensions [29; 30] along with the high bacillary load in the nasal secretions of
lepromatous patients [25] support the role of the nasal tract as a major port of entry and
exit of *M. leprae*. Another plausible route of entry is the skin with epidemiological studies
showing the development of the first leprosy lesions on the extremities where injury to the skin is common [31].

**Clinical features of leprosy – the leprosy spectrum and reactions**

The cardinal sign of leprosy is a hypopigmented or erythematous skin lesion accompanied by loss of sensation. Patients commonly present with skin lesions, numbness or weakness caused by peripheral nerve involvement, or more rarely, a painless burn or ulcer in an anaesthetic hand or foot [32]. Patients may also present initially with a leprosy reaction [33]. During the chronic course of leprosy, acute increases in disease activity called reactions may occur which may be either type I (reversal reaction, RR) or type II (erythema nodosum leprosum, ENL) as will be described subsequently.

The wide range of clinical manifestations of leprosy forms an unique spectrum which correlate with the magnitude of the host immune response. The pathology of leprosy has been fairly well characterized on basis of clinical, bacteriological and histopathological characteristics and forms the basis of the Ridley-Jopling system of classification of leprosy [34]. The Ridley-Jopling system still remains the basis of a classification of leprosy for scientific purposes. However, the need for histological facilities and skilled, trained personnel meant that this system was complicated for use under field conditions. More recently, a simplified system of classification has been recommended by the WHO for use in the field where slit-skin smears for bacteriology are not available [35].

![Figure 1. Leprosy is classified according to the Ridley-Jopling (R-J) 5-group classification (LL – TT) for scientific purposes or according to the simplified WHO 2 group system (multibacillary-paucibacillary) for treatment purposes. Recently, the WHO system has been further modified into a 3 group system based on the number of lesions. The lepromatous pole of the R-J classification is associated with the abundant presence of M.leprae specific antibodies (humoral immunity, HI) whereas cell-mediated immunity (CMI) becomes stronger towards the tuberculoid pole. Chronic leprosy is also associated with acute reactions – erythema nodosum leprosum (ENL) or Type II reaction mainly in LL/BL patients and reversal reaction or Type I reaction associated with the borderline leprosy forms (BL/BB/BT).](image)
**Ridley-Jopling classification, 1966**

The Ridley-Jopling classification is based on the spectral manifestation of leprosy and has at its one pole lepromatous leprosy (LL), which shows multiple, symmetrically distributed lesions throughout the body (skin, nerves, eyes and internal organs). Histopathologically, LL lesions show an infiltrate largely composed of macrophages, showing varying degrees of foamy changes, and few, scattered lymphocytes, predominantly of the CD8⁺ subset [32]. Acid-fast \( M. leprae \) are numerous within and outside macrophages in LL lesions and may aggregate to form globi. Nerves may show some structural damage and cellular infiltration but not cuffing. In addition, Schwann cells, perineural cells, axons, intraneural macrophages of dermal nerves may contain bacilli. Nerve destruction is gradual, slow and frequently unnoticed until late. Immunologically, LL is associated with a complete absence of \( M. leprae \) specific cell mediated immune responses (CMI) [36; 37]. This is also apparent in the lack of delayed type hypersensitivity (DTH) response to subcutaneous challenge with lepromin, a preparation from highly bacilliferous LL lesions or from \( M. leprae \) infected armadillo tissues [38]. Interestingly however, patients with LL leprosy are able to mount a normal CMI response against other infectious agents including other mycobacteria [36; 37] suggesting that the immune deficiency is leprosy-specific. Furthermore, antibodies to \( M. leprae \) are abundant in LL sera but are ineffective in controlling the progress of the disease.

On the other hand, tuberculoid leprosy (TT) shows few lesions with well defined margins, which are markedly anaesthetic and do not show the presence of acid-fast bacteria. Lesional infiltrate primarily consists of foci of well developed epithelioid macrophages, with or without Langhans' type of multi-nucleated giant cells surrounded by a cuff of lymphocytes. The T-lymphocyte subsets are predominantly of the CD4⁺ type. Within the granulomas, small nerves may be destroyed beyond recognition and a thickened peripheral nerve is usually palpable in the vicinity of a lesion. Immunologically, TT shows a strong CMI response to \( M. leprae \) with an intense DTH response to lepromin [39]. This is also reflected clinically and histopathologically in tuberculoid lesions, which are restricted in distribution and may even heal spontaneously [34]. \( M. leprae \) specific antibodies are usually absent or present at very low levels in these patients.

In-between the two polar leprosy forms are the immunologically unstable borderline forms including borderline lepromatous (BL), mid-borderline (BB) and borderline tuberculoid (BT), showing clinical and histopathological characteristics intermediate to the polar forms. Within these groups, there is a gradual decrease in CMI from BT to BL and this is inversely correlated with the bacillary load within the lesions. About 20-30% of these borderline patients develop acute reactions during or after the course of the disease [40; 41].

Due to the complexity of the Ridley-Jopling classification, the WHO recommended an operational classification to serve as a basis for chemotherapy [42]. Since the transmission of leprosy was thought to occur largely through the highly bacilliferous cases of leprosy the WHO recommended all LL, BL and BB leprosy under the Ridley-Jopling classification with a bacterial index (BI) in slit-skin smears $\geq 2$ to be grouped as multibacillary leprosy (MB) [42]. On the other hand, I, BT and TT leprosy with a BI < 2 under the Ridley-Jopling system were thought to pose fewer problems of transmission due to the lower bacterial load and were grouped as paucibacillary (PB). For further convenience, revised recommendations were issued by the WHO in 1988 whereby all smear positive cases were included as MB whereas I, BT and TT cases except those with smear positivity were included as PB [43]. In 1998, based on the fact that services for processing skin smears may not be available or reliable in the field, the 7th WHO expert committee on leprosy recommended a further simplification of classification [35]. This new classification groups leprosy patients into (a) paucibacillary (PB) single lesion leprosy - only one skin lesion, (b) PB leprosy - 2-5 skin lesions; and (c) multibacillary (MB) leprosy - more than 5 skin lesions. A case of leprosy was defined as a person with one or more of the following features – i) hypopigmented or reddish skin lesion(s) with definite loss of sensation, ii) involvement of the peripheral nerves, as demonstrated by definite thickening with loss of sensation, iii) skin smear positive for acid-fast bacilli [35]. This case definition did not include cured persons with late leprosy reactions or with residual disabilities.

In practice, the different diagnostic criteria used complicate the comparison of data among countries and even within a country, and increase the risk of misclassification as has been suggested from various studies [44; 45; 46]. Hence the use of a standard classification such as the clinico-histopathological Ridley-Jopling system should be a pre-requisite to allow comparison of different studies across the globe and even over time [47].

**Reactions in leprosy**

A significant proportion of the leprosy patients, especially in the borderline region of the spectrum, develop leprosy reactions either sometime during the course of the disease or even after the completion of multi-drug therapy (MDT) [48; 49; 50; 51]. Reactions are thought to be immune exacerbations in response to *M. leprae* and its antigenic components [51; 52] and may be either type I/reversal reaction (RR) – with acute increase in *M. leprae* specific CMI or type II/erythema nodosum leprosum (ENL) – an immune complex mediated condition with some involvement of CMI [51; 52].
Introduction and outline of the thesis

Type I reaction or Reversal reaction

About 30% of individuals with borderline leprosy are at risk for type 1 reaction which is characterized clinically by acute inflammation of skin and/or nerves leading to nerve function impairment if not treated rapidly [32; 53]. The characteristic signs of RR are an erythematous swelling of existing lesions, appearance of new lesions and the onset or worsening of neuritis [51; 52]. Histopathologically, RR is characterized by a shift of classification towards the tuberculoid end of the spectrum with increased infiltrate of lymphocytes, epitheloid cells, giant cells, oedema and a decrease in bacterial load. A sudden increase in *M. leprae*-specific CMI accompanied by an influx of CD4+ T cells at the lesional site are some of the immunological characteristics of RR [51; 52]. The immune response is characteristic of a delayed-type hypersensitivity (DTH) reaction with peripheral blood lymphocytes demonstrating an increased reactivity to *M. leprae* antigens in a lymphocyte transformation test (LTT) [54]. This may lead to local decrease in bacillary load and augmentation of T cell reactivity leading to nerve damage [51].

Incidence of Type I reactions is significantly higher in BB and BL patients as compared to BT patients [55; 56; 57]. De Rijk *et al.* [56] noted that 88% of reactional events in MB patients and 52% in PB patients involved neuritis. Moreover, in 40-50% of both groups, neuritis occurred in the absence of skin involvement [56]. Furthermore, in 80% of the cases, RR occurred within the first year of treatment or the first 6 months after completion of MDT [56; 58; 59].

Type II reaction or Erythema Nodosum Leprosum

ENL, on the other hand, affects 20% of lepromatous and 10% of borderline lepromatous cases, with a high bacterial load and greater infiltration of lesions as important risk factors [32; 53]. ENL is characterized by painful and tender red papules or nodules of the skin accompanied by fever, joint pain, oedema of the hands, feet, and face, proteinuria and malaise [32; 52; 53]. Other manifestations include iritis, episcleritis, arthritis, dactylitis, lymphadenopathy, organomegaly and orchitis [33]. Neuritis may be part of ENL but is usually milder than in RR [60]. Most patients experience multiple acute episodes or chronic ENL lasting more than 6 months or even years [61; 62]. The inflammatory infiltrate is usually in the deeper layers of the dermis and subcutis [60]. In acute lesions within 72 hours the predominant cell type is the polymorphonuclear leukocyte (PMN) whereas between 72-96 hours equal numbers of neutrophils, lymphocytes and plasma cells are seen along with persistent mast cells [60]. Chronic lesions > 9 days on the other hand have fewer neutrophils and eosinophils but an increased number of lymphocytes, plasma cells and histiocytes [60]. Vasculitis appears to be a major pathological event in ENL along with interstitial oedema and
necrotizing changes [63; 64]. A large proportion of patients with ENL showed deposition of Ig and complement in the skin, with 70% showing *M. leprae* antigens within the complexes, which was absent in patients without ENL [65]. Furthermore, immune complex deposition was significantly higher in skin but similar in serum to patients without ENL [65], suggesting that ENL was caused by local deposition of immune complexes. Laal et al. [66] provided evidence for CMI in ENL by demonstrating a strong leukocyte migration inhibition and antigen-induced lymphoproliferation in PBMCs from ENL patients. In addition, several studies have shown an important role for TNF-α in the pathogenesis of ENL [67; 68; 69; 70] suggesting that CMI may be a crucial component of this type of reaction. Later studies have shown that IFN-γ and IL-12 are also important in addition to TNF-α and may contribute to the deleterious effects classically attributed to TNF-α [71].

A wide variation has been reported in the incidence of ENL in leprosy patients globally ranging from 2% to 31% of MB patients [55; 72; 73; 74]. The most important risk factor was found to be a high bacillary load while HIV positivity was found to be another risk factor [74]. A majority of the cases presented 2-3 years after diagnosis of leprosy with some patients developing episodes as late as 7 years after starting treatment [74]. Sixty-three percent of the cases had multiple episodes of ENL, which required management with long courses of prednisolone and additional clofazimine for periods of upto 5 years [74].

**Host cell parasitism and bacterial survival**

Macrophages are known to be important host cells for *M. leprae* and *M. tuberculosis* [75]. On the other hand, *M. leprae* is also known to promote its survival in the mammalian host by entry into immunologically privileged sites such as the peripheral nerve and selectively parasitizing non-professional phagocytic cells such as MHC Class II negative Schwann cells [76; 77]. Rambukkana and co-workers [78; 79; 80] elegantly demonstrated one possible mechanism of *M. leprae* invasion of the Schwann cells of the peripheral nerves using laminin α2 (LN-α2) as a bridge [80]. A 21-kD laminin binding protein on the *M. leprae* cell wall was shown to bind specifically to LN-α2 in the basal lamina [78; 81] which in turn bound to α-dystroglycan on the Schwann cell [82]. In addition, other non-laminin receptors such as the 25-kD glycoprotein from the human peripheral nerve [83] may serve to strengthen this interaction with Schwann cells [78]. Later studies showed a role for the *M. leprae* specific PGL-I antigen in binding to the native laminin-2 in the basal lamina of the Schwann cell-axon units through specific trisaccharide units [84]. PGL-I was also shown to play a role in *M. leprae* induced contact-dependent demyelination of nerve fibres in a time and dose dependent manner, thus contributing to the pathology of leprosy [85].

Within the host the bacterium may adopt different mechanisms to avoid or
circumvent the host immune responses [86; 87; 88]. Phagocytosis thorough receptors which bypass bactericidal activities of macrophages may provide an opportunity for pathogens to manipulate the host environment to their own advantage. Caron and Hall [89] demonstrated the advantage of using complement receptor (CR) 3 as a receptor for bacterial entry which avoided the usual inflammatory response associated with the uptake of IgG-opsonized targets through FcγII. Phagocytosis using CR1 [90; 91] or the mannose receptor (MR) [92] prevented fusion of lysosomes with phagosomes preventing O2⁻ radical production thus may allow for survival of mycobacteria. Inhibition of phagosome-lysosome fusion by \( M.\text{leprae} \) was demonstrated by Sibley \textit{et al.} [86] and confirmed by Frehel and Rastogi [93] who also suggested a role for mycobacterial surface components in this event since antiserum coating of \( M.\text{leprae} \) partly reversed the fusion inhibition event. Furthermore, Sturgill-Koszycki \textit{et al.} [94] reported a selective inhibition of fusion of Mycobacterium containing vacuoles with vesicular proton ATPase preventing phagosome acidification and hence mycobacterial killing. Another mechanism of mycobacterial escape suggests a progressive translocation of \( M.\text{tuberculosis} \) and \( M.\text{leprae} \) from phago-lysosomes into the cytoplasm of phagocytes dependent on the secretion of the mycobacterial antigens CFP-10 and ESAT-6 [88].

**The host immune response in leprosy**

Intracellular pathogens, which include different species such as \textit{Listeria, Leishmania, Mycobacteria, Salmonella, Chlamydia, Rickettsia} and \textit{Trypanosoma}, live inside the host cells where they are protected from the effector cells of the immune system. The preferred host cells for the pathogens are the mononuclear phagocytes, which are also an important component of the innate immune system. In a constant competition between the pathogen and the host these phagocytes can restrict the intracellular growth of the pathogen by phagosome lysosome fusion, generation of reactive oxygen and nitrogen intermediates or restricting nutrient availability to the pathogen. At the same time, these cells secrete soluble mediators such as cytokines and chemokines to increase trafficking of T- and B-lymphocytes and concentrate them at the sites of infection. Moreover, the phagocytes along with other specialized antigen-presenting cells like the dendritic cells ensure that protein and lipid antigens from the pathogens are processed and presented leading to activation of the host T-lymphocytes [95]. These T-lymphocytes are thought to be crucial in determining the resistance, susceptibility, and often, the immunopathogenesis of intracellular infections. Although the CD4⁺ lymphocytes are primarily implicated in acquired immunity, increasing evidence points to a role for CD8⁺ T-cells as well as unconventional T-cells such as the CD4⁺CD8⁻TCRαβ⁺ T cells recognizing lipid antigens, TCRγδ⁺ T-cells or natural killer (NK) T-cells [96; 97; 98].
Interestingly, *M. leprae* in itself is a rather innocuous organism as suggested by the fact that about 95% of patients infected with the bacterium do not develop overt disease [99]. This also suggests the importance of the host immune response in controlling disease progression although much of the pathology of leprosy is also thought to be related to an inappropriate immune response.

**Genetics of the host response in leprosy**

The low genetic diversity of *M. leprae* and the fact that only about 0.1 – 1% of the infected population develops overt disease [100], indicate the capacity of the host immunity to control the infection. This suggests differences in host susceptibility to the pathogen which may also explain the clinical spectrum of leprosy. There is increasing evidence for association of the genetic background of the host with development of leprosy or its different spectral forms [100]. A strong argument in favour of the role of host genetic background comes from twin studies where a higher concordance rate for leprosy was found amongst monozygotic twins [60-85%] than dizygotic twins [5-20%] [100; 101]. A previous report suggested that populations with different ethnic backgrounds living in the same endemic areas exhibit distinct prevalence rates for leprosy [100]. Moreover, vaccine studies showed the highly variable protection rates of BCG amongst different populations [102]. Two approaches have been commonly used to test genetic associations in leprosy – the candidate gene approach and, more recently, genome wide scans [100].

**The candidate gene approach**

Since the host immune response plays a crucial role in the development of the disease, the most common candidate genes studied in relation to leprosy are genes related to the immune system, such as the human leukocyte antigen complex (HLA), cytokines and receptors. The HLA loci are traditional candidates for association studies with leprosy with the class I HLA-A*1102-B*4006-Cw*1502 haplotype showing a highly significant association with leprosy susceptibility [103]. Among the class II genes the HLA-DRB1 locus, more specifically DRB1*15 and DRB1*16 are associated with leprosy susceptibility in India [104; 105], Thailand [106] and Brazil [107] while the HLA-DQw1 locus was found to be associated with LL patients [108]. A single nucleotide polymorphism (SNP) resulting in a G-A substitution in the promoter region of TNFA gene encoding for TNF-α showed protection against leprosy [109; 110; 111]. However, contradictory results were shown for this SNP in other studies [112; 113]. Similarly, an SNP at the +252 position (A-G) in the
Introduction and outline of the thesis

first intron of the lymphotoxin (LT)-a gene together with the -308A SNP as a haplotype was also associated with protection against leprosy in a Brazilian population [111] possibly by production of higher levels of TNF-α and LTα [100]. Similarly a -819T SNP in the promoter region of the IL-10 gene was found to be associated with susceptibility to leprosy [110; 114]. However, while there is confirmatory epidemiological evidence for the association of this SNP in linkage with other haplotypes in leprosy susceptibility [114], no biological evidence linking these genotypes/haplotypes with IL-10 production in leprosy has been obtained to date [100]. The vitamin D receptor (VDR) was associated with leprosy in an Indian population where a T-C substitution at codon 352 at the 3’ gene region resulted in susceptibility to lepromatous and tuberculoid leprosy in the presence of genotypes TT and CC respectively [115] whereas in a Malawian population the CC genotype was associated with susceptibility to leprosy per se [113]. However, the biological relevance of this SNP on VDR function in leprosy is not known. The NRAMP-1 gene has also been studied as a candidate gene for leprosy resistance/susceptibility based on studies in mice [116]. Within humans however, evidence for association of this gene with resistance to leprosy is controversial [117; 118; 119; 120] although a 4 nucleotide deletion in the 3’ untranslated region (UTR) was more frequently encountered in multibacillary leprosy patients [121]. Other candidate genes tested for leprosy susceptibility include the IL-12Rβ1 and 2 [122; 123; 124], laminin [125] and Toll-like receptor (TLR)-2 [126]. Studies on a small Indonesian population revealed that a polymorphism at the +7809 position in the laminin-α2 gene substituting valine for alanine (V2587A) resulted in a missense mutation which was strongly associated with tuberculoid leprosy [125]. On the other hand, whereas missense mutations of IL-12Rβ1 were not associated with LL [122], IL-12Rβ2 was found to be highly expressed in TT leprosy [124] and missense mutations in the 5’ flanking region of the gene could affect its expression and hence increase susceptibility to the LL form of leprosy [123]. Despite the initial enthusiasm about the association of the Arg677Trp polymorphism in the toll-like receptor (TLR)-2 gene with approximately 25% of LL patients by Kang and co-workers [127], it was subsequently shown to be the result of an exon duplication event rather than a genuine polymorphism [128]. However, more recent studies have shown the association of a TLR2 microsatellite marker with increased risk for development of reversal reaction [129] suggesting that TLR2 may indeed have a role in leprosy.

Genome wide scan

In recent years, the use of genome wide scans have enabled genetic linkage studies to be performed in leprosy to uncover regions of the genome which may harbour potential candidate genes. Early studies showed major leprosy susceptibility loci in the 10p13 region,
containing the macrophage mannose receptor gene [101] and the 20p12 region [130], also found to be associated with atopic dermatitis and psoriasis in other studies [131; 132; 133]. Later studies showed linkage of the 10p13 region with TT leprosy rather than leprosy in general [134; 135]. A recent study mapped the leprosy susceptibility locus to the chromosome 6q25 in Vietnamese families affected with leprosy [134]. Further analysis using a systematic association scan showed a significant association between leprosy and 17 markers located in a block of approximately 80 kilobases overlapping the 5‘ regulatory region shared by the Parkinson’s disease gene PARK2 and the co-regulated gene PACRG [136]. Possession of as few as two of the 17 risk alleles was highly predictive of leprosy which was also confirmed in unrelated leprosy cases and controls from Brazil. The 17q11 locus, which contains the NOS2A and CCL2 (MCP-1) genes among others, was implicated in leprosy susceptibility in other genome wide scans [137]. However there is no independent confirmation of the association of these genes with leprosy.

A major limitation of genetic association studies are the conflicting results, primarily as a result of biases of design including the choice of appropriate statistical tools, selection of controls and low power of the statistics due to insufficient sample size. However, at least part of the discrepancies may also be attributed to the ethnic specificity of the susceptibility/resistance variants being selected by different environmental pressures, the outcome of which is the disease leprosy. Moreover, relatively few studies have explored the physiological relevance of the associated genetic variants to susceptibility/resistance in leprosy.

The immune system and leprosy

Innate immunity

The innate immune response is composed principally of phagocytic cells and is capable of immediately recognizing and responding to microbial invasion. While professional phagocytes such as macrophages and neutrophils act as effector cells to resolve the infection, macrophages and professional antigen-presenting cells like dendritic cells (DC) are also crucial for the initiation of adaptive immunity and the generation of specific immunity.

Macrophages

Macrophages play a crucial role in host defence against intracellular pathogens including \textit{M. leprae}. LL leprosy is characterized by the accumulation of macrophages which are unable to restrict the multiplication of \textit{M. leprae}. Attempts to induce activation of LL macrophages by local administration of antigens or IFN-\gamma have proven to be ineffective [138]. In contrast,
within TT lesions, hardly any bacteria are seen within macrophages [34]. In recent times, fundamental differences have been reported in the macrophages from LL and TT lesions apart from differences in functional capacities [139; 140]. Expression of toll-like receptor (TLR)-2 and 1 are stronger in macrophages from TT than LL [139]. On the other hand, macrophages from MB lesions reportedly expressed higher levels of DC-SIGN which was suggested to be associated with a Th2 environment in these lesions [140]. Thus, the MB lesions represent a progressive reduction of Th1 activation resulting in a state of antigen-specific tolerance [141; 142]. Macrophages in LL lesions may downregulate CMI by reducing antigen-presenting function and secreting T-helper (Th) 2 cytokines or by secreting suppressive factors [142; 143]. In this regard, adherent cells from MB lesions have been shown to reduce proliferation of lymphocytes from healthy individuals through secreted factors [143]. Similarly, monocytes from LL patients were shown to be involved in the IL-10 and PGE-2 mediated T-cell suppression [142]. Recent advances have suggested a dichotomy of macrophage phenotypes mimicking the Th1-Th2 paradigm of the T-cells with classical activation of the macrophage by microbial products leading to a pro-inflammatory phenotype (MΦ1) while alternative activation may result in an anti-inflammatory phenotype (MΦ2) characterized by IL-10 as the hallmark cytokine [144]. Using in vitro generated MΦ1 and MΦ2 from healthy donors, Verreck and co-workers showed that while mycobacteria can grow in both macrophage subtypes, they induce IL-23 in MΦ1 in contrast to IL-10 in MΦ2 [144]. These results suggest an inherent plasticity of the human macrophage component which along with active interference by pathogens in macrophage signalling pathways may critically affect host defence against mycobacteria. In other studies, M.leprae infected macrophages were refractory to IFN-γ induced activation [145; 146] and manifested aberrant effector functions including impaired microbicidal capacity, decreased oxidative state and lower MHC Class II expression which was related to the length of the infection [146; 147]. A similar phenotype was also associated with M.tuberculosis infection of macrophages where it was shown to be a consequence of disrupted signal transduction, impaired CD64 expression and IL-6 induction [148; 149; 150]. However, although such a mechanism might be plausible in leprosy, evidence for such a hypothesis is yet to be presented.

**Neutrophils**

Although neutrophils are generally the first cell type to reach the site of inflammation, the chronic course of leprosy is characterized by an absence of neutrophils in situ. ENL lesions, however, show a massive infiltrate of polymorphonuclear (PMN) cells, especially within 72 hours after onset of reaction, suggesting a functional role in this phenomenon [60; 151]. Oliveira et.al.[151] showed that apoptosis of PMNs is significantly increased in
ENL patients. Furthermore, PMNs could be stimulated by *M. leprae* and *M. leprae* derived-lipoarabinomannan (LAM) to secrete TNF-α and IL-8 [151]. This suggested that PMNs could contribute to the recruitment of lymphocytes to the lesional sites and also to the TNF-α induced tissue damage. However, at present, it is unclear if a more significant role is associated with neutrophils in the pathogenesis and the immune response in leprosy.

**NK cells**

Similar to neutrophils, the role of NK-cells *in vivo* in leprosy patients is not well established. One of the earliest reports observed no significant difference in NK cell activity between lepromatous and borderline leprosy and healthy individuals [152]. However, a depression of NK cell activity was reported in LL patients with ENL which appeared to be a result of monocyte activity but not due to NK cell dysfunction [152]. In contrast, a later study reported lower NK activity in untreated and non-reactional lepromatous leprosy patients compared to healthy controls [153]. However patients with RR had normal NK activity whereas no significant difference was found between RR and non-reactional BT patients with respect to NK activity [153]. Similarly, Chiplunkar et.al. [154] also observed that NK cells from untreated and MDT treated LL patients were less effective in mediating antibody-dependant cell cytotoxicity (ADCC) compared to TT patients or healthy controls. Treatment of the NK cells with IL-2 or IFN-α was shown to increase cytotoxicity in these patients [154]. Similarly, intradermal administration of IL-2 in LL patients resulted in an 8-fold increase in the NK-cells in these patients, along with increased T-cell and monocyte infiltration into the dermis, upgradation of the lesional infiltrate to a granulomatous tuberculoid type but no reversal in the anergy to *M. leprae* antigens [155]. Viable *M. leprae*, on the other hand, were found to confer resistance to NK-cell mediated killing on macrophages and Schwann cells probably by a mechanism involving heat shock protein (hsp) induction although this was not further elaborated by the authors [156]. In contrast, heat shock protein 10 could induce cytotoxic activity in normal individuals and PB patients, whereas MB patients showed variable lytic activity depending on bacterial load [157]. This was related to the ability of hsp10 to induce both Class I and Class II restricted effector αβ and γδ T cells and/or CD16⁺CD56⁺ NK cells in PB patients, normal individuals and bacteriologically negative MB patients. In contrast, only Class II restricted CTL activity was observed in bacteriologically positive MB (MB⁺) patients [157]. Moreover, IL-4 generated in MB⁺ patients may play a crucial role in downmodulating the activity of the CTLs and NK cells by antagonizing IL-12 induced IFN-γ production [157]. Another important cytokine, which, along with IL-12, plays an important role in induction of IFN-γ production, is IL-18 [158; 159]. IL-18 mRNA expression is elevated in PBMCs of TT but not LL patients stimulated with *M. leprae*, which
also correlated with expression in lesions [158]. Moreover, IL-18 induced IFN-γ production in PBMCs from TT but not LL patients [158] and this could be reduced by 50-60% with anti-IL-12 antibodies suggesting a collaborative role for these cytokines in inducing an IFN-γ response [158; 159]. However, in contrast to the results from Garcia et al. [158], the later study [159] reported that IL-18 was a strong inducer of IFN-γ in response to hsp65 in LL patients which may be related to the different antigens used in the two studies. NK cells were involved early (within 24 hrs) in the IFN-γ response while at later intervals (48 hrs to 5 days) CTLs played a more significant role [158; 159]. De la Barrera et al. [159] elaborated these results further showing that endogenous IL-13 production by NK cells and T cells in MB patients resulted in a decreased CTL activity and IFN-γ production in these patients. They further speculated that the presence of IL-4 in MB patients may lead to accumulation of IL-13+ NK cells due to an impaired maturation of NK cells or a decline in the IFN-γ+ NK cells [159].

Mast cells

As with other aspects of immunity in leprosy, various studies have presented contradictory results of the role of mast cells in leprosy [160]. Higher numbers of mast cells were found in lepromatous as compared to tuberculoid leprosy by some researchers [161; 162] while the opposite was reported by Cree et al. [163]. Antunes et al. [160] speculated that an immunohistochemically observed increase in the tryptase-positive subset of mast cells as compared to the tryptase- and chymase-positive subset in reactional lesions may be related to the onset of the reaction. However these observations have not been validated by other reports and the role of specific mast cell subsets in reactions and in chronic leprosy remains enigmatic.

Complement

The complement system (CS) represents an important first line of defence in resistance to pathogens and is involved both in recognizing pathogens and in inducing inflammation [164]. Initiation of the CS results in a series of protein-protein interactions, some involving enzymatic activation of key CS proteins, with the release of biologically active peptides and the deposition of fragments on target cells. The activation of complement through either the classical, lectin or alternative pathway converges on the deposition of C3b on the pathogen surface, a key event in complement mediated lysis. The activation of the CS also results in the release of small peptides such as C3a and C5a derived from the cleavage of CS proteins which have important biological properties such as mast cell degranulation and
release of vasoactive amines resulting in vasodilatation and also chemotaxis of immune cells. Deposition of C3b on the pathogen/target cell initiates a cascade resulting in the assembly of the membrane-attack complex (MAC), forming pores on the surface of the target cell and its eventual osmotic death.

A recent study reported on the levels of complement components and the total complement haemolytic activity in the sera of leprosy patients [165]. A reduction in complement haemolytic activity was observed in the sera of LL patients as compared to TT, dimorphous leprosy (DL – including the borderline phenotypes) and healthy controls. Moreover, while a lower level of C4 was observed in LL patients, a lack of significant difference in the different leprosy patient groups in a haemolytic assay using non-sensitised rabbit erythrocytes under conditions favouring activation of the alternate pathway of complement activation suggests a greater role for the antibody-dependant classical or the lectin pathway in leprosy [165]. The lectin pathway of complement activation involves direct interaction of mannan-binding proteins/lectins (MBP/MBL), which have a structural similarity to C1q of the classical pathway, with carbohydrates on the target cell leading to C3 cleavage. Interestingly, the highest median levels of mannan-binding lectin (MBL) were observed in the sera of LL and DL patients which had lower haemolytic activity whereas TT patients with lower MBL levels showed higher haemolytic activity [165]. These results are in concordance with previous reports of increased protection against leprosy [166; 167] and tuberculosis associated with MBL deficiency in the sera [168]. Similarly, a recent genetic analysis showed the association of haplotypes/compound genotypes resulting in low levels of MBL with protection against development of LL and BL [169]. Moreover a strong association was found between the haplotype LYPA, associated with high MBL protein levels, and leprosy per se and its LL and BL forms with a 2-fold increased risk of developing the MB forms of the disease [169]. Functionally, MBL has also been shown to bind M. leprae and enhance phagocytosis and uptake of mycobacteria in vitro [166; 170] suggesting a role in uptake, pathogen spread and establishment of leprosy.

Opsonization of microbes by complement protein components promote their uptake by complement receptors on phagocytic cells such as monocytes, macrophages, neutrophils and B cells [171; 172]. CR1, CR2 and CR3 show specificity for partially cleaved C3 fragments which are deposited on the microbial cells on activation. With respect to leprosy, whole M. leprae bacilli incubated with human serum as well as circulating immune complexes from leprosy patients [173] have been shown to activate the complement in vitro. As a consequence, the bacteria are opsonised by C3 fragments and taken up by monocytes expressing CR1 and CR3 [174].
Dendritic cells: the bridge between innate and adaptive immunity

Figure 2 Dendritic cells act as bridges between innate and acquired immunity by recognizing conserved sequences called pathogen associated molecular patterns (PAMPs) on pathogens through pattern recognition receptors (PRRs). This information is then transmitted on to naïve T cells together with co-stimulation and a polarizing signal resulting in the development of the different T cell subsets.

To activate or to suppress

Dendritic cells (DCs) are the sentinels of the innate immune system, sensing pathogen and host derived signals through various pattern recognition receptors (PRRs) to influence the subsequent development of innate and adaptive immune responses [175]. DCs are the main antigen presenting cells involved not only in T cell priming but also in establishing tolerance even in mature T cell compartments [175; 176; 177]. How antigen presentation selects between productive immunity and the non-responsiveness associated with tolerance induction is still a matter of considerable debate. Two important schools of thought prevail. Whilst it has been suggested that this choice is determined by the maturation state of the DC [178; 179], other reports have shown evidence to the contrary [176]. DC subsets have been identified which respond to pathogenic antigens by differential production of cytokines [180]. However whether the DC subsets are differentially selected between immunity and tolerance or act at a more subtle level of modulation of type 1 or type 2 helper T cell responses is at present a matter of considerable debate.

Receptors associated with antigen recognition

The most extensively studied PRRs is the TLR family comprising 10 members (in humans) with different ligand specificities [181]. LPS is known to signal through TLR4 whereas
mycobacterial lipopeptides signal through TLR2 [182]. TLRs can recognize antigens like mycobacterial lipoarabinomannan (LAM) including uncapped (AraLAM), mannose-capped or phosphoinositide-capped LAM (PILAM), the 19-kDa lipoprotein, soluble tuberculosis factor (STF) and whole mycobacteria [183;184]. Mycobacterial PILAMs induce production of TNF-α and IL-12 in DCs [185] whereas phosphatidylinositide dimannoside (PIM), abundantly found in M.bovis, BCG and M.tuberculosis, was shown to activate macrophages through TLR2 to secrete TNF-α [186]. Another major immuno-stimulatory component of M.tuberculosis, the 19-kDa lipoprotein was shown to trigger TLR2 to activate NF-κB and secrete IL-12 [187]. Tri-acylated lipopeptides, representing the 19- and 33-kDa lipoproteins of M.leprae, can also activate DCs through TLR2 [139].

Recent data seem to suggest that distinct TLRs may be associated with specific groups of the leprosy spectrum. Expression of TLR2 and TLR1 was found to be much stronger in skin lesions from the resistant tuberculoid form of leprosy as compared to the lepromatous form [139]. On the other hand, an Arg677Trp mutation in the intracellular domain of hTLR2 resulting in an impairment of NF-κB activation was associated with subset (22%) of lepromatous leprosy (LL) patients from Korea [126;188].

Other receptors such as the C-type lectin DC-SIGN are important in the binding to mycobacteria and viruses such as HIV [189]. DC-SIGN may also be involved in suppression of TLR mediated inflammation in response to mycobacterial infections [189]. DC-SIGN has been shown to bind to the mannose-capped cell wall component lipoarabinomannan (ManLAM) of slow growing virulent mycobacteria such as M.tuberculosis and M.leprae [190] triggering production of IL-10 and inhibiting IL-12 and TNF-α production by DCs or monocytic cell lines [189; 191; 192]. In another study, M.tuberculosis ManLAM could also induce production of TGF-β, another immunosuppressive cytokine, by human monocytes [193]. Recently, ManLAM was shown to block BCG mediated maturation of DCs and this effect could be reversed by antibodies specific for DC-SIGN [191]. This suggests that pathogen recognition through TLRs or DC-SIGN could have opposing effects on DC maturation [189]. In contrast, other C-type lectins such as dectin-1 can act together with TLR2 to enhance the production of IL-12 and TNF-α by DCs facilitating a Th1 response [194]. This suggests that the inflammatory responses may be fine-tuned by the balance between TLR and C-type lectin activation. Recent studies have also showed association of DC-SIGN with lepromatous leprosy (LL) involved both in the uptake of M.leprae and the Th2 environment associated with LL [140; 195]. Other receptors on immature DCs such as the mannose receptor (MR) [196], CD11b and CD11c [197] were reported to mediate to mediate binding of mycobacteria to macrophages [198; 199] and probably DCs.

It is estimated that 25% of circulating monocytes differentiate into migrating DCs whereas others give rise to resident macrophages [200; 201]. It was recently reported that in
vitro incubation of inflammatory monocytes with *Salmonella typhimurium* blocked nearly 50% of their differentiation into DC [202]. Another study reported that pre-incubation of monocytes with BCG did not affect their differentiation into DCs, but resulted in IL-1β mediated blocking of their further maturation (CD83 and CD86 expression) and IL-12p70 production by these DC’s [203]. However, pre-incubation of monocytes with *M.leprae* cytosolic protein up to a concentration of 1μg/ml failed to block maturation of DCs and the production of IL-12p70 [203]. In contrast, our preliminary results showed that pre-incubation of monocytes with *M.leprae* sonicate antigen (MLS) blocked differentiation to CD1a+ DCs and further maturation in response to LPS stimulation. The effect was not so pronounced with LPS or BCG. A recent study [195] showed that activation of human monocytes through TLR2/1 heterodimer induced their rapid differentiation into two distinct subsets: DC-SIGN’CD16+ “macrophages” and DC-SIGN/CD1b- “DCs”. The latter induced stronger T-cell proliferation and IFN-γ production as compared to the DC-SIGN’CD16+ cells. Moreover, the in vivo association of DC-SIGN+ cells predominantly with LL lesions and the CD1b+ cells with TT and reversal reactions suggested an association with the spectral pathology of leprosy [195]. Similarly, DC-SIGN was reported to be associated with Th2 environment associated with LL in another study [140]. A subsequent study [204] reported that macrophage derived DCs (MACDC) were more efficient than monocyte derived DCs in stimulating T cell responses at low bacterial multiplicity of infection (MOI) with *M.leprae* [204]. Hence the origin of DCs may also influence the subsequent response to infection.

The presence of CD1+CD83+ mature DCs in tuberculoid leprosy lesions suggests a role for DCs in the immune response against leprosy [97; 195]. In in vitro cultures integral *M.leprae* caused down-regulation of MHC class I and II on DCs and induced expression of the maturation marker CD83 only at very high bacterial doses [205]. Furthermore, *M.leprae* was less efficient in DC-mediated induction of T cell responses as compared to *M.bovis* BCG or *M.avium* [205]. In contrast, our preliminary observations showed strong induction of DC maturation by *M.leprae* whole sonicate (MLS). Moreover, these mature DCs were able to induce proliferation of autologous T cells and could produce IL-12 on CD40 ligation. Other groups studying different sub-cellular fractions of *M.leprae* found the cell wall fraction to be toxic to DCs while the cytosol fraction was less efficient in comparison to the membrane fraction in stimulating DCs [206]. The cell membrane fraction, on the other hand, was shown to upregulate MHC Class II and CD86 expression in DC. Furthermore, it could induce strong IFN-γ production in CD4+ and CD8+ T cells and preforin production in *M.leprae* specific CD8+ cytotoxic T lymphocytes [206].
The adaptive immune system

The adaptive immune response requires the specific recognition of foreign antigens which activates the humoral response, resulting in B-cell maturation and antibody production, and the cell-mediated mechanisms, focusing mainly on T-cell activation. These divergent arms of the host response to pathogens are not mutually exclusive as T-cell help is required for antibody maturation and isotype switching while B cells can function as antigen-presenting cells in the induction of specific T cells.

Humoral Immunity (HI)

The study of the humoral immunity in leprosy has largely been restricted to antibodies in immunodiagnosis or monitoring of leprosy and are dealt with in the subsequent sections. Although the in situ presence of plasma cells and B-cells has been reported in leprosy [207; 208; 209], not much is known of the relation to the pathology of leprosy lesions. Recently, the presence of B-cells were reported in lesional skin from both the lepromatous and tuberculoid regions of the leprosy spectrum and this was associated with the detection of *M. leprae*-specific antibodies in supernatants of organotypic cultures of the corresponding lesional biopsies [209]. It was speculated that these lesional B-cells could influence T cell-responses and/or play a role in maintaining the inflammatory reaction in leprosy partly through the local secretion of antibodies [209]. However, functional data supporting such a hypothesis are lacking.

It is generally thought that antibodies against *M. leprae* components do not play a significant role in protection against leprosy. However antibodies may play a role in the uptake of *M. leprae* by mononuclear phagocytes and hence the pathogenesis of the disease [199]. Natural antibodies from non-immune serum were found to be critical for the binding of complement component C1q to PGL-I and the fixation of C3 to *M. leprae* [199]. Previously, C3 was shown to be fixed to PGL-I on *M. leprae* and mediate its uptake through complement receptors on mononuclear phagocytes [174; 210; 211]. These studies together showed that natural antibody promoted complement receptor-mediated uptake of *M. leprae* by host cells. This also suggests that the natural antibody repertoire of an individual may determine the predisposition to leprosy and may even determine the form of leprosy [199]. In this regard, Caron and Hall [89] showed that uptake of IgG-opsonised targets by FcγRII activated the Cdc42, Rac, Rho cascade of Rho GTPases triggering an inflammatory response and respiratory burst within macrophages. In contrast, uptake of complement C3bi-opsonised organisms through CR3 activates only Rho, but not Cdc42 and Rac, with a resultant lack of inflammatory response which might be a plausible mechanism in mycobacterial infections like leprosy.
**Cell-Mediated Immunity (CMI)**

**T-cell subsets**

The induction of a specific T-cell response occurs primarily in the peripheral lymphoid organs, the lymph node and the spleen, where circulating naïve T cells from the blood come in contact with antigens presented by APCs such as dendritic cells. CD4+ T cells which are the dominant players in both the induction and effector phases of the adaptive immune response are activated by presentation of peptides derived from exogenous antigens in the context of MHC Class II molecules. These cells differentiate into T-helper (Th) cells which carry out their effector functions by producing distinct patterns of cytokines as is discussed subsequently. CD8+ T cells comprise the other arm of the effector response of the CMI which recognize antigenic peptides in the context of Class I MHC. Effector CD8+ T cells, also called cytotoxic T lymphocytes (CTL), exert antimicrobial effects either by secretion of cytokines such as IFN-γ and TNF activating T cells or by direct mechanisms such as the perforin/granzyme pathway-mediated lysis of pathogen-infected cells. However, at least a subset of the CD8+ T cells was considered to be of the “suppressor” (Ts) phenotype [212; 213; 214; 215], although, in practice, CTLs and Ts were distinguished primarily on the basis of their phenotype since with the exception of, possibly, the CD28 marker, no specific surface markers have been described for these two subtypes [215; 216].

Histopathologically, CD4+ T cells are the predominant subset in TT leprosy whereas the majority of the T lymphocyte population in LL leprosy are of the CD8+ subtype [212; 213; 214]. Moreover, CD4+ T cell clones from TT individuals proliferated in response to *M. leprae* antigens whereas CD8+ T cell clones from lepromatous individuals did not proliferate but suppressed the proliferation of CD4+ cells [215; 217; 218].

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**Figure 3.** Naïve T cells may develop into various T cell subsets under the influence of polarizing signals (Signal 3, in grey). Furthermore, cytokines secreted by the T cell subsets may act in autocrine or paracrine manner to reinforce the polarization in addition to their effects on other cells.
**Effector mechanisms of cell-mediated immunity: The Th1/Th2 paradigm**

Since the initial description of T-helper 1 (Th1) and Th2 patterns of cytokine production in mouse CD4+ cell subsets [219] and in humans [220; 221] attempts have been made to delineate all immune responses in disease along these lines. In general, Th1 cells are thought to be involved in CMI-DTH reactions and are important in responses against intracellular pathogens. In contrast, Th2 cytokines encourage antibody production, particularly IgE responses, enhance eosinophil proliferation and are effective against extracellular pathogens, parasites and in development of allergic responses [221]. Although the proper balance of Th1 and Th2 immunity is as important for the success of an immune response as its specificity and overall magnitude, it still remains unclear how the Th1/Th2-response profile is matched to distinct pathogens and to particular affected tissues [177]. Leprosy, which displays a spectrum of immune responses ranging from a strong CMI at the TT pole to an almost exclusively humoral response at the LL pole, has been analysed as a model system to study the Th1/Th2 paradigm. Yamamura et al. [222] demonstrated an increased expression of T-helper 2 (Th2) cytokines interleukin-4 (IL-4), IL-5 and IL-10 in skin lesions from LL patients at the mRNA level. In contrast, “protective” Th1 cytokines IL-2, IFN-γ and TNF-α are associated with TT leprosy [222]. Contemporary studies with *M. leprae* responsive CD4+ T-cell clones (TCC), generated from peripheral blood or lesional skin of polar TT patients showed a distinct Type 1 cytokine secretion profile, producing IL-2, IFN-γ and GM-CSF, but little or no IL-4 and IL-5 [215; 223]. In contrast, *M. leprae* non-responsive CD4+ TCC produced little IL-2 and IFN-γ but showed a Type 2 profile secreting IL-4, IL-5 and GM-CSF [215]. Interestingly, polar LL patients showed a subset of IL-4-producing CD8+ clones which suppressed proliferation of *M. leprae* responsive CD4+ clones in an IL-4-dependant manner [215]. Immunohistochemical studies on cytokines in leprosy lesions showed significantly higher expression of IFN-γ in tuberculoid lesions but not lepromatous lesions [224; 225]. The latter studies, however, need to be interpreted cautiously since the specificity of some of the antibodies used may not be optimal [226]. The seemingly clear cut relationship between Type 1- and Type 2-like T cell phenotypes and the polar forms of leprosy has however been disputed subsequently with studies reporting that almost half of the patients showed co-expression of both IL-4 and IFN-γ irrespective of clinical classification [227; 228; 229]. It must be noted that these latter studies involved bulk peripheral blood mononuclear cells stimulated with various *M. leprae* antigens and may not necessarily reflect the situation of cells at the clonal level or within lesions. Our own studies on *in situ* cytokines within borderline leprosy lesions showed the presence of both IFN-γ and IL-4 mRNA and protein [unpublished observations]. RR lesions on the other hand showed increased expression of both cytokines while in ENL, the IL-4 expression was relatively higher than IFN-γ [unpublished observations]. At the clonal level, apart from the polarised Type 1- or Type 2-like phenotypes, a significant
A proportion of the lesional *M. leprae*-responsive TCC showed a Type 0-like cytokine profile, producing both IFN-γ and IL-4 [230]. This shifted to a polarised Type 1-like phenotype during the development of RR, whereas, the *M leprae* non-responsive T cells from the same lesions were heterogeneous consisting of either Type 1-, Type 0- or Type 2-like phenotype [230; 231]. The above results suggest that although there is some evidence for association of polarized Type 1 or Type 2 responses with the leprosy spectrum especially in isolated clonal T cell populations, such distinctions may be less discernable *in vivo* due to the complexity of the induced immune response.

**Regulatory/suppressor cell responses in leprosy**

In addition to the “classical” functional subsets of CD4+ T cells, two additional populations have attracted growing attention, the T-regulatory (Treg) cells and the IL-17 producing Th cells. Tregs represent the anti-inflammatory or suppressive group of CD4+ T cells which may be divided into two main groups: the adaptive Tregs consisting of the IL-10 producing Treg 1 (Tr1) cells and the TGF-β-producing Th3 cells, and the naturally occurring CD4+CD25+ Tregs [232]. Tr1 and Th3 cells are the primary mediators of anti-inflammatory responses against exogenous antigen such as those associated with mucosal immunity. Tr1 and Th3 cells develop through the differentiation of uncommitted naïve Th precursors whereas exposure of CD4+CD25- naïve T cells to TGF-β can mediate their transition towards a CD4+CD25+ regulatory T cell phenotype with similarly potent immunosuppressive qualities, and are more often associated with tolerance to self antigen [232; 233]. While Tregs require antigen specific activation to carry out their effector function, their suppressive effects are mediated in a non-specific manner inhibiting both innate and adaptive immune responses.

Although studies on the association of the “classical” regulatory T cell phenotypes with leprosy are lacking, a *M. leprae* specific suppression of effector cell responses has been described in LL [234]. One of the early studies to report active immunosuppression in leprosy observed the suppression of proliferative responses to Con A in the presence of lepromin in LL and BL patients which was attributed to adherent monocytes and Tγ cells [235]. A different set-up was used by Nath et.al. [236] who studied suppression of lymphoproliferative responses to ConA by *M. leprae* specific lymphocytes from leprosy affected HLA-D identical siblings. Interestingly, lymphocytes from TT but not LL patients whereas macrophages from LL but not TT patients suppressed lymphoproliferation in HLA-D identical normal siblings [236]. Stoner et.al. [234] demonstrated the specific suppression of lymphoproliferative responses to *M. leprae* antigen with some degree of cross reactivity to BCG but not to an unrelated antigen in healthy contacts of leprosy patients. They suggested T-suppressor (Ts) cells as possible candidates associated with this suppression, although they did not directly
demonstrate presence of such cells in their cultures. In a histopathological study, Van Voorhis et al. [212] showed that the T cell infiltrate in LL lesions consisted almost exclusively of OKT8/Leu-2a cells which they considered as “suppressor” T cell populations in contrast to the predominance of OKT4/Leu-3a helper T-cells in TT lesions. This skewed distribution of T cell subsets was also demonstrated in subsequent studies [213; 214]. However no attempt was made in the above mentioned studies to demonstrate the “suppressor” phenotype of the T cell subset in question. Using \textit{M.leprae} stimulated Th and Ts clones from a BL patient, Ottenhoff et al. [237] showed that Ts clones suppressed responses of Th clones to \textit{M.leprae} and other mycobacteria but not to unrelated antigens or mitogens demonstrating the specific nature of the suppression. Subsequent studies however demonstrated that the CD8+ population consisted of distinct subsets which may either be cytotoxic or mediate suppression through IL-4 [215]. Apart from the suppressor role of CD8+ subsets, the induction of CD4+ suppressor T cell subsets by a specific hsp65 peptide (between amino acids 439-448) – HLA-DRB1*1503 combination was also demonstrated indicating that specific peptide-HLA Class II combinations could exclusively activate Ts cells [238]. Despite these indications, the presence and activity of the conventionally accepted Tr1, Th3 and Treg subsets has not been conclusively demonstrated thus far.

\textbf{Th17 cells in leprosy}

Recently, a subset of non-classical IL-17 producing Th cells were shown to contribute to the development of some Type 1 organ specific autoimmune [239] inflammatory or allergic diseases including systemic lupus erythematosus [240]. These cells may represent a separate lineage of Th cells distinct from either of the classical Th1 or Th2 subsets [241; 242]. IL-17 is a member of a family of cytokines with at least 6 members in the human genome, including IL-17, IL-17B, IL-17C, IL-17D, IL-17E and IL-17F [243]. IL-17 is considered a pro-inflammatory cytokine because it increases IL-6, IL-8, nitric oxide, TNF-\(\alpha\) and IL-1\(\beta\) production by various cell types [244; 245; 246; 247; 248]. In addition, IL-17 elicits the secretion of granulocyte-colony stimulating factor (G-CSF) and CXC chemokines that stimulate granulopoiesis and recruitment of polymorphonuclear neutrophils into tissues [249; 250]. IL-17 was expressed in psoriatic lesions; a disease associated with increased expression of Type 1 cytokines [251]. Interestingly, with respect to leprosy, preferential expression of IL-17 mRNA was seen in association with LL lesions [251]. This probably suggests that IL-17 is secreted by Type 0, Type 1 and Type 2 T cells [245; 246]. An alternative explanation could be the lack of specific production of Th1 cytokines such as IFN-\(\gamma\) in LL patients which are involved in the reported suppression of IL-17 [252]
Cross-talk between the cells

Cytokines and chemokines as signaling molecules

The generation and maintenance of immune responses is controlled by a network of small, non-structural, intercellular regulatory proteins, the cytokines and chemokines, which mediate multiple immunologic and non-immunologic functions and are involved in the cross-talk between the different cells of the immune system. These molecules play a crucial role in the recruitment of the immune cells, the clonal expansion of lymphocytes as well as in the innate immune response and the effector response of most immune cells. This results in a complex, fine-tuned regulatory network of cytokines which often determines the clinical course of the infection and the outcome. The role of cytokines in immune regulation and inflammation is fairly well studied as is the role of inflammation as a driver of pathology.

Cytokine cascades and their role in leprosy pathology and immunity

With respect to leprosy, research has focussed on the association of differential cytokine profiles with the spectral pathology [215; 222; 253]. However results from the studies have been varied and conflicting and in retrospect it is difficult to associate distinct cytokine patterns with different spectral forms of leprosy or reactions. Initial studies looked at the association of leprosypathological pathology with the Th1-Th2 cytokine profiles [215; 222; 253], which has been described in detail in the preceding sections. However later studies increasingly demonstrated the lack of a clear cut dichotomy in the cytokine profiles associated with the leprosy spectral forms and reactions [227; 228; 229; 230; 231].

Analyses of leprosy sera showed increased expression of cytokines (except IL-2 in all patients, IFN-γ in LL patients and IL-10 in TT patients) as compared to healthy controls [254; 255] suggesting activation of the immune cells by M.leprae antigens in all leprosy patients. IFN-γ and TNF-α were elevated in TT patients as compared to LL patients which also showed a significant negative correlation with BI in these patients [254]. These results correlated with in vitro studies showing that a vast majority of the T cells in tuberculoid leprosy are of the CD4+ subset and of the Th1 phenotype producing IFN-γ, IL-2 and TNF-α but little or no IL-4, IL-5 and IL-6 on in vitro stimulation with M.leprae or its antigenic fractions [215; 223; 256]. Furthermore, in vivo analyses showed the presence of cytokine mRNA for IFN-γ, IL-2, lymphotoxin (LT), TNF-α and GM-CSF [222; 253; 257] whereas, in mice, mRNA for IFN-γ, IL-2 and TNF-α is detected in the lymph nodes draining the site of vaccination with killed M.leprae [258]. This suggested that mycobacteria preferentially induce Th1 cells for an effective response in both humans and mice. Interestingly, no significant difference was found between levels of IL-2 in TT and LL patients in serum [254], in contrast to the results...
using PCR and in vitro stimulation of PBMCs.

TNF-α is considered to be crucial in host defence against intracellular pathogens [259] and anti-TNF-α antibodies have been shown to suppress CMI in vivo [260]. Moreover, TNF-α was shown to be necessary in granuloma formation and mycobacterial elimination [261]. LL patients showed higher levels of IL-10 and IL-1β as compared to TT patients [254]. In vivo studies have demonstrated the predominance of IL-4, IL-5 and IL-10 in LL lesions previously [222]. IL-10 has been shown to suppress macrophage-mediated destruction of intracellular microorganisms [262]. This was in line with the positive correlation between IL-10 levels and BI in LL patients [254]. Interestingly, IL-4 was reportedly one of the important cytokines in LL patients which induced a suppression of *M. leprae* induced immune reactivity of CD4+ T cell clones in vitro [215]. However such a suppressive activity of IL-4 has not been confirmed by other studies [256], although IL-4 has been shown to play a role in the Th2 polarization of CD4+ T cells [263; 264].

**Recombinant cytokines as an adjunct to MDT in leprosy**

Since activation of macrophages is important in killing of the intracellular *M. leprae*, the role of macrophage activating cytokines like IFN-γ have been studied in this respect. Initial studies suggested that the defective CMI in LL was caused by defective IFN-γ activity in these patients [224; 265]. This defective activity was rather a result of the failure of lymphocyte to produce macrophage activation products in response to *M. leprae* rather than the inability of macrophages to get activated by these products [266; 267; 268]. This was supported by experiments where monocytes from LL patients could inhibit growth of *Legionella pneumophila* when treated with supernatants of Con A activated lymphocytes suggesting that monocyte-activating molecules like IFN-γ from these cultures could activate the LL monocytes normally [266]. Further direct evidence was provided by a study in which in vitro monocyte-derived macrophage cultures from LL patients responded to treatment with recombinant human IFN-γ (rhIFN-γ) in the presence of *M. leprae* by producing H2O2 to the same extent as normal healthy donors [267]. Intralesional injection of rhIFN-γ caused an accumulation of T-cells predominantly of the CD4+ helper phenotype and monocytes at the site which was increased by a second injection of the cytokine, although this could not conclusively be attributed to a direct effect of the IFN-γ [138]. Interestingly, prolonged treatment with rhIFN-γ induced ENL in 60% of the LL patients as compared to 15% of the patients receiving MDT alone [269]. In vitro analyses showed a 3 - 7.5 fold increase in IFN-γ induced TNF-α production by LL patient monocytes which could be suppressed by thalidomide [269]. Similarly, low dose inoculation of rhIL-2 intradermally had clear systemic effects including an elevated number of circulating mononuclear cells, generalized
infiltration into the skin, and subsequent rapid five-fold increase in clearance of \textit{M. leprae} bacilli as compared to MDT alone, without significant side-effects [270]. Considering the reported beneficial effects of these recombinant cytokine therapies, it is surprising that such a line of therapy has not been pursued further.

**Chemokines in cell migration and tissue immunity in leprosy**

Chemokines are potent chemoattractants of various leukocyte subsets and play an important role in migration of effector cells in inflammatory diseases such as tuberculosis [271], sarcoidosis [272], cutaneous leishmaniasis [273] and psoriasis [274]. However, not much is known about the chemokine profiles in tissues and in circulation in leprosy patients. Some of the early studies looked at the induction of IP-10 (CXCL10) on local administration of recombinant human IFN-\(\gamma\) or PPD in LL lesions [275]. Although LL lesions did not express IP-10 constitutively, administration of PPD or IFN-\(\gamma\) into the lesions resulted in a strong induction of IP-10 expression especially by keratinocytes followed by monocytes, fibroblasts and endothelial cells. In contrast, TT lesions and lesions of cutaneous leishmaniasis showed intense IP-10 expression by keratinocytes and infiltrating cells suggesting a differential expression of IP-10 across the leprosy spectrum associated with IFN-\(\gamma\) expression [275]. A subsequent study showed IP-10 to be important in the migration of monocytes and activated T-lymphocytes and also enhanced T-cell adhesion to endothelial cells [276]. However, in contrast to the chemokine RANTES (CCL5), which was a chemoattractant for unstimulated and stimulated T cells, IP-10 appeared to be specific for the CD4^+CD29^+ activated T cell subtype [276] suggesting a distinct role in inducing migration of these cells to the site of inflammation. More recently, Kirkaldy \textit{et.al.} [277] studied the expression of the chemokines MCP-1 (CCL2), RANTES and IL-8 (CXCL8) within leprosy lesions across the spectrum by \textit{in-situ} hybridization (ISH). Although all chemokines were elevated in leprosy lesions, no differences in the level of expression were noted across the spectrum. However, MCP-1 and RANTES were elevated in reversal reactions as compared to non-reactional leprosy suggesting a role for these chemokines in migration and activation of the monocytes and T-lymphocytes in these lesions [277]. In subsequent studies, MCP-1 and IL-8 were found to be elevated in circulation in LL patients [278; 279]. Hasan \textit{et.al.} [280] recently demonstrated elevated levels of MCP-1 in sera of LL patients as compared to endemic healthy controls (EC) or pulmonary tuberculosis patients which was probably related to the extent of dissemination of the disease. In contrast, RANTES levels were significantly lower in the LL patients as compared to EC or TB patients suggestive of a shift away from the Th1 phenotype of these patients [280]. Mendonca \textit{et.al.} [281] reported elevated levels of only CCL3 (MIP-1\(\alpha\)) and CCL11 (Eotaxin) but not CCL2, CXCL9 or CXCL10 in leprosy patients as compared to non-
infected individuals in a Brazilian population. They suggested the utility of CCL11 monitoring in plasma as an aid to the diagnosis of leprosy patients from non-infected populations.

**Treatment of leprosy and reactions**

The present treatment of leprosy using multiple drug treatment (MDT) was in response to increasing rates of primary and secondary dapsone resistance and was based on the experience with tuberculosis. Over the years, the duration of intake of the drugs has been shortened from treatment to skin smear negativity [WHO, 1982] to the present fixed-duration treatment (FDT) [32; 53]. The present recommended standard regimen for MB leprosy is: rifampicin 600 mg. once a month, dapsone 100 mg. daily, clofazimine 300 mg. once a month and 50 mg daily for a duration of 12 months. The treatment regimen for PB is: rifampicin 600 mg. once a month and dapsone 100 mg. daily, whereas PB single lesion is treated with a single dose of rifampicin 600 mg., ofloxacin 400 mg. and minocycline 100 mg. (ROM) [35].

Prompt and adequate treatment of leprosy reactions is essential to avoid permanent damage to peripheral nerves and deformities. Prednisolone is widely used in the control of reactions both RR and ENL. RR and neuritis are treated with a standard 12-week course of prednisolone starting at 40-60 mg. daily and then in gradually reduced dosages every week till the end. Mild ENL is treated with non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin while severe ENL is treated with prednisolone as for RR. Alternatively, thalidomide is also used in the treatment of severe ENL although it is contraindicated in women of child-bearing age due to its teratogenic effects. Clofazimine, which is a component of MDT is also effective against ENL although it is less potent than corticosteroids. The dose of clofazimine used is 300 mg. daily for a duration not exceeding 12 months [35].

**Biomarkers in leprosy**

*Antibody detection in diagnosis and monitoring*

Serology in leprosy has largely focussed on identifying markers that facilitate the diagnosis of cases with few or no clinical symptoms since early detection and treatment can significantly reduce the risk of deformities and disease transmission [282]. Antibody levels have been used as a surrogate marker of bacterial load in numerous studies with widely varying but positive correlation between seropositivity and BI [283]. A significant decrease in misclassification of MB patients was achieved by combining serology with lesion counting [284]. Moreover, since antibody levels decline with treatment at the rate of about 25-50% per
year, measurement of serum antibodies may have some value in monitoring the treatment in leprosy [285; 286; 287; 288; 289].

Detection of serum antibodies directed against various *M. leprae* components have been evaluated for their utility in the diagnosis and monitoring of treatment in leprosy. The most widely used antigens include the *M. leprae* specific phenolic glycolipid (PGL)-I, the 35kD and the cross reactive antigen 85 complex (Ag 85). Since the demonstration of the species-specific PGL-I in the cell wall of *M. leprae* [290], various studies have shown seropositivity in 90% of untreated MB patients (BL/LL) and upto 50% of PB (BT/TT) patients while the proportion of seropositive healthy endemic controls was around 5-10% [282]. The demonstration by Brett *et al.* [291] and Fujiwara *et al.* [292; 293] that the major epitope of PGL-I was the 3,6-di-o-methyl-glucopyranosyl residue situated at the distal disaccharide region led to the development of test systems using synthetic analogues of PGL-I such as ND-o-BSA and NT-p-BSA. In general, the PGL-I ELISA is highly effective in diagnosis of MB patients (LL/BL) showing 90-100% sensitivity whereas its sensitivity for PB (BT/TT) patients is low [282]. Moreover a high variability in seropositivity rates for PGL-I antibodies in different studies may be a reflection of the different antigen types used (native antigen vs neo-conjugates), the criteria used to define a positive result and the inclusion of treated cases in the patient groups [282]. Modifications of anti-PGL-I antibody assays including the *M. leprae* gelatin particle agglutination test (MLPA) [294; 295] and the latex agglutination test (LAT) [296] were evaluated and found to have a good concordance with the conventional PGL-I ELISA. Recently, a simple and rapid immunochromatographic flow test was developed for detection of IgM anti-PGL-I antibodies in serum and whole blood samples from leprosy patients. The flow test was comparable to the PGL-I ELISA in detection of 97% MB patients and 40% PB patients whereas only 9.8% of endemic controls showed seropositivity [297]. However, apart from the PGL-I ELISA, which is used mainly in research settings, none of the above mentioned tests have been implemented widely.

The monoclonal antibody ML04 reacting against the My2a epitope on the 35kD antigen [298; 299], widely used in an inhibition ELISA [287; 300] or a serum antibody competition test (SACT) could detect almost 100% of active BL/LL patients, 40% tuberculoid patients while showing seropositivity in 10% of healthy contacts, It also correlated better with the BI and clinical score when compared with a PGL-I ELISA. A modified dipstick assay for the detection of anti-35kD IgG showed a higher sensitivity as compared to the anti-PGL-I dipstick assay although the latter demonstrated a higher specificity as compared to the former [301]. However, as with the *M. leprae* flow test, these dipstick assays were not developed further probably on account of the limited commercial value, as leprosy is still largely a disease associated with poverty [302].

With the sequencing of the complete *M. leprae* genome [303] a comparative genomics
approach was used to select *M. leprae* candidate antigens which had no known homologue in other mycobacteria and that contained peptide binding motifs covering more than 75% of the known HLA-DR alleles [304]. Five of the twelve selected candidate genes recognised by patient T cells induced significantly higher IFN-γ production in PB and reactional patients as compared to MB patients and negative controls. The additional value of the five post-genomic antigens lies in the ability to detect 71% of exposed healthy contacts which were not identified by the PGL-I IgM antibody assay. Such tests may facilitate development of new tools for the detection of specific *M. leprae* infection.

**DNA and RNA markers in leprosy**

Standard immunological and histological approaches for assessing leprosy have limited value for diagnosing new cases at early stages and for monitoring treatment whereas the availability of the *M. leprae* genome sequence has led to the development of molecular techniques which can reportedly confirm 40-50% of the cases missed by standard histology [305]. Several polymerase chain reaction (PCR) methods have been developed to amplify genes encoding various *M. leprae* proteins such as the 18kD [306], 36kD [307], 65kD [308], leprosy serum reactive protein (LSR) [309], rRNA [310; 311] and repetitive sequences [312]. These assays have been reported to be sensitive to 1-10 organisms and to be positive in 95-100% of BL/LL and 50-70% of TT, BT and I specimens. Using a combination of multiplex-PCR to amplify the 16S rDNA and reverse cross-blot hybridization technique with mycobacterial and species specific probes, Kox *et al.* [313] could rapidly identify mycobacteria as the species level including *M. leprae* with a sensitivity of 97.9% and a specificity of 96.9%. However, comparison of conventional PCR with more recent quantitative real-time PCR techniques did not suggest a significant increase in detection of *M. leprae* using the latter approach [314; 315]. Other techniques such as reverse transcription (RT)-PCR and nucleic acid sequence-based amplification (NASBA) targeting 16S rRNA are reportedly useful in determining viability of *M. leprae* [316; 317].

**Cytokines as biomarkers**

Several studies have been carried out to assess the validity of measuring serum cytokines for detection and monitoring the leprosy spectrum and reactions. Many studies have presented contradictory results with respect to the predominant cytokines involved, which may be related to the different assay conditions, samples and populations examined [318; 319]. Moubasher *et al.* [254] observed that while leprosy patients irrespective of the spectrum showed elevated levels of IL-1β and TNF-α as compared to healthy controls [254]
some degree of differential expression was noted with IFN-γ and TNF-α being elevated in TT as compared to LL sera whereas the opposite response was seen with respect to IL-10 and IL-1β respectively. Interestingly, although no significant difference was observed in IL-2 levels between the patients and controls, IL-2R was found to be elevated in LL as compared to TT patients [254]. Type I reactional patients showed elevated levels of IFN-γ, IL-2R and IL-1β as compared to non-reactional patients liable to such reaction [254; 320; 321; 322] whereas in Type II reactional patients, in addition to the above mentioned cytokines, IL-10 levels were also elevated [155; 223; 254]. Moreover, patients who developed reactions had significantly higher IL-1β levels as compared to those who did not, suggesting a prognostic value of IL-1b measurement in serum in predicting reactions [323].

**Markers of cellular activation**

Besides T cell cytokines, other indicators of cellular activation such as neopterin [324; 325], soluble CD27 (sCD27) [255], chitotriosidase [in this thesis] and acute phase proteins [69] have been used as a marker for cell mediated immune activity in leprosy and other inflammatory diseases. Neopterin belongs to the class of pteridines derived from guanosine triphosphate (GTP) due to the activity of GTP cyclohydrolase I coupled with a relative deficiency of 6-pyruvoyl tetrahydropterin synthase in human and primate macrophages [325]. Interferon-γ is the central stimulus for GTP cyclohydrolase I mediated production of neopterin in macrophages which may also be super-induced by LPS, TNF-α or IL-2 induced IFN-γ production or by GM-CSF-mediated monocyte/macrophage accumulation. Thus the presence of neopterin in body fluids was suggested to be evidence for the activation of the CMI response [325]. Neopterin production in pulmonary tuberculosis correlated with the extent and activity of the disease [326] and was useful in the monitoring of treatment [327]. Elevated levels of serum neopterin were previously reported in 75% of leprosy patients including lepromatous (LL-BL) patients [255] and in particular in reactions [318; 324] as compared to healthy controls. Furthermore, levels of neopterin were found to be significantly elevated in reactional as compared to non-reactional leprosy patients [255; 318]. It is paradoxical that the elevated neopterin, associated with increased IFN-γ production, is also associated with lepromatous forms of leprosy suggesting that the CMI response may not be completely defective in these patients.

Recent studies have shown that human phagocyte-specific chitotriosidase, the first discovered mammalian chitinase, is an important component of the innate immune response [328]. Chitotriosidase, an endoglucosaminidase belonging to family 18 of glycosylhydrolases, cleaves chitin [329]. Chitotriosidase was discovered in sera of Gaucher patients, unable to degrade the glycosphingolipid glucosyleramide within the lysosomes due to a lack of the
lysosomal enzyme acid β-glucocerebrosidase [329; 330]. Consequently, glucosylceramide predominantly accumulates in lysosomes of macrophages; the Gaucher cells [331]. Chitotriosidase serves as a crucial macrophage-derived biomarker to monitor disease onset, progression and therapeutic response in Gaucher disease [332]. Increased chitotriosidase activity has been found within atherosclerotic lesions, in which cholesterol-laden foam cells reside [333]. Elevated serum chitotriosidase activity has been reported in malaria [334] and sarcoidosis [335; 336] as well, but not in pulmonary tuberculosis, a mycobacterial disease [336]. However, chitotriosidase activity has been reported in tuberculous pleural effusions [337], suggesting that chitotriosidase production may be local in these patients. Since the macrophage plays a central role in the control of \textit{M.leprae} infection, evaluation of markers of macrophage activation in body fluids may contribute significantly to the diagnosis and classification of leprosy patients.

Acute phase proteins (APP), which are systemic markers of inflammation, have been evaluated in diagnosis, classification and monitoring of leprosy and reactions. The most frequently assessed APPs in relation to leprosy include serum amyloid A (SAA) and C-reactive protein (CRP). Various studies have shown the limited value of CRP in identification or classification of non-reactional leprosy patients [338; 339; 340; 341]. With respect to SAA there are contradictory results with elevated levels reported in LL as compared to TT patients by Scheinberg \textit{et.al}. [338], whereas other studies did not show a significant difference [340; 341]. However, ENL patients were unanimously shown to have elevated levels of SAA and CRP as compared to non-reactional LL/BL patients and controls suggesting their utility as bio-markers [338; 339; 340; 341]. An important limitation of the use of biomarkers such as CRP, SAA, neopterin, chitotriosidase along with cytokines and other soluble/cellular markers is that they reflect the general inflammatory response and would be expected to change in all immune-mediated conditions, thus lacking disease specificity. Hence, the use of such markers needs to be combined with other markers more specific for the disease such as the anti-PGL-1 assay and with clinical observations in order to obtain a more accurate and global view of the progression of the disease.
Outline of the thesis

Leprosy is a chronic mycobacterial disease which displays a clinical and immunological spectrum determined by the host immunological response against *M. leprae* and its antigenic components. The spectral pathogenesis of leprosy is associated with the differential activation of the immune system. The accepted dogma associates lepromatous leprosy with a predominantly Th2 cytokine profile, favouring a strong humoral immune response, tuberculoid leprosy shows a potent cell-mediated immune response associated with a Th1 cytokine profile. Furthermore, both leprosy reactions, RR and ENL, are reportedly associated with changes in cytokine activity whereas, *M. leprae*-specific T-cell clones isolated from RR lesions showed a polarized Th1-like cytokine profile. The association of discrete cytokine profiles with the spectral forms of leprosy suggests that identification of cytokine profiles associated with spectral leprosy and reactions might be useful in their detection and monitoring of treatment. Chapters 2, 3, 4 and 5 deal with the assessment of various cytokines, cellular activation markers and acute phase proteins in early diagnosis, classification and monitoring of treatment in leprosy and reactions. Chapter 6 deals with an aspect of immunology which is often neglected in leprosy i.e. the presence of B cells in lesional skin across the leprosy spectrum especially in tuberculoid leprosy. This is on account of the prevailing thought that immunity to intracellular infections is almost exclusively a T-cell mediated event. The present study used immunohistochemistry to demonstrate the presence of B cells in leprosy lesions, including BT lesions, together with a human organotypic skin explant model to demonstrate the secretion of cytokines and *M. leprae*-specific antibodies in the lesional skin. The possible implications of the presence of intralesional B-cells and the role of *in situ* antibody secretion in leprosy pathology are speculated upon in Chapter 6. Antigen presenting cells such as dendritic cells (DC) are central to antigen presentation and in polarizing and regulating the types of T-cell mediated immune response to infectious agents and in the induction of peripheral immunological tolerance. The diverse functions of DCs in immune regulation depend not only on the diversity of DC subsets and lineages but also on the functional plasticity of DCs at the immature stage. Chapter 7 pursues the hypothesis that modulation of maturation of dendritic cells, by *M. leprae* components may influence T-cell responses thus determining the spectral pathology of leprosy. Finally, Chapter 8 is a summary of the results presented in the thesis along with a discussion of some of the salient observations in the context of leprosy pathology.
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Immunopathology of leprosy

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Serum levels of interferon-γ, tumour necrosis factor-α, soluble interleukin-6R and soluble cell activation markers for monitoring response to treatment of leprosy reactions

*Clinical and Experimental Immunology
Serum levels of interferon-γ, tumour necrosis factor-α, soluble interleukin-6R and soluble cell activation markers for monitoring response to treatment of leprosy reactions

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Summary
Identifying pathogen and host-related laboratory parameters are essential for the early diagnosis of leprosy reactions. The present study aimed to clarify the validity of measuring the profiles of serum cytokines [interleukin (IL)-4, IL-6, IL-10, interferon (IFN)-γ and tumour necrosis factor (TNF)-α], the soluble IL-6 receptor (sIL-6R), soluble T cell (CD47) and macrophage (neopterin) activation markers and Mycobacterium leprae-specific anti-PGL-1 IgM antibodies in relation to the leprosy spectrum and reactions. Serum samples from 131 Indonesian leprosy patients (82 non-reactional leprosy patients and 49 reactive) and 112 healthy controls (HC) from the same endemic region were investigated. Forty-four (89-8%) of the reactive patients had erythema nodosum leprosum (ENL) while only five (10-2%) had reaction (RR). Follow-up serum samples after corticosteroid treatment were also obtained from 17 of the patients with ENL and one with RR. A wide variability in cytokine levels was observed in the patient groups. However, IFN-γ and sIL-6R were elevated significantly in ENL compared to non-ENL patients. Levels of IFN-γ, TNF-α, and sIL-6R declined significantly upon corticosteroid treatment of ENL. Thus, although the present study suggests limited applicability of serial measurement of IFN-γ, TNF-α and sIL-6R in monitoring treatment efficacy of ENL, reactions it recommends a search for a wider panel of more disease-specific markers in future studies.

Keywords: cytokine measurement, leprosy, neopterin, reactions, soluble receptors

Introduction
Leprosy is a chronic disease displaying an immunological spectrum ranging from tuberculoïd (TT) leprosy, with strong cell-mediated immunity (CMI) against Mycobacterium leprae, to lepromatous (LL) leprosy, showing a complete absence of M. leprae-specific CMI [1]. This spectral pathology of leprosy is associated with differential activation of immune cells in parallel with the production of cytokines, which are the signals between the immune and the resident cells [2]. Previous studies suggested that the T cell responsiveness to M. leprae in LL was caused by defective interferon (IFN)-γ activity in these patients [3,4]. In addition, increased expression of mRNA for T helper 2 (Th2) cytokines interleukin (IL)-4, IL-5, and IL-10 has been shown in skin lesions from LL patients. In contrast, Th1 cytokines (IL-2), IFN-γ and tumour necrosis factor (TNF)-α are associated with TT leprosy [3,6], suggesting that differential cytokine profiles are associated with the leprosy spectrum.

Furthermore, leprosy reactions, namely type 1 or reversal reaction (RR) and type 2 or erythema nodosum leprosum (ENL), are associated reportedly with changes in cytokine activity [2,5]. M. leprae-specific T cell clones generated from RR lesions showed a polarized Th1-like profile [7]. In contrast, a predominant Th2 cytokine profile was observed in LL patients [8]. This suggests that identifying cytokine profiles associated with reactions may help in their early diagnosis and eventual monitoring of treatment efficacy. Many studies have presented contradictory results with respect to the predominant cytokines associated with reactions which may be related to the different assay conditions, samples and populations examined [9-12]. Moreover, in view of the difficulty in obtaining lesional biopsies and peripheral blood mononuclear cells (PBMC) under field conditions
and the relative ease of serum immunosassays, measurement of cytokines and other soluble cellular products in circulation has been used as an alternative in various studies [9-12].

Due to the uncertainty created by the often contradictory previous studies [9-12], the primary aim of the present study was to assess the measurement of a broad panel of soluble ‘biomarkers’ in serum in relation to the leprosy spectrum and reactions. The soluble markers studied included the cytokines (IL-4, IL-6, IL-10, IFN-γ and TNF-α), the soluble IL-6 receptor (sIL-6R), a soluble T cell activation marker (sCD27), a macrophage activation marker (neopterin) and the M. leprae-specific anti-PGL-I IgM antibodies.

Materials and methods

Patients and controls

The study included 131 leprosy patients and 112 normal healthy controls (HC) attending the leprosy clinic at the Hasanuddin University hospital in Makassar, Indonesia. The study was approved by the ethical committee of Hasanuddin University and informed consent was obtained from the patients included. The median age of the patients was 31 years (range: 9–88 years) and included 90 males and 41 females. The median age of the HC was 31 years (range: 19–41 years) and included 91 males and 21 females.

Every patient was assessed clinically by detailed history, medical and dermatological examinations. Bacteriological examination of skin-smear was carried out to determine the bacteriological index (BI). The patients were classified according to Ridley and Jopling’s five subgroup classification [13] as 34 lepromatous (LL), 78 borderline lepromatous (BL), three mid-borderline (BB), six borderline tuberculoid (BT) and 10 tuberculoid (TT) patients. Forty-nine of the aforementioned patients were diagnosed with reactions, of whom 44 were ENL and five RR. ENL was diagnosed by the acute appearance of nodular skin lesions, accompanied by fever with or without peripheral nerve pain and nerve dysfunction. RR reactions presented typically as an acute inflammation of pre-existing lesions and onset of new erythematous skin lesions. For the purpose of comparisons the BL and LL patients without ENL reactions were grouped together as NE (non-ENL, BL/LL, n = 68), as this group of patients is prone to ENL. Similarly, BL, BB and BT patients without RR were grouped as NRB (non-reactional borderline, n = 82), as this group is prone to RR. Thus the BL patients were common to both NE and NRB groups, as potentially they might develop either ENL or RR. The serum profiles of these groups were compared with ENL and RR patients, respectively.

Leprosy was treated with multi-drug treatment (MDT) according to World Health Organization (WHO) guidelines [14]. Reactions were treated using prednisolone, starting at 40 mg/day and gradually tapering off over a period of 12 weeks [15]. Clinical improvement of reactions was defined as complete subsidence of all reactional symptoms. In the absence of biopsy samples, the clinical assessment was performed by the clinician-in-charge, although no elaborate scoring system was used to grade improvement of reactions. Follow-up samples at the end of corticosteroid treatment were obtained from 17 patients with ENL and one patient with RR only.

After informed consent, blood samples were collected by venipuncture, the serum was separated, aliquoted and stored in liquid nitrogen at Makassar until transported to the Netherlands for analysis.

Cytokine assays

The cytokines (IL-4, IL-6, IL-10, IFN-γ, TNF-α), sIL-6R and sCD27 were estimated according to the manufacturer’s instructions using PeliKine enzyme-linked immunosorbent assay (ELISA) kits (Sanquin Reagents, Amsterdam, the Netherlands). Neopterin was estimated using the Brahms ELISA kit also according to the manufacturer’s instructions (Brahms, Henningsdorf, Germany) [16].

Anti-PGL-I IgM assay

Anti-PGL-I IgM antibodies were detected as described in Brett et al. [17] using natural trisaccharide linked to bovine serum albumin via a phenolic ring (NT-P-BSA) as a semi-synthetic analogue of PGL-I.

Statistical analysis

The differences in cytokine levels were compared within the leprosy spectrum, reactional patients and HC. Because the data did not follow Gaussian distribution, the Kruskal–Wallis test was performed to test the differences in cytokine levels across the leprosy spectrum, reactions and HC. Dunn’s post-hoc rank test was used to compare each group of the leprosy spectrum and reactions with HC. As NE and NRB patient groups are prone to ENL and RR, respectively, ENL was compared with NE while RR was compared with NRB and the differences in cytokine levels were analysed using the Mann–Whitney U-test. Correlations between different cytokines were analysed using Spearman’s rank correlation coefficient. A correlation was assumed when r ≥ 0.3 with P < 0.05. The paired t-test was used to compare cytokine levels before and after corticosteroid treatment.

Results

Preliminary statistical analyses suggested no statistically significant effect of MDT status on the cytokine levels in the patients. Hence the patients were grouped into NE, NRB,
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Fig. 1. Levels of interleukin (IL-6) (a), interferon (IFN-γ) (b), tumour necrosis factor (TNF-α) (c) and soluble IL-6 receptor (sIL-6R) (d) in sera of NE (non-eruption nodolous lepromatous leprosy (ENL), NRB (non-reaction borderline), ENL (•) and RR (○) (reversal reaction) leprosy patients and HC (●) (healthy controls). Median value indicated by line (-).

ENL and RR, irrespective of their MDT status to increase the number of patients within each group.

Cross-sectional study of cytokine profiles in the various disease groups

Proinflammatory cytokines (IL-6, IFN-γ, TNF-α)

Significant difference was seen with respect to the overall levels of cytokines IL-6 (P < 0.0001), IFN-γ (P < 0.0001) and TNF-α (P < 0.001) across the patient groups by the Kruskal–Wallis test (Fig. 1–c). Dunn’s post-hoc test showed significantly higher IL-6 and TNF-α compared to HC in NE (P < 0.001), ENL (P < 0.001; P < 0.0001, respectively), and RR (P < 0.001; P < 0.001, respectively). Patients IFN-γ was higher compared to HC in only ENL (P < 0.01) and RR (P < 0.05) patients. No significant difference in IL-6 levels was observed either between NE and ENL (P = 0.91) or NRB and RR patients (P = 0.15), respectively. Similarly, no significant difference in TNF-α levels was observed between either NE and ENL (P = 0.35) or NRB and RR patients (P = 0.19), respectively. A significant difference was observed, however, between IFN-γ levels in NE and ENL patients (P = 0.001) but not between NRB and RR patients (P = 0.13).

Cytokine receptor (IL-6R)

A significant difference was seen with respect to overall levels of soluble IL-6R (P = 0.0011) across the patient groups by the Kruskal–Wallis test. Dunn’s post-hoc test showed significant differences between HC and NE (P < 0.05), but not the other patient groups. ENL patients showed significantly higher levels of sIL-6R compared to NE (P = 0.006), while no significant difference was observed between NRB and RR patients (P = 0.46) (Fig. 1d).

Cytokines IL-10 and IL-4

IL-10 levels showed significant difference across the patient groups (P < 0.001) by the Kruskal–Wallis test. Dunn’s post-hoc test showed significantly higher IL-10 levels in ENL patients compared to HC (P < 0.05) (Fig. 2a). However, no significant difference was observed in IL-10 levels between either NE and ENL (P = 0.43) or NRB and RR (P = 0.37) patients, respectively. A significant difference was found with respect to IL-4 levels across the patient groups (P < 0.0001) by the Kruskal–Wallis test, which could be attributed to a significant difference between BL patients (within the NE group) and HC. However, no difference with respect to IL-4 levels was seen in NE compared to ENL patients (P = 0.53).
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![Diagram with graphs showing interleukins and neopterin levels across different groups.](image)

Fig. 2. Levels of interleukin (IL) 10 (a), IL-4 (b), neopterin (c) and sCD27 (soluble T cell activation marker) (d) in sera of NE (●) (non-erythema nodosum leprosy (ENL)), NRB (■) (non-reactive borderline), ENL (▲) and RR (○) (reversal reaction) leprosy patients and HC (●) (healthy controls). Median value indicated by line (+).

The number of RR samples (n = 2) was not sufficient for a comparison of RR with NRB patients.

**Cellular activation markers**

Neopterin levels showed a significant difference across the patient groups (P < 0.001) by the Kruskal-Wallis test and was higher in NE (P < 0.001), ENL (P < 0.001) and RR (P < 0.05) compared to HC (Fig. 2c) by Dunn’s post-hoc test. However, no significant differences were found in neopterin levels between either NE and ENL (P = 0.56) or NRB and RR (P = 0.67) patients, respectively. Similarly, sCD27 levels showed a significant difference across the study groups (P < 0.001) and was higher in all patient groups compared to HC by Dunn’s post-hoc test (Fig. 2d). No significant difference, however, was observed in sCD27 levels between NE and ENL (P = 0.14) or NRB and RR (P = 0.22) patients, respectively.

**Anti-PGL-1 antibodies**

Anti-PGL-1 IgM antibodies were assayed in sera from 72 patients and eight HC. A significant difference was seen in anti-PGL-1 antibody levels across the patient groups (P = 0.008) by the Kruskal-Wallis test with Dunn’s post-hoc test showing higher levels in ENL (P < 0.01) patients compared to HC. On the other hand, no difference was observed between NE and ENL (P = 0.46) or NRB and RR (P = 0.82) patients, respectively.

**Correlations between different cytokine levels**

**Correlation between proinflammatory cytokines**

A positive correlation was noted between the levels of IL-6 and TNF-α in NE (r = 0.561, P < 0.001) and in ENL (r = 0.534, P = 0.0002) patients. On the other hand, IL-6 correlated with IFN-γ in NE (r = 0.593, P < 0.001) but not in the ENL patients (r = 0.04, P = 0.78). A correlation was also observed between IFN-γ and TNF-α in NE (r = 0.472, P < 0.0001) but not in ENL patients (r = 0.012, P = 0.94).

**Correlation between IL-6 and its soluble receptor (sIL-6R)**

Although IL-6 levels correlated with sIL-6R levels in NE patients (r = 0.393, P = 0.002), no such correlation was observed among ENL patients (r = 0.272, P = 0.07).
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Correlation between IL-10, IL-4 and proinflammatory cytokines

IL-10 correlated significantly with IL-6 in both NE (r = 0.537, P = 0.0065) and ENL patients (r = 0.552, P = 0.0031). However, while a weak correlation was seen between IL-10 and TNF-α in ENL patients (r = 0.343, P = 0.026), no correlation was seen in NE patients (r = 0.251, P = 0.07). IFN-γ levels did not correlate with IL-10 in either patient group (data not shown). None of the proinflammatory cytokines correlated with IL-4 (data not shown).

Correlation between T cell cytokines and soluble T cell activation product (sCD27)

Interestingly, a negative correlation was seen between the T cell cytokine IFN-γ and the sCD27 in both NE (r = -0.5126, P = 0.0027) and ENL (r = -0.6065, P < 0.0001) patients. No correlation was noted between IL-10 and sCD27 serum levels for either patient group (data not shown).

Correlation between the macrophage activation product neopterin and macrophage cytokines

Neopterin levels correlated with IL-6 (r = 0.337, P = 0.047) as well as TNF-α (r = 0.398, P = 0.019) in ENL patients only, although the correlation was not highly significant.

Follow-up of ENL patients during corticosteroid treatment

Cytokine levels were tested at the onset of ENL and at the completion of corticosteroid treatment in 17 patients. Levels of IFN-γ (P < 0.0001), TNF-α (P = 0.02) and IL-6 (P < 0.0001) (Fig. 3a-c), but not anti-PCL-1 antibodies (P = 0.14) (data not shown), declined significantly with corticosteroid treatment and paralleled the clinical improvement of the patients as assessed by the clinician-in-charge.

Discussion

The present study was undertaken to assess the validity of measuring serum cytokines for detection and monitoring the leprosy spectrum and reactions in a field setting. A wide variability was seen in serum cytokine levels within the patient groups and HC. Nevertheless, IFN-γ was associated significantly with reactions and was higher in ENL compared to NE patients. These results are consistent with previous observations in serum [9], as well as those in culture supernatants and mRNA from M. leprae-stimulated and freshly isolated PBMs from ENL patients [8,18,19]. Concurrently, however, another study reported contradictory results showing low serum IFN-γ levels at the onset of ENL [20]. On the other hand, in contrast to the undetectable levels of IFN-γ reported in our previous study [16], a majority of the RR (three of five) patients in the present study showed detectable serum IFN-γ levels. Such variable results could be attributed to the different patient populations involved in the studies. However, no significant difference was observed in IFN-γ levels between RR and NE, as was observed in the previous report [10]. It must be stressed that not too much significance can be attached to the results in RR patients in the present study, due to the low sample size. Despite the above-mentioned conflicting results, elevated levels of IFN-γ in ENL, encountered in this study, suggest involvement of the CM1 response in ENL pathology which is compatible with the findings of previous reports [19]. The association of IFN-γ with ENL pathology is supported further by studies demonstrating development of ENL in LL patients on administration of IFN-γ [21].

The serum levels of IL-6, TNF-α and the macrophage activation product neopterin were higher in leprosy patients compared to HC, which is consistent with increased immune activity in patients. However, in the present study no significant difference in IL-6, TNF-α and neopterin levels was observed between reactive (ENL and RR) and the non-reactive patients (NE and RR, respectively), which is in contrast to previous reports [11,18,22,23]. Soluble IL-6R levels, although lower in patients compared to HC, were elevated in ENL compared to NE. It could be speculated that lower levels of sIL-6R in patients compared to HC may be caused by formation of complexes with IL-6 in these patients, as has been suggested previously in a study of patients with systemic juvenile rheumatoid arthritis [24]. The absence of a significant difference in IL-10 levels between ENL and NE patients is in contrast to previous reports [8,9]. Moreover, unlike the negative correlation observed previously between levels of IL-10 and IFN-γ in
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leprosy sera [9], no such correlation was observed in the present study. Interestingly, the positive correlation between IL-10 and TNF-α in ENL patients is similar to the previous observation of simultaneous expression of IL-10 and TNF-α in skin lesions of patients with RR [25]. This was suggested to be indicative of simultaneous activation of proinflammatory and regulatory pathways through suppressive/regulatory cytokines such as IL-10 [25] and probably reflects a control mechanism to prevent the excessive tissue-damaging effects of the proinflammatory cytokines.

A negative correlation was found between sCD27 and IFN-γ within the patient groups, which is surprising given the fact that both IFN-γ and sCD27 [26,27] are secreted by activated T cells. On the other hand, functional studies have shown loss of CD27 in CD8+ T cells as a result of differentiation to effector cell populations [28,29]. Other studies have reported down-regulation of CD27 expression with repeated antigenic stimulation of T cells [30]. Similar situations could be envisaged in a chronic disease such as leprosy, where T cells are likely to be exposed repeatedly to antigenic stimulation, although in the context of the present study this is merely speculative.

Serum levels of sIL-6R, IFN-γ and TNF-α declined with corticosteroid treatment and paralleled clinical improvement in ENL patients. The one RR patient with a follow-up serum sample also showed a decline in IFN-γ, TNF-α and neopterin with corticosteroid treatment (data not shown). Corticosteroids, the primary treatment modality for reactions [31], cause a decrease in the number of circulating lymphocytes and monocytes and decrease production of cytokines such as IFN-γ [32,33], IL-1 [34], TNF-α [32,35] and IL-2R [34] and neopterin expression [10,23]. However, in our previous study, TNF-α appeared to persist even after the completion of corticosteroid treatment in the majority of RR patients [19]. In contrast, in the present study TNF-α declined significantly in all but two of 17 ENL patients, where an increase with treatment was seen. In this regard, an ongoing study in our laboratory suggested the association of persistent high serum levels of TNF-α at the end of corticosteroid treatment with a probable risk for development of subsequent episodes of RR (unpublished observations), although this observation needs further validation. The increase in TNF-α at the end of corticosteroid treatment in the two exceptional patients is also similar to observations of a previous study, where TNF-α was observed to decline with treatment in RR while in ENL patients it was found paradoxically to increase at the end of steroid treatment [17]. No decline in serum neopterin levels was seen at the end of corticosteroid therapy in ENL patients in the present study, in contrast to previous observations in RR [10,23] and ENL patients [23].

Taken together, the variable results from different studies suggest that the regulation of cytokine secretion is more complex than commonly recognized and can be influenced by the method of analysis. A major limitation of serum analysis is that serum measurements may not reflect adequately the tissue immune response [25]. This may be addressed by alternative approaches, such as human organotypic culture of lesions where biopsies are available [36]. The presence of soluble receptors and other inhibitors could influence the detection of soluble markers which can be assessed using special ELISAs to detect complex formation [24]. The serum biomarkers used in the present study could be supplemented with more conventional markers of inflammation, such as C-reactive protein (CRP) [37]. However, it appears from our ongoing study that serum CRP, despite being associated with ENL, showed no decline during treatment (unpublished observations), suggesting that CRP may not be valuable for monitoring leprosy patients as is usually anticipated with other inflammatory diseases. Moreover, CRP, along with cytokines and other soluble markers used in the present study, reflect the general inflammatory response and would be expected to change in all immunemediated conditions, thus lacking disease specificity. In this regard, the patients in the present study were examined medically for signs of other concomitant infections and skin inflammations and controls from the same area were used to generate baseline values for cytokines and other soluble markers.

In conclusion, the study suggests limited applicability of serial measurement of the cytokines IFN-γ, TNF-α and sIL-6R in monitoring therapeutic efficacy of ENL patients. However, a cautious approach to interpreting serum cytokine profiles and a further search for a wider panel of more disease-specific markers is recommended in future studies.

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References

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Serial measurement of cytokines, cytokine receptors and neopterin in leprosy patients with reactions

Serial measurement of serum cytokines, cytokine receptors and neopterin in leprosy patients with reversal reactions


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Summary Serum levels of cytokines (IL-4, IL-5, IFN-γ, TNF-α), cytokine receptors (TNFR I and II) and one monokine (neopterin) were estimated in seven leprosy patients to establish disease associated markers for reversal reactions (RR). Sera were collected at diagnosis of leprosy, at the onset of reversal reaction and at different time points during and at the end of prednisone treatment of reactions. It was expected that the serum cytokine and monokine profile before and at different time points during reactions would provide guidelines for the diagnosis and monitoring of reversal reactions in leprosy. The cytokines and cytokine receptors were measured by ELISA, whereas a radioimmunoassay was used for neopterin measurement. Six of the seven patients showed increased levels of neopterin either at the onset of RR or 1 month thereafter, and levels declined on prednisone treatment to that seen at the time of diagnosis without reactions. No consistent disease associated cytokine profile was observed in these patients. Interestingly, serum TNF-α levels were increased in the same patients even after completion of prednisone treatment, indicating ongoing immune activity. In conclusion, this study demonstrates that despite cytokines levels in leprosy serum being inconsistent in relation to reversal reactions, serum neopterin measurement appears to be an useful biomarker in monitoring RR patients during corticosteroid therapy.
Cytokines and neopterin in reactions

Introduction

The immunopathology of leprosy is primarily due to immune interaction between subsets of T cells, antigen presenting cells and Mycobacterium leprae antigens. Such interactions produce type I/type 2 cytokines\(^1\)\(^-\)\(^6\) and activated macrophage products (monokines),\(^7\) which act primarily as molecular signals for communication between immune cells and target resident cells. They play a pivotal role in the dynamics of the host immune response and tissue damage. During the chronic course of leprosy, sudden increases in immune activity may occur which are called reactions. These are either type I (reversal reaction, RR), due to an acute increase in the cell mediated immune response, or type II (erythema nodosum leprosum, ENL), described as an immune complex mediated disease.\(^8\) In respect to T cell cytokine responses in leprosy, it was demonstrated that M. leprae responsive T cell clones from RR lesions were polarized to a type 1-like cytokine profile.\(^9\) Similarly, peripheral blood mononuclear cells (PBMC) from ENL patients also displayed a type 1 cytokine secretion profile.\(^10\) These approaches have been useful in understanding the immunopathology of the different disease states of leprosy. However, they are difficult for routine monitoring of clinical states of patients and in aiding diagnosis. In this respect, measurement of serum cytokines provides a simpler and cheaper alternative. Besides T cell cytokines, detection of neopterin, a monocyte/macrophage activation product,\(^7\)\(^,\)\(^11\)\(^-\)\(^13\) was used as a marker for cell mediated immune activity in leprosy and other diseases\(^5\)\(^,\)\(^14\)\(^,\)\(^15\) with elevated levels detected in 75% of leprosy patients.\(^7\) Levels of neopterin were found to be significantly elevated in reactional as compared to non-reactional leprosy patients.\(^11\)

Prednisolone, the drug of choice in the treatment of type 1 reactions, suppresses inflammatory processes, and is of great importance in the recovery of nerve function after the reaction.\(^16\) Corticosteroids usually influence the cytokine milieu in patients with RR, causing a decrease in the pro-inflammatory cytokines IFN-\(\gamma\) and TNF-\(\alpha\) while in one study, levels of the anti-inflammatory IL-10 were increased.\(^6\) High levels of neopterin, seen in four patients with RR and two patients with ENL in another study, were found to decline on corticosteroid therapy.\(^11\) Hence measurement of cytokine levels along with neopterin might be useful in the monitoring of corticosteroid therapy during leprosy reactions.

The goal of the present study was to identify cytokine/monokine profiles in sera or plasma of leprosy patients associated with the onset of reversal reactions and to assess change in these profiles with corticosteroid treatment in the hopes of providing indicators for diagnosing reactions and, further, to monitor the patients during corticosteroid therapy.

Materials and methods

PATIENTS

Seven leprosy patients, classified clinically and histologically according to Ridley–Jopling criteria\(^17\) at the Leonard Wood Memorial Centre for Leprosy Research, Cebu, Philippines were studied. Six of the patients were classified clinically as BL and one as LL. Histopathologically five patients were classified as BL and two as LL subpolar. The BI of the patients at diagnosis varied between 2:8 and 4. Details of the patients are given in Table 1. The age of the patients ranged between 20 and 56 years. These patients, who developed reversal reactions, were studied serially. Reversal reactions were diagnosed clinically and graded as mild, moderate or severe according to the following criteria: mild (1+), slight erythema, slight swelling of
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Table 1. Patients included in the study. ROM = rifampicin (600 mg) + ofloxacin (400 mg) + minocycline (100 mg) monthly. MDT = WHO multibacillary MDT, BI = Bacterial Index, LLs = LL subpolar

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existing lesions, may or may not have slight nerve tenderness; moderate (2+), erythema, oedema of existing lesions with development of new oedematous/erythematous lesions associated with oedema of hands and feet and definite nerve tenderness; severe (3+), marked erythema and oedema of existing lesions, some ulcerating, new oedematous/erythematous skin lesions, some erythematous, definite pain and tenderness of peripheral nerve trunks with fever of 39°C and above, joint pains and muscle pain. Then, six of the patients had reactions of moderate severity and 1 had a reaction of mild severity. Reactions were treated using prednisone, at a dose of 25–30 mg per day, in divided doses. The treatment was gradually tapered off and stopped when the reaction subsided. All patients showed marked improvement on prednisone treatment with complete subsidence of reversal lesions.

Approximately 20 ml of blood was obtained from the patients, with informed consent, under the supervision of one of the investigators (TTF). Serial samples were obtained from the patients at diagnosis and subsequently on manifestation of RR. 1 month, 2 months at the end of steroid treatment and then again 1–2 months after steroid treatment. Collected sera or plasma were shipped in dry ice to the Academic Medical Centre, Amsterdam and stored at −80°C until further analysis.

CYTOKINE/MONOKINE MEASUREMENTS

Cytokines

The assayed cytokines included IFN-γ, TNF-α, IL-4 and IL-5 and cytokine receptors TNF-αR (p55 and p75). The cytokine levels were measured using commercially available kits (Pelikine, CLB, The Netherlands) according to manufacturer’s instructions.

Neopterin

Plasma neopterin levels were measured using a commercially available RIA kit as described previously. The assay is based on the competition of unlabelled neopterin of the plasma or standards and radiolabelled neopterin for the binding sites of a neopterin specific antibody. The concentration of unlabelled neopterin in serum is inversely proportional to the amount of radioactivity in the neopterin-antibody complex. The upper limit of the normal range is approximately 10 nmol/l in serum.
**STATISTICAL ANALYSIS**

The Wilcoxon rank test for paired samples was used to analyse the differences in cytokine/monokine levels in the patients at the different disease stages.

**Results**

Sera of each study patient before the onset of reactions served as controls. The levels of cytokines/monokine in the sera were also compared with levels of these cytokines/monokine in normal healthy individuals irrespective of ethnic origin as reported in literature. The results of the cytokine/monokine assays are shown below.

**CYTOKINES**

Assessment of inflammatory cytokines TNF-α and IFN-γ showed that four of seven patients had TNF-α concentrations between 80 and 130 pg/ml at diagnosis. However, only two of these patients showed sustained high levels when developing RR. Conversely, two patients who did not show detectable TNF-α at diagnosis, had increased levels at onset of RR. No significant difference was found in TNF-α levels before and at onset of RR or during corticosteroid treatment of the patients since higher TNF-α levels were seen at different time points during treatment of reactions and even after completion of treatment in four of the patients (Figure 1). IFN-γ was below the detection limits of the ELISA in most sera and plasma samples tested (data not shown). IL-4 levels varied between 0 and 200 pg/ml at the onset of the disease but no significant difference was noted with the onset of reactions or with the institution of corticosteroid treatment. IL-5 levels were low in all the sera tested (data not shown). TNF-α receptor levels (p55 and p75) showed minor increases at onset of RR though the differences were not statistically significant (data not shown). To sum up, no consistent

![Figure 1](image.png)  
**Figure 1.** Trends in serum TNF-α concentrations in individual patients. CS = corticosteroid (prednisolone).
Immunopathology of leprosy

Figure 2. Trends in serum neopterin concentrations in individual patients CS = corticosteroid (prednisolone). Line indicates cut-off based on a previous study.11

cytokine profile within patient serum could be associated with development of RR or response to corticosteroid treatment of these patients.

NEOPTERIN

The levels of plasma neopterin were elevated in six of seven patients at onset of RR as compared to the levels at disease diagnosis ($P = 0.028$), although the absolute amounts of neopterin detected varied among the patients (Figure 2). These elevated levels dropped significantly at the end of prednisolone treatment to levels found at disease diagnosis in 5/6 patients ($P = 0.018$) (Figure 2). Two patients showed increased neopterin levels after 1 month of corticosteroid treatment. However, this decreased to levels seen at disease diagnosis in both patients after 2 months of corticosteroid treatment. Thus a general trend could be observed, whereby appearance of RR correlated with elevation of neopterin levels, which declined with the institution of corticosteroid treatment. No correlation was observed between levels of neopterin at diagnosis and the duration to manifestation of RR.

Discussion

A panel of cytokines, a monokine and cytokine receptors was assessed in leprosy patients to identify patterns of expression that might be associated with detection and/or monitoring of reversal reactions. The cytokines and receptors were selected to represent commonly known type-1 (IFN-γ, TNF-α) or type-2 T cell cytokines (IL-4, IL-5), cytokine receptors (TNF-R I and II) and a macrophage activation product (neopterin) known to be involved in the immunopathology of leprosy. In the present study, IFN-γ was undetectable in most of the sera
Cytokines and neopterin in reactions

in contrast to a previous study, which reported high serum IFN-γ levels on manifestation of RR. Moderate levels of IL-4, a predominantly type-2 T cell dependent cytokine, were observed during the course of reversal reaction. The presence of IL-4 in the sera of RR patients can be justified on the basis of findings in literature. Although it was difficult to establish a clear pattern, TNF-α concentrations tended to be elevated at different time points during treatment of RR and even at the end of corticosteroid treatment in four of the cases (Figure 1). This probably indicates ongoing immune activity in the patients, though clinically these patients showed complete subsidence of RR with the administration of prednisone.

Nevertheless, our results show that no consistent cytokine profile within individual patient’s serum could be associated with manifestation of RR or response to corticosteroid treatment. In this respect, previous studies on serum cytokine detection in leprosy reactions have often shown contradictory results. Elevated levels of TNF-α were observed in type 1 reactions and found to decline with corticosteroid treatment, which was contradicted by a subsequent study. A very high concentration of circulating IL-2 receptor (IL-2R) was reported in RR patients and marked reduction of this level was observed with corticosteroid treatment. Although elevated IL-2R was also reported in a subsequent study, the level appeared unaffected by corticosteroid treatment. Our study along with that of Moubasher and co-workers are among a few to have assessed a panel of cytokines for obtaining a broader view of the immunological responses associated with reactions. Furthermore, in contrast to most other studies, our study involved longitudinal sampling of the patients before and at fixed time points during the course of RR.

In this study, neopterin showed increasing trend with manifestation of RR and declined with prednisone treatment. A similar trend was reported previously in a retrospective study. The present study thus showed the utility of plasma neopterin assay in the monitoring of RR patients, though not as a predictive parameter. However elevated serum or plasma neopterin levels need to be interpreted with caution especially in view of its reported association with a variety of conditions involving activated cell mediated immunity. Increased neopterin concentrations are prevalent in asymptomatic HIV antibody seropositive individuals and even in apparently healthy subjects from rural Africa showing evidences of subclinical parasitic infections.

In conclusion, it appears from our results that measurement of cytokine profiles within serum/plasma of patients is of limited value in monitoring disease progression or prediction of reactions in leprosy. On the other hand, measurement of macrophage activation markers like neopterin would provide useful supportive data for diagnosis and to monitor response to steroid treatment in reversal reactions.

Acknowledgements

The authors are grateful to the late Professor G. P. Walsh for his unfailing interest and inspiration for the continuation of this work. Further the support of Professor T. van der Pol of the Department of Internal Medicine, AMC-UvA at the initial period of this work is duly acknowledged. This work forms a part of the research programme of ODP-1/ODP2 of the Departments of Dermatology and Pathology, AMC-UvA, the Netherlands, which was supported, jointly by the Netherlands Leprosy Relief Organization, the Q.M. Gastmann Wickers Stichting and the Royal Netherlands Academy of Sciences (KNAW). Finally, the KNAW is duly acknowledged for maintenance support for A. Iyer.
Immunopathology of leprosy

References


Cytokines and neopterin in reactions

Utility of measuring serum levels of anti-PGL-I antibody, neopterin and C-reactive protein in monitoring leprosy patients during multi-drug treatment and reactions

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Utility of measuring serum levels of anti-PGL-I antibody, neopterin and C-reactive protein in monitoring leprosy patients during multi-drug treatment and reactions


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2 Department of Pathology, Academic Medical Centre, Amsterdam, the Netherlands
3 Faculdade de Odontologia da USP, Baia, Brazil
4 Department of Dermatology, Leiden University Medical Centre, Leiden, the Netherlands

Summary

OBJECTIVE To verify the validity of measuring the levels of Mycobacterium leprae-specific anti-phenolic glycolipid (PGL-I) antibody, neopterin, a product of activated macrophages, and C-reactive protein (CRP), an acute phase protein, in serial serum samples from patients for monitoring the leprosy spectrum and reactions during the course of multi-drug treatment (MDT).

METHODS Twenty-five untreated leprosy patients, 15 multi-bacillary (MB) and 10 paucibacillary (PB), participated. Eight patients developed reversal reaction and five developed erythema nodosum leprosum (ENL) during follow-up. The bacterial index (BI) in slit-skin smears was determined at diagnosis and blood samples collected by venipuncture at diagnosis and after 2, 4, 6 and 12 months of MDT. PGL-I antibody and neopterin were measured by enzyme-linked immunosorbent assay, whereas the CRP levels were measured by the latex agglutination method.

RESULTS The levels of PGL-I antibodies and neopterin were higher in the sera of MB than PB patients, which correlated with the patients’ BI. The serum levels of CRP did not differ significantly between the MB and PB patients. The serum levels of PGL-I and neopterin were no higher in reactive patients than non-reactive patients prone to such reactions. However, ENL patients had higher serum CRP levels than non-reactive MB patients. The serum PGL-I antibody levels declined significantly during MDT, in contrast to neopterin and CRP levels.

CONCLUSION Measuring the serum levels of PGL-I antibodies and neopterin appeared to be useful in distinguishing MB from PB patients, whereas monitoring the levels of PGL-I antibodies appeared to be useful in monitoring MB patients on MDT. Measuring serum CRP, although not useful in monitoring the patients, has limited significance in detecting ENL reactions.

Keywords leprosy, Mycobacterium leprae, neopterin, C-reactive protein, multi-drug treatment

Introduction

Leprosy is a chronic infectious disease with a clinical spectrum determined by the host’s cell-mediated immunity (CD4) towards Mycobacterium leprae (Harboe 1994; Modlin 1994). The prevalence of leprosy worldwide has declined significantly since the introduction of the World Health Organization (WHO) recommended multi-drug treatment (MDT) in 1982 (WHO 1982). However, areas of hyper-endemic infection and the high number of new cases detected in many countries (WHO 2006) remain a cause for concern.

The tissue damage occurring in leprosy is exacerbated during episodes of reactions, namely type 1 (reversal reaction (RR)) and type 2 (erythema nodosum leprosum (ENL)) reactions (Naa’s 1994). The acute inflammation associated with reactions causes irreversible tissue damage and nerve destruction; thus, early detection of leprosy reactions is a key priority (Jacobson & Krahenbuhl 1999; Britton & Lockwood 2004). Permanent nerve damage can be prevented provided reactions are detected early and adequately treated (Naa’s 1996).

With the current emphasis on the integration of leprosy control activities into the general health-care services (WHO 2006), we need laboratory markers to detect leprosy patients at the early stages, to aid clinical diagnosis and to monitor treatment efficacy. Several approaches have been attempted, with mixed results. The measurement of serum antibodies to PGL-I, an M. leprae cell wall antigen is specific (Brust et al. 1983) but has limited sensitivity in
Neopterin, PGL-I and CRP in leprosy

Table 1: Severity score of reacational patients

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Table 1: (Continued)

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BI, bacterial index; LL, lepromatous; ENL, erythema nodosum leprosum; TT, tuberculosis; RR, reversal reaction; BL, borderline lepromatous; BB, borderline.

A1, degree of inflammation of skin lesions: 0 = none; 1 = erythema; 2 = erythema and raised; 3 = edema. A2, peripheral oedema (rating to reaction): 0 = none; 1 = mild; 2 = visible, but not affecting function; 3 = oedema affecting function.
A3, nerve pain: 0 = none; 1 = pain on activity; 2 = pain at rest; 3 = pain disturbing sleep.
A4, nerve tenderness, worst affected nerve only: 0 = none; 1 = mild tenderness; 2 = withdrawal or winching; 3 = not allowing palpation.

has been previously used as a marker for increased cell-mediated immune activity in leprosy and other diseases (Marr et al. 2002; Hoffmann et al. 2003). Increased levels of neopterin are associated with both ENL and RR (Hamerla et al. 1999; Fabel et al. 2004). Similarly, increased levels of the acute phase C-reactive protein (CRP) have been associated with ENL (Foss et al. 1993). Other studies looked at the profiles of serum cytokines associated with the leprosy spectrum and reactions with often contradictory results (Moubasher et al. 1998; Fabel et al. 2004). Thus, our aim was to verify the validity of measuring anti-PGL-I antibody, neopterin and CRP levels in monitoring the leprosy spectrum and reactions during the course of MDT in serial serum samples from patients.

Materials and methods

Patients

Twenty-five untreated patients (13 females; age range 38–76 years), attending the leprosy clinic at the Instituto Lauro de Souza Lima, Rova, Brazil, agreed to participate in the study. Every patient was clinically assessed by taking a detailed history and thorough medical and dermatological examination. Slit-skin smears were also bacteriologically examined to determine the bacterial index (BI). Patients were classified based on the clinical findings and histopathological examination (Ridley & Jopling 1966) and included lepromatous (LL, n = 7), borderline lepromatous (BL, n = 2), borderline (BB, n = 6), borderline tuberculoid (BT, n = 5) and tuberculoid (TT, n = 5)
Table 2. Median values of PGL-I, neopterin and CRP in the sera of MB and PB leprosy patients. Values in parentheses indicate the range.

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<th>MDT (months)</th>
<th>PGL-I (OD)</th>
<th>Neopterin (nm)</th>
<th>CRP (mg/dl)</th>
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<td>PB</td>
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PGL-I, CRP, C-reactive protein; MB, multi-bacillary; PB, paucibacillary; MDT, multi-drug treatment.

*Significant values.
Neopterin, PGL-I and CRP in leprosy

Wells of ELISA plates were coated with either the semi-synthetic antigen PGL-I - NTP-BSA (natural trisaccharide coupled to bovine serum albumin through a phenol group) (0.01 mg/ml carbonate buffer per 50 μl per well, overnight at 4 °C) or with coating buffer (control). After blocking with 1% milk-PBST (phosphate buffered saline tween 20), a dilution of each sample equivalent to 1:500 serum in PBST containing 10% normal goat serum (NGS) was included (50 μl per well, 37 °C, 60 min) in four wells (a pair of each of antigen coated and buffer coated). The plates were washed with PBST and incubated with 1:10 000 dilution of peroxidase-conjugated goat anti-human IgM (Cappel/Organon Teknika, Turnhout, Belgium) in PBST-10% NGS (50 μl per well, 37 °C, 60 min).

After another wash, the colour reaction was developed with 50 μl of substrate solution, o-phenylenediamine, was added to the wells (at room temperature for about 20 min). The reaction was stopped with 50 μl of 2.5 N H₂SO₄ and the absorbance was read at 490 nm. The mean absorbance of the control wells subtracted from that of the wells with NTP-BSA. The result was regarded positive if the optical density (OD) exceeded 0.200.

The serum neopterin levels of the patients were assessed using a commercially available ELISA kit (Neopterin ELISA, BL, Germany) according to the manufacturer’s instructions (Westermann et al. 2000). This ELISA is based on the competition of unlabelled neopterin from the patients’ sera with enzyme-labelled neopterin for the binding sites of a neopterin-specific antibody. The upper limit of the normal range is approximately 10 nm (Hamelink et al. 1999).

The serum levels of CRP were assayed by the latex agglutination method using the rapid-latex CRP kit (Omega Diagnostics, Scotland, UK) according to manufacturer’s instructions. The sera that showed agglutination were diluted for measuring the titre. A positive result for CRP was reported if the level was at least 0.6 mg dl.

Statistical analysis

The differences in the anti-PGL-I IgM, neopterin and CRP levels were compared within the leprosy spectrum and in reactional patients. As the data did not follow Gaussian distribution, the Kruskal–Wallis test was performed to test the differences in anti-PGL-I IgM, neopterin and CRP levels across the leprosy spectrum and reactions. The NE patients are prone to ENL and NRB patients to RR. Hence ENL was compared with NE, while RR was compared with NRB. The differences in anti-PGL-I IgM, neopterin and CRP were analysed using the Mann–Whitney U test. Repeated measures of analysis of variance (ANOVA) was performed to test whether a significant change occurred in

Figure 1 Comparisons of PGL-I (a), neopterin (b) and C-reactive protein (c) in the sera of reactional and non-reactional patients.
the levels of anti-PGL-I IgM, neopterin and CRP in serial serum samples. Correlations between anti-PGL-I IgM, neopterin, CRP and 3H were analysed using the Spearman's rank correlation coefficient. Differences were considered significant when $P < 0.05$.

Results

Of the 25 patients, 13 developed a reaction during the 12 months of follow-up. Eight had type 1 and five had type 2 reactions. Six of the patients who developed type 1 reaction and all patients with type 2 reaction were MB.

Serum PGL-I

The PGL-I levels were significantly higher in MB patients before and at all time points during treatment (Table 2). In untreated patients, the serum PGL-I levels and 3H correlated strongly ($r = 0.72, P < 0.0001$). However, the serum PGL-I levels did not differ significantly between NE and ENL ($P = 0.90$) or NRB and RR patients ($P = 0.85$) (Figure 1). The PGL-I levels fell significantly ($P = 0.006$) in MB but not in PB patients during the 12-month follow-up with MDT treatment (Figure 2). In PB patients, the levels remained low throughout the period of follow-up.

Neopterin

The serum neopterin showed a similar trend to PGL-I, with levels being significantly higher in MB than in PB patients before and throughout treatment (Table 2). There was a significant correlation between the serum levels of neopterin and 3H of the untreated patients ($r = 0.60, P = 0.001$), but not between NE and ENL ($P = 0.11$) or NRB and RR (Mann-Whitney, $P = 0.1$) (Figure 1). The neopterin levels did not fall significantly during MDT (Figure 3).

C-reactive protein

In contrast to PGL-I and neopterin, the CRP levels did not differ significantly between MB and PB patients (Table 2). Moreover, no significant correlation was seen
Neopterin, PGL-I and CRP in leprosy

Figure 3 Serial measurements of neopterin levels in NE (a), NRB (b), ENL (c) and RR (d) patients during MDT. NE, non-reactional leprosy; NRB, non-reactional borderline; ENL, erythema nodosum leprosum; RR, reversal reaction; MDT, multi-drug treatment.

between the CRP levels and BL in the patients (r = 0.26).
ENL patients, however, had significantly higher levels of
CRP than NE patients (P = 0.03). No such difference
was noted between NRB and RR patients (P = 0.49)
(Figure 1). Furthermore, the CRP levels did not decline
during MDT (Figure 4).

Discussion
We assessed the laboratory markers, which can be used as
an adjunct to the clinical monitoring of leprosy patients on
MDT and those developing reactions. The PGL-I IgM
levels were significantly higher in MB patients than PB
patients and showed a strong association with the bacterial
load, confirming the findings of Oskam et al. (2003),
Roche et al. (1993) and Stefani et al. (1998). The PGL-I
levels showed a trend towards significant decline during
the 12 months of follow-up (Cho et al. 1991; Roche et al.
1993; Stefani et al. 1998). Our results suggest an important
role for the measurement of anti-PGL-I antibodies in
distinguishing MB from PB leprosy, as reported previously
(Oskam et al. 2003).

Neopterin showed a trend similar to PGL-I with
significantly higher levels in MB than PB patients and
correlating significantly with the bacterial load. This is
contradictory to a previous report where no such
difference between neopterin levels in MB and PB was
observed (Hamerlink et al. 1999). We found no signif-
cant difference in the neopterin levels between non-
reactional and reactional patients, which contrasts with
previous reports where ENL (Hamerlink et al. 1999)
and RR (Hamerlink et al. 1999; Faber et al. 2004)
patients had significantly higher serum levels of neopterin
than non-reactional leprosy patients. In contrast to
Hamerlink et al. (1999), we exclusively compared ENL
with NE (LL and BL without reactions), while RR was
compared with NRB (BB and BT) as NE and NRB
are prone to ENL and RR, respectively. The neopterin
levels did not decline significantly during the 12 months of follow-up of patients on MDT.

Neopterin is a monocyte/macrophage activation product and often used as a marker of CMI activity (Mürr et al. 2002; Hoffmann et al. 2003). The CMI plays an important role in determining the leprosy spectrum. PB leprosy is associated with strong CMI, which declines progressively towards the lepromatous end of the spectrum (Riley & Jopling 1966; Medlin 1994; Jacobson & Krabenhohl 1999; Britton & Lockwood 2004). Hence, it seems paradoxical that we detected higher levels of neopterin in MB patients. These higher neopterin levels could be a result of the sheer large numbers of macrophages recruited in MB leprosy, a more generalized disease. On the other hand, PB leprosy has a more limited tissue distribution of macrophages resulting in lower overall neopterin levels in circulation despite a strong activation of the CMI.

C-reactive protein, the prototypical acute phase protein in humans, has been used to detect acute infections, assess response to treatment and evaluate the inflammatory response in chronic diseases, such as vasculitis and rheumatoid arthritis (Marnell et al. 2005). The serum CRP has been reported to be elevated during ENL reaction (Foss et al. 1993; Hussain et al. 1995; Merton et al. 1996). Similarly, we saw a significantly higher level of CRP in ENL patients than in NE patients (Merton et al. 1996). However, no difference in the serum CRP levels was observed either between MB and PB or RR and NRB patients nor was there any significant decline in the levels during the course of the MDT.

A major limitation of the measurement of serum neopterin and CRP in leprosy patients is the lack of disease specificity, as both products are indicative of general inflammation and likely to be elevated in all immune-mediated diseases (Hussain et al. 1995; Mürr et al. 2002; Hoffmann et al. 2003; Marnell et al. 2005). Hence, the significance of serum neopterin and CRP measurement need to be interpreted with caution. Nevertheless, on the basis of previous studies (Foss et al. 1993; Faber et al. 2004; Marnell et al. 2005), measurement of neopterin and
Neopterin, PGL-I and CRP in leprosy

CRP levels are likely to be valuable in monitoring the treatment of acute reactional episodes in serial samples, rather than as diagnostic biomarkers. Similar limitations are also involved in the measurement of other markers of cellular activation, such as cytokines, as indicators of reactions (Faber et al. 2004). Hence, further studies are needed to search for other bio-markers which would help in early detection of reactions in leprosy.

A limitation of our study is the small sample size, which may have resulted in some of the smaller differences between the groups to be missed owing to the lack of power of the statistical analysis. A study involving a larger number of patients is therefore desirable to unveil subtle changes in the level of the soluble products associated with the different patient groups.

In conclusion, PGL-I and neopterin serum measurements may be useful in distinguishing MB from PB patients, which can aid in choosing treatment. The CRP levels appear to have some value in the detection of ENL reactions. The results suggest a need for further research to identify other new laboratory markers for diagnosis and monitoring of leprosy and reactions.

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Immunopathology of leprosy


Increased chitotriosidase activity in serum of leprosy patients: Association with bacillary leprosy

Submitted
Increased chitotriosidase activity in serum of leprosy patients:

Association with bacillary leprosy

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Running title: Chitotriosidase activity in leprosy serum

Key words: Chitotriosidase, leprosy, macrophages, neopterin

Abbreviations: Bacteriological index (BI), mid-borderline (BB), borderline lepromatous (BL), borderline tuberculoid (BT), cell-mediated immune response (CMI), erythema nodosum leprosum (ENL), healthy controls (HC), lepromatous leprosy (LL), lipoarabinomannan (LAM), multibacillary (MB), multi-drug therapy (MDT), non-ENL BL/LL (NE), non-reactional borderline (NRB), paucibacillary (PB), phenolic glycolipid - 1 (PGL-I), reversal reaction (RR), tuberculoid leprosy (TT), Mycobacterium leprae (M.leprae).
Summary

Human phagocyte-specific chitotriosidase is associated with several diseases involving macrophage activation. Since macrophage activation plays an important role in the control of *Mycobacterium leprae* infection, we studied the association of chitotriosidase with leprosy both in serum and in situ in lesional skin biopsies from patients. Serum samples from 77 Indonesian leprosy patients (38 non-reactional and 39 reactional leprosy patients) and 39 healthy controls (HC) from the same endemic region were investigated.

The patients were classified as multibacillary (MB, *n* = 69) or paucibacillary (PB, *n* = 9) based on the bacterial index in slit-skin smears. Thirty-six of the reactional patients had erythema nodosum leprosum (ENL), while only 3 had reversal reaction (RR). Follow-up serum samples after corticosteroid treatment were also obtained from 17 patients with ENL and one with RR. Multibacillary (MB) patients showed increased chitotriosidase activity in serum as compared to paucibacillary (PB) patients and healthy controls. Although no significant difference was observed between reactional and the corresponding non-reactional groups, ENL showed significantly higher chitotriosidase activity as compared to HC. Furthermore, corticosteroid treatment resulted in significant decline of enzyme activity in ENL sera. Chitotriosidase activity correlated with levels of neopterin, another macrophage activation marker, but not with IL-6, IFN-γ, TNF-α and IL10. Immunohistochemical staining of 6 MB (LL = 5, BL = 1) lesional skin sections from archival material showed positive staining for chitotriosidase within lipid-laden macrophages suggesting that macrophages are the source of the enzyme detected in serum. Thus, serum chitotriosidase activity is potentially useful in distinguishing MB from PB leprosy and in monitoring response to therapy in ENL.

Introduction

An important aspect of leprosy is its unique spectral pathology [1,2,3]. At one pole lepromatous leprosy (LL) presents with disseminated lesions characterized histopathologically by a predominant macrophage infiltrate with foamy changes and a high load of acid-fast *Mycobacterium (M.) leprae* [3]. Tuberculoid (TT) leprosy, at the other end of the spectrum, has a limited distribution, with lesions showing granulomas of epithelioid macrophages and multi-nucleated giant cells surrounded by a cuff of lymphocytes, with no detectable *M. leprae* [3]. During the chronic course of leprosy, sudden increases in immune activity may occur which are called reactions. These are either type I (reversal reaction, RR), due to an acute increase in the cell mediated immune response (CMI), or type II (erythema nodosum leprosum, ENL), described as an immune complex mediated disease with involvement of the CMI.

Early studies provided evidence for a defect in the CMI in LL using an in vitro lymphocyte transformation assay [4]. A strong CMI response to *M. leprae* was however
found in TT and contacts of leprosy patients (except those of LL patients treated less than 6 months) [5]. Since the CMI response is the primary line of defence against intracellular pathogens like mycobacteria, its defect in LL patients has important consequences for disease progression. In TT patients, the strong CMI limits the spread of *M. leprae*, but sometimes results in damage to nerves as a bystander effect [3].

It is ironical that the macrophage, which is an important component of the CMI response, is also one of the preferred host cells for *M. leprae* [6]. Different survival mechanisms have been described for *M. leprae*, either through a defective host macrophage [7] or an active subversion of the host defences [8,9]. Indirect evidence, however, suggests that macrophage activation is not completely defective in lepromatous leprosy patients since elevated levels of neopterin, a marker of macrophage activation, are observed in sera of LL – borderline lepromatous (BL) patients [10] and leprosy reactions [10,11,12], as compared to healthy controls.

Recent studies have shown that human phagocyte-specific chitotriosidase, the first discovered mammalian chitinase, is an important component of the innate immune response against fungal pathogens [13]. Chitotriosidase is an endoglucosaminidase belonging to family 18 of glycosylhydrolases and cleaves chitin [14]. The enzyme was discovered in sera of Gaucher patients, who lack the lysosomal enzyme acid β-glucocerebrosidase and therefore are unable to degrade the glycosphingolipid glucosylceramide within the lysosomes [14,15,16]. Chitotriosidase serves as a crucial macrophage-derived biomarker to monitor disease onset, progression and therapeutic response in Gaucher disease [17]. Increased chitotriosidase activity has been found within atherosclerotic lesions, in which cholesterol-laden foam cells reside [18]. Elevated serum chitotriosidase activity has been reported in malaria [19] and sarcoidosis [20,21] as well, but not in pulmonary tuberculosis, a mycobacterial disease [21]. However, chitotriosidase activity has been reported in tuberculous pleural effusions [22], suggesting that chitotriosidase production maybe local in these patients.

Since macrophage activation plays an important role in the control of *M. leprae* infection, we studied the association of chitotriosidase with leprosy both in serum and *in situ* in lesional skin biopsies from patients.

**Materials and Methods**

**Patients and controls**

The study included 78 leprosy patients of which 72 attended the leprosy clinic at the Hasanuddin University hospital in Makassar, Indonesia. Thirty-six normal healthy controls (HC) residing in the same area as the patients were also included in the study. The
Chitotriosidase in leprosy

study was approved by the ethical committee of the Hasanuddin University and informed consent was obtained from the patients and HC. In addition, serum and skin biopsy specimen were also available from 6 patients from archival material at the Academic Medical Center, Amsterdam, The Netherlands which were used for concomitant immunohistochemistry to study the localization of chitotriosidase within the immune cells and chitotriosidase activity within serum (Table 1). The median age of the patients was 29 years (range: 14-80 years) and included 51 males and 27 females. The median age of the HC was 28 years (range: 19-41 years) and included 28 males and 8 females.

Every patient was clinically assessed by detailed history, medical and dermatological examinations. Bacteriological examination of slit-skin smears was carried out to determine the bacteriological index (BI). The patients were classified according to Ridley and Jopling’s five sub-group classification [1] as 23 lepromatous (LL), 43 borderline lepromatous (BL), 3 mid-borderline (BB), 2 borderline tuberculoid (BT) and 7 tuberculoid (TT) patients. Patients with a BI > 0 were were further grouped as multibacillary (MB, n = 69) and included all the LL, BL and BB patients, whereas those with BI = 0 were grouped as paucibacillary (PB, n = 9) and included the BT and TT patients. Thirty-nine of the patients were diagnosed with reactions of which 36 had type II /erythema nodosum leprosum (ENL; LL=17, BL=19) and 3 had type I / reversal reaction (RR; BB=2, BT=1). ENL was diagnosed by the acute appearance of nodular skin lesions, accompanied by fever with or without peripheral nerve pain and nerve dysfunction. RR reactions typically presented as an acute inflammation of pre-existing lesions and/or onset of new erythematous skin lesions. For the purpose of comparisons the BL and LL patients without ENL reactions were grouped together as NE (non-ENL BL/LL, n= 34) since this group of patients is prone to ENL. Similarly, BL, BB and BT patients without RR were grouped as NRB (non-reactional borderline, n= 25) since this group is prone to RR. Thus the BL patients were common to both NE and NRB groups since potentially they might develop either ENL or RR [10]. The serum profiles of these groups were compared with ENL and RR patients respectively.

Leprosy was treated with MDT according to World Health Organization guidelines [23]. The study group thus consisted of 30 untreated and 48 treated patients (Fig. 1b). Reactions were treated using prednisolone starting at 40 mg/day and gradually tapering off over a period of 12 weeks [23]. In the absence of biopsy samples in the Indonesian patients, the initial clinical assessment as well as assessment of improvement was done by the clinician-in-charge. Clinical improvement of reactions was defined as complete subsidence of all reactional symptoms. Follow-up samples at the end of corticosteroid treatment were obtained from 17 patients with ENL and 1 patient with RR.

After informed consent, blood samples were collected by venipuncture, the serum was separated, aliquoted and stored in liquid nitrogen at Makassar until transported to the
Netherlands for analysis. No concomitant biopsy specimen could be obtained from the Indonesian patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Classification</th>
<th>Reaction</th>
<th>B.I.</th>
<th>Chitotriosidase n.mol.mL⁻¹.hr⁻¹</th>
<th>Immunostaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>LL</td>
<td>ENL</td>
<td>n.a.</td>
<td>396</td>
<td>+</td>
</tr>
<tr>
<td>A2</td>
<td>LL</td>
<td>ENL</td>
<td>n.a.</td>
<td>115.3</td>
<td>+</td>
</tr>
<tr>
<td>BR15</td>
<td>LL</td>
<td>ENL</td>
<td>4</td>
<td>531.3</td>
<td>+</td>
</tr>
<tr>
<td>BR39</td>
<td>BL</td>
<td>-</td>
<td>4</td>
<td>136.2</td>
<td>+</td>
</tr>
<tr>
<td>BR43*</td>
<td>LL</td>
<td>-</td>
<td>5</td>
<td>251.4</td>
<td>+</td>
</tr>
<tr>
<td>BR47</td>
<td>LL</td>
<td>ENL</td>
<td>5</td>
<td>456</td>
<td>+</td>
</tr>
</tbody>
</table>

**Key**

n.a. : not available  
* : representative immunostainings depicted in Fig. 3

Table 1. Chitotriosidase levels in 6 MB patients

**Cell culture**

Monocytes were isolated and cultured as described previously (13). Briefly, the cells were cultured for 7 days in RPMI 1640 (BioWhittaker, Viviers, Belgium) supplemented with 10% human serum (HS) (BioWhittaker) to allow for chitotriosidase induction in the presence or absence of dexamethasone at the following pre-determined optimal concentration of 2 nM (Sigma-Aldrich Chemie BV, The Netherlands). CD163 cell surface expression was determined by flow cytometry according to standard procedures on a FACS Calibur and data were analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA, USA).
**Chitotriosidase assay**

Chitotriosidase enzyme activity was determined in sera of leprosy patients, or in cell lysates, using the fluorescent substrate 4-methyl umbeliferyl (4-MU) β-D-N,N’,N’’-(MU)-triacylchitotriose [24]. Activities were calculated as nanomoles per millilitre per hour. Due to the absence of a cDNA source of the leprosy and control patients we were not able to screen for chitotriosidase deficiency and have decided to use actual enzyme activities. To exclude chitotriosidase deficient individuals from our analysis we did not take into account enzyme activities below 5nmol/ml*h.

**Cytokine and soluble serum marker assays**

The measurement of cytokines IL-6, IL-10, IFN-γ, TNF-α, cytokine receptor (sIL-6R), sCD27 and neopterin has been described previously [10,25].

**Anti-PGL-I IgM assay**

Anti-PGL-I IgM antibodies were detected as described in Brett et al. 1986 [26] using natural tri-saccharide linked to bovine serum albumin via a phenolic ring (NT-p-BSA) as a semi-synthetic analogue of PGL-I.

**Immunohistological techniques**

Formalin-fixed, paraffin embedded tissues (6μm) were stained by the Haematoxylin- Eosin (HE) technique for a light microscopic examination of the tissue morphology. The immune infiltrate was characterized by immunohistochemical staining as described earlier [27]. Briefly, the sections were deparaffinized and preincubated with 3% H2O2 in 0.01% sodium azide to inhibit endogenous peroxidase activity. Antigen retrieval for CD68 staining was carried out by treating the slides with 0.25% pepsin in 0.01M HCl for 10 minutes at 37°C. The sections were blocked with normal goat serum. The antibodies used for the stainings were CD68 (Dako, Denmark), mycobacterial lipoarabinomannan (LAM, clone F30-5, from Dr. A. Kolk, KIT-Biomedical Center, The Netherlands), M.leprae specific phenolic glycolipid -I (PGL-I, clone 47-21, from Dr. A. Kolk, KIT Biomedical Center, The Netherlands) and chitotriosidase (mouse monoclonal clone CH1, IgG2b). Primary mouse monoclonal antibodies were applied to the specimen followed by incubation with biotinylated rabbit anti-mouse Ig and subsequently horse radish peroxidase (HRP) labelled streptavidin. The colour reaction was developed using 3 amino-9 ethyl carbazole (AEC) as a substrate. The sections were counterstained with haematoxylin. The percentage of the various cellular populations in the lesions were determined by light microscopy in three randomly selected fields of sequential sections.

The double stainings were carried out to study the localization of chitotriosidase in
relation to the macrophages. The staining methods were similar to that used by Verhagen et al [27]. The antibodies used in the staining were chitotriosidase (mouse monoclonal clone CH1, IgG2b) and the HAM-56 clone (Dako, Denmark) to stain macrophages, (since staining for CD68 on paraffin sections required antigen retrieval, which might affect the staining for chitotriosidase).

Statistical Analysis
Since the data did not follow Gaussian distribution, the Mann-Whitney U test was performed to compare levels of the serum markers and chitotriosidase activity between MB and PB patients and HC. Moreover, since NE and NRB patient groups are prone to ENL and RR respectively, ENL was compared with NE while RR was compared with NRB and the differences in levels of serum markers and chitotriosidase activity were analyzed using the Mann-Whitney U test. Chitotriosidase activity in sera of patients before and after treatment of ENL were compared by the Wilcoxon Rank Test for paired samples. Correlations between different serum markers and chitotriosidase activity were analysed using the Spearman’s rank correlation coefficient. A correlation was assumed when \( r > 0.4 \) with \( P < 0.05 \).

Results

**Increased chitotriosidase activity in serum of leprosy patients**

Serum chitotriosidase activity was significantly elevated in leprosy patients as compared to healthy controls from the same leprosy endemic area (\( P < 0.0001; \) Fig 1a). The patients were classified as MB, PB, ENL, RR, NE and NRB as described in detail in materials and methods. Previously we have shown that multi drug therapy (MDT) status did not significantly change levels of serum cytokines and receptors [10]. Similarly, in the present study, statistical analysis revealed that the MDT status did not significantly affect chitotriosidase activity in the patients (Fig. 1b). Hence, phenotypic patient groups were assigned irrespective of their MDT status to have statistically significant numbers of patients within each group. Median activity of serum chitotriosidase was significantly higher in MB (median: 100.5, range 3-2362) as compared to HC (median: 16, range 2.3-16) (\( P < 0.0001 \)), but no difference was found between PB (median: 24, range 8-83) and HC. A significant difference in chitotriosidase activity was also observed between MB and PB patients (\( P = 0.011 \)) (Fig. 1c). Whereas serum chitotriosidase activity was elevated in ENL (median: 105, range 3-2362) (\( P < 0.0001 \)) as compared to HC, no significant difference was noted between RR (median: 8, range 4-24) and HC. On the other hand, while RR showed significantly lower chitotriosidase activity as compared to NRB (median: 99, range 10-544) (\( P = 0.031 \)), no difference was noted between NE (median: 99, range 6-544) and ENL (Fig. 1d). The latter observation should be interpreted with caution however, because the number of RR patients in the study is low.
Chitotriosidase in leprosy

Serum samples from 17 patients with ENL and 1 with RR were available at the onset of reaction and at the end of corticosteroid treatment. Chitotriosidase activity was observed to decline significantly following corticosteroid treatment in ENL (median: 24; range: below detection-360) \((P = 0.002)\) (Fig. 2a) and in the single RR patient (data not shown) and was not significantly different from the activity observed in HC. Furthermore, we found that
in vitro polarization of human monocytes towards macrophages with the corticosteroid dexamethasone prevented chitotriosidase induction (Fig 2b). The dexamethasone-driven polarization of the macrophages was confirmed, for instance, by analysis of the induction of CD163 at the cell surface (Fig 2c).

**Fig. 2.** Effect of corticosteroid treatment on chitotriosidase activity. Chitotriosidase activity in ENL before (■) and after (▲) treatment with prednisolone in comparison with HC (▼). Chitotriosidase activity declined on prednisolone treatment of ENL to levels observed in HC (A). Chitotriosidase activity during dexamethasone induced polarization of human monocytes towards macrophages. Chitotriosidase activity was suppressed (B) concomitantly with induction of CD163 (C) at the macrophage cell surface

**Chitotriosidase activity correlates with neopterin in leprosy serum**

The results of a cross-sectional analysis of serum focusing on cytokines and soluble cellular markers have been described elsewhere [10]. A summary of the serum cytokines and soluble cellular markers is included in Table 2. Here, we analysed whether serum chitotriosidase activity correlated with any of the soluble markers of the above mentioned cross-sectional analysis. Serum chitotriosidase activity correlated well with another macrophage activation product, namely neopterin (r = 0.48, P = 0.0003) (Table 3). Chitotriosidase activity did not correlate with the cytokines IL-6 (r = 0.02), IL-10 (r = 0.19), IFN-γ (r = 0.16), TNF-α (r = -0.10), the soluble cytokine receptor sIL-6R (r = 0.21) or sCD27 (r = 0.10) (Table 2). Neither BI (r = 0.36) nor levels of anti-PGL-I IgM antibodies (r = 0.11) correlated with chitotriosidase activity in sera of the patients (Table 3).
### Table 2: Median levels of cytokines, cytokine receptor, cellular activation products, PGL-I and chitotriosidase across the disease groups

<table>
<thead>
<tr>
<th>Classification</th>
<th>IL-6 pg/ml</th>
<th>sIL-6R ng/ml</th>
<th>IL-10 pg/ml</th>
<th>IFN-γ pg/ml</th>
<th>TNF-α pg/ml</th>
<th>sCD27 U/ml</th>
<th>Neopterin n.mol/L</th>
<th>Chitotriosidase n.mol.mL⁻¹.hr⁻¹</th>
<th>PGL-I O.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB</td>
<td>54.1</td>
<td>39.6</td>
<td>10.9</td>
<td>6.3</td>
<td>17.4</td>
<td>142.6</td>
<td>24.2</td>
<td>100.5</td>
<td>0.091</td>
</tr>
<tr>
<td>0-8002</td>
<td>0-106.8</td>
<td>0-410.8</td>
<td>0-20.9</td>
<td>0-613.2</td>
<td>55.3-655.5</td>
<td>3.7-116.2</td>
<td>(3-2362)</td>
<td>0.003-2.812</td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>20.2</td>
<td>51.5</td>
<td>0</td>
<td>9.3</td>
<td>0</td>
<td>157.5</td>
<td>9.8</td>
<td>24</td>
<td>0.015</td>
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<td>0-147.2</td>
<td>15.7-84</td>
<td>0-34.6</td>
<td>0-13.9</td>
<td>0-379.5</td>
<td>121.2-220.8</td>
<td>5.4-24.9</td>
<td>(8-83)</td>
<td>0.005-0.207</td>
<td></td>
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<td>NE</td>
<td>113.6</td>
<td>31.39</td>
<td>23.2</td>
<td>0</td>
<td>0</td>
<td>260.2</td>
<td>22.6</td>
<td>99</td>
<td>0.091</td>
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<tr>
<td>(0-8002)</td>
<td>(5.4-88.4)</td>
<td>(0-623.2)</td>
<td>(0-127.7)</td>
<td>(0-613.2)</td>
<td>(108.3-1062)</td>
<td>(4.3-116.2)</td>
<td>(6-544)</td>
<td>(0.004-1.396)</td>
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<td>ENL</td>
<td>72.7</td>
<td>44.96</td>
<td>14.7</td>
<td>2.5</td>
<td>15.8</td>
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<td>(5.6-186.5)</td>
<td>(0-171.3)</td>
<td>(0-15.4)</td>
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<td>(728.6)</td>
<td>(3.9-11.9)</td>
<td>(23-42)</td>
<td>(0-0.027)</td>
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Key: The values represent the median of the different parameters measured with ranges in parentheses.
Chitotriosidase

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<td>PGL-I</td>
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Table 3  Correlation of chitotriosidase activity with cytokines, PGL-I and BI in leprosy patients

**Immunohistochemical analysis of chitotriosidase in tissues of MB and PB patients**

As chitotriosidase is expressed by human macrophages and also stored in specific granules of polymorphonuclear neutrophils [13], immunohistochemical staining was performed to identify the source of the observed increase in chitotriosidase activity in leprosy patients. Positive staining for chitotriosidase protein, which appeared to be localized within macrophages, was seen in lesional skin biopsies from 6 MB patients (Figs. 3a and 3e). However, observation of serially stained sections showed that only a sub-population of the CD68+ cells expressed chitotriosidase protein, suggesting that chitotriosidase activity was only upregulated in a subset of macrophages. This positive staining in tissue sections corresponded to the high serum levels of the enzyme seen in these patients as depicted in Table 1. Immunohistochemical double staining of corresponding serial sections with antibodies to chitotriosidase and the macrophage-specific antibody HAM56 showed co-localization, confirming the macrophage localization of the enzyme (Fig. 3b). This suggests that the macrophages, present in the skin, are the source of the chitotriosidase activity detected in serum of leprosy patients. To study the localization of *M. leprae* associated antigens in relation to chitotriosidase, serial tissue sections were also stained for mycobacterial LAM (Fig. 3c) and the *M. leprae* specific glycolipid PGL-I (Fig. 3d). Positive staining for both antigens was seen in foam cells, which were also positive for the macrophage marker CD68 in serial sections (Fig. 3e), suggesting that these antigens are also localized within macrophages.
Fig. 3. Immunohistochemical staining of lesional skin from a MB patient (BR43). Single staining for chitotriosidase (A); double staining for chitotriosidase (blue) and macrophages (HAM56) (red), ↑ shows double stained cells (B); single staining for LAM (C); PGL-I (D) and CD68 (E). Magnifications 20X
**Discussion**

Human phagocyte-specific chitotriosidase activity is associated with several diseases involving macrophage activation [14,28,29] and is a valuable tool for monitoring the efficacy of therapy in Gaucher’s disease [15]. The crucial role of the macrophage in leprosy prompted us to look at chitotriosidase activity in the sera of leprosy patients and healthy controls mainly from leprosy endemic areas in Indonesia. The present report is, to our knowledge, the first one to describe an association of serum chitotriosidase activity with leprosy.

Serum chitotriosidase activity was elevated in MB as compared to PB patients or healthy controls in the present study. MB leprosy is characterised by an abundant macrophage infiltrate with many macrophages showing foamy morphology depending on the chronicity of the disease [1,3]. Representative immunohistochemical staining of skin biopsies from 6 MB patients (LL/BL) with high serum chitotriosidase activity showed positivity for chitotriosidase, which was localized within the macrophages (Fig. 3a, b), suggesting that these macrophages are the active producers of chitotriosidase activity detected in MB serum although observations on single stained serial sections showed only a subset of the CD68+ macrophages to be positive for chitotriosidase. Similar observations have also been reported for macrophages in atherosclerotic plaques, where only a sub-population of the cells are positive for chitotriosidase [18]. Chitotriosidase activity was higher in ENL as compared to HC although no significant difference was observed between ENL and non-ENL MB which suggests that chitotriosidase activity is independent of the reaction state of the patient. However, surprisingly, RR patients showed a trend towards decreased chitotriosidase activity as compared to NRB patients. Although this apparently contradictory result may reflect the inherently different nature of the two reactions, where RR is associated with an increased CMI whereas ENL is thought to be an immune complex disease with some involvement of the CMI, it needs to be interpreted cautiously on account of the low number of RR patients in the study.

Recently, it has been suggested that chitotriosidase RNA expression levels, derived from Kupffer cells in the liver from individuals suffering from non-alcoholic steatohepatitis and simple steatosis, correlate amongst others with TNF-α RNA expression [30]. In the present study, serum chitotriosidase activity did not correlate at all with serum cytokines (IL-6, IL-10, IFN-γ, TNF-α), the IL-6 receptor, or the T-cell activation marker CD27, in leprosy patients (Table 3). The lack of association between chitotriosidase and other indicators of inflammation suggested that chitotriosidase activity could be regulated differently. It could be speculated that chitotriosidase induction is a response to lipid accumulation within the macrophages [31] irrespective of the cytokine environment in the surrounding tissue. A positive correlation was observed, however, between chitotriosidase activity and serum levels of neopterin, another macrophage activation associated molecule (Table 3) [32]. Elevated
Chitotriosidase in leprosy

levels of serum neopterin were previously reported in leprosy patients in general [10,11] and in particular in reactions [11,12]. As observed for chitotriosidase [17-20], elevated levels of serum neopterin were also reported in diseases associated with macrophage activation like malaria [33,34], tuberculosis [35] and coronary artery disease [36]. Serum neopterin levels were significantly higher in sarcoidosis patients with progressive disease (Stage II) as compared to patients with no indications for corticosteroid therapy (Stage 0 or Stage I) [37]. Similarly, increased chitotriosidase activity was observed in Stage II and Stage III sarcoidosis patients in a separate study [20]. Serum chitotriosidase activity also correlated with neopterin levels in Gaucher’s patients although it was weaker as compared to the correlation with angiotensin converting enzyme (ACE), adenosine deaminase (ADA) and β-hexosaminidase respectively [38]. Interestingly, in the present study, serum chitotriosidase activity did not correlate with either anti-PGL-I IgM levels or with the BI of the patients. This suggests also that the chitotriosidase activity does not reflect the actual bacterial load, but is a measure of accumulation of the lipid antigens such as LAM and PGL-I (Figs. 3d, e) in the macrophages. This may in turn explain why chitotriosidase activity persists even after completion of MDT in the patients, since M. leprae antigens such as LAM and PGL-I are known to persist even after completion of MDT in the patients [27]. However, in the absence of functional data, these suggestions remain speculative.

Chitotriosidase activity declined in patients with ENL on treatment with prednisolone. This was supported by in vitro data showing lack of induction of chitotriosidase during dexamethasone-induced polarization of monocytes to macrophages. Our previous study with the same patient group [10] showed a decline in serum IFN-γ, TNF-α and sIL-6R but not neopterin on corticosteroid treatment of ENL. This suggests that although chitotriosidase and neopterin are both products of macrophage activation, regulation of their expression may be different as also reported elsewhere [38]. In this regard, neopterin has been proposed as a marker of an active CMI response [32] since it is induced in macrophages in response to IFN-γ from T-cells. On the other hand, chitotriosidase is elevated in response to lipid accumulation within alternatively activated macrophages as is seen in Gaucher disease [31].

We have emphasised in an earlier report that a major limitation of analysis of cytokines and activation markers is that serum measurements may not adequately reflect the tissue immune response [10,27]. However, in the present report, high serum levels of chitotriosidase corresponded to positive staining for the enzyme within foamy macrophages in the MB leprosy patients. Another limitation is a lack of disease specificity whereby cytokines and other soluble markers studied in the present report may change in other immune-mediated conditions [10]. Similarly, whereas both neopterin and chitotriosidase be elevated in diseases involving macrophage activation, chitotriosidase may also be elevated in other lipid accumulation disorders [15,17-20]. Hence, neither cytokines, neopterin nor
chitotriosidase activity measurements can be used for the diagnosis of leprosy and associations of these markers with disease conditions should be interpreted with caution. However, these molecules may be useful prognostic markers in monitoring the response to corticosteroid therapy in reactions. In this regard, the patients in the present study were medically examined for signs of other concomitant infections and skin inflammations and general health conditions and controls from the same area were used to generate baseline values.

The present study does not shed any light on the role of chitotriosidase in leprosy pathology. In this respect, van Eijk et. al. [13] previously demonstrated the anti-fungal activity of human chitotriosidase in vitro and in vivo in mouse models of systemic Candidiasis and systemic Aspergillosis. Another report observed that deficiency in chitotriosidase was also associated with infections with Wuchereria bancrofti, the causative agent of filariasis [39]. This led to the suggestion that human chitotriosidase is a component of the innate immunity involved in protection against chitin containing pathogens. However, M.leprae is not known to contain chitin in its cell wall/membrane fraction [40]. It is interesting to note that variants of chitotriosidase affecting its activity are associated with Gram-negative bacteremia in children undergoing therapy for acute myeloid leukemia, suggesting more pleiotropic effects of chitotriosidase [41]. Alternatively, increased chitotriosidase in leprosy may be an indirect phenomenon related to lipid overloading of lysosomes in macrophages. However, in the context of the present study, these suggestions remain purely speculative and need to be studied further at a functional level.

In conclusion, serum chitotriosidase activity was observed to be associated with multibacillary leprosy and may be potentially useful in monitoring response to therapy in ENL reactions.
Acknowledgements
George Gussenhoven is acknowledged for performing the anti-PGL-I antibody ELISAs. Dr. Michael Tank is gratefully acknowledged for help with the statistical analysis. The Royal Netherlands Academy of Sciences (KNAW), the Q.M.Gastmann Wichers Stichting and the Netherlands are kindly acknowledged for maintenance support for Anand Iyer. The study will form a part of the Ph.D. thesis of Anand Iyer at the University of Amsterdam, the Netherlands.
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Immunopathology of leprosy
Leprosy-specific B-cells within cellular infiltrates in active leprosy lesions

Leprosy-specific B-cells within cellular infiltrates in active leprosy lesions

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Keywords: B-cells; Leprosy lesions; Cytokines

Summary Leprosy is a spectrum disease with polar lepromatous and tuberculoid forms correlating with enhanced humoral and cell-mediated immunity, respectively, against Mycobacterium leprae and the borderline forms, borderline lepromatous, multiborderline, and borderline tuberculoid showing intermediate clinical and immunological characteristics. Histopathologically, the cellular infiltrates of leprosy lesions show predominantly the presence of interacting T-cells and antigens presenting cells like macrophages, whereas the presence of B-cells has only been sporadically reported. The present study demonstrates by immunohistochemical techniques the presence of B-cells, including plasma cells, in active lesiona from lepromatous leprosy, skin smear negative borderline lepromatous, and paucibacillary borderline tuberculoid leprosy. Furthermore, the study demonstrates the in situ production of M. leprae—specific antibodies from BT lesions using an organotypic skin explant culture model. Finally, analysis of the cytokine release profile in supernatants of lesional organotypic skin cultures showed a microenvironment conducive to the differentiation and maturation of B-cells. The results demonstrate the presence of different functionally active B-cell stages within lesions of patients with leprosy, including borderline tuberculoid patients, which could secrete anti-M. leprae—specific antibodies. However, their role in leprosy pathology remains to be elucidated.

1. Introduction

Leprosy is a spectrum disease with the polar lepromatous (LL) and tuberculoid (TT) forms and the borderline forms including borderline lepromatous (BL), multiborderline (MB), and borderline tuberculoid (BT) forms showing clinical and immunological characteristics between the polar forms [1]. Immunity to intracellular infections like leprosy is largely regarded as a T-cell-mediated event. TT characterized by a strong T-cell-mediated immunity resulting in restriction of
the spread of Mycobacterium leprae by granuloma formation and is often self-resolving [2]. On the other hand, lack of specific T-cell-mediated immunity results in the growth of M leprae associated with LL [3]. Several studies have reported high levels of circulating antibodies to M leprae antigens such as phenolic glycolipid (PGL)-1, 18-, 38-, and 36- kDa in LL and BL leprosy [4], which is thought to be a consequence of a polarized T helper 2 response in lepromatous patients. However, the role of antibodies in the pathogenesis of leprosy is poorly understood, although contacts of patients with leprosy who were positive for anti-PGL-1 antibodies were thought to be at an increased risk for developing clinical leprosy [5]. Furthermore, immune complexes consisting of M leprae-specific antibodies with M leprae antigens have been implicated in the pathogenesis of acute exacerbations of leprosy such as erythema nodosum leprosum reactions [6].

The orchestrated role of T-cells, macrophages, and antigen-presenting cells, such as dendritic cells, are well known to be of primary importance in leprosy pathology [7]. Although presence of plasma cells in LL and BL lesions has been reported [8], the importance of their in situ presence related to the pathology of leprosy has never been elucidated. Interestingly, the presence of plasma cells has also been occasionally reported in histological sections of leprosy reactions and TT/BT lesions [9] despite anti-M leprae antibodies being undetectable in circulation among 50% to 60% TT and BT patients with leprosy [10]. Lai et al. [11,12] reported previously the detection of M leprae-specific antibodies in supernatants of cultured leprosy lesions from patients with leprosy incubated in medium for 48 hours. However, the experimental setup did not clarify whether active release of antibodies into the supernatant was actually involved. A later study demonstrated secretion of anti-M leprae antibodies from full-thickness skin cultures of lesions from BL/LL patients [13]. Although these studies indicated the presence of M leprae-specific antibodies in lesional tissues, no systematic attempt was made to relate the presence of antibodies with histological or immunohistochemical identification of B-cells/plasma cells within tissue sections of the lesions. Moreover, secretion of antibodies in paucibacillary (BT/TT) lesions has seldom been studied.

Against this background, the present study explored, by immunohistochemical analysis, the in situ presence of B-cell subsets within active lepromatous skin of patients with leprosy on multidrug treatment (MDT), particularly in those of a BT or bacteriologically negative BL classification. The functional entity of the B-cell infiltrates within the lesions was determined by studying M leprae-specific antibody release and the local cytokine milieu within lesional biopsies using the human organotypic skin culture model established in our laboratory [14].

2. Materials and methods

2.1. Patients

Lesional skin biopsies were obtained from a total of 15 patients with leprosy (1 LL, 4 BL, and 10 BT patients) with active lesions attending the outpatient department of the National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra, India. The clinical diagnosis of the patients was confirmed histopathologically according to the Ridley-Jopling classification [11]. Patients were treated with World Health Organization-MDT regimens for a minimum of 6 and 12 months, respectively, for the paucibacillary (BT) and multibacillary (BL/LL) patients [15]. All borderline (BT/BL) patients had active lesions at the time of the biopsy, as defined symptomatically by the appearance of swollen lesions with well-demarcated margins, histopathologically showing large numbers of infiltrating cells, despite being bacteriologically negative. Only the LL patient was bacteriologically positive with bacteriological index of 3+ (indicating 110 acid fast bacilli in 1 oil immersion field). Three of the BT patients had reversal reaction at the time of biopsy. All the patients, except one untreated BT, were under MDT treatment, having received between 2 and 6 months of therapy at the time of the biopsy. Skin biopsies from 10 normal individuals, neither having leprosy nor any other skin lesions, being subjected to cholecytectomy, served as healthy controls (HC). Ethical clearance for the present study was obtained following the guidelines of the Indian Council for Medical Research, and informed consent of the patients was obtained before inclusion in the study.

2.2. Antigens

M leprae isolated from armadillo liver were kindly supplied by Prof P.J. Brunner, College of Veterinary Medicine and Biomedical Sciences, Department of Micro-

Table 1: Characterization of the immune infiltrates

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</tr>
<tr>
<td>T-cell</td>
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<td>Macrophages</td>
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</tr>
<tr>
<td>CD68</td>
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<td>PGL-1</td>
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* Semiquantitative scores based on distribution of positive cells in the infiltrate: 0 = negative; 1 = 1 to 3 cells in the section; 2 = 3 to 10 cells per granuloma, few granulomas; 3 = 10 to 50 cells per granuloma; 4 = 50 to 100 cells per granuloma; many granulomas.

* Membrane and cytoplasmatic staining within macrophages.

* Diffused cytoplasmatic staining in few epitheloid cells.

* Strong membrane and cytoplasmatic staining of macrophages and some extracellular staining.
Fig. 1  Representative immunohistochemical stainings of skin sections from a LL (left panel) and a BT patient (right panel) for B-cell subsets: CD20 (A and E), CD79 (B and F), CD138 (C and G), isotype controls (D and H) (magnification, ×10; areas enclosed by boxes enlarged ×40 in insets).
2.3. Processing of skin biopsies

Skin biopsies (1 × 1 cm) were obtained from the periphery of lesions and divided into 2 equal halves. One half was fixed in 10% formaldehyde and processed further for histopathological and immunohistochemical analysis. The other half was used for organotypic culture as described previously [14].

2.4. Immunohistological techniques

Formalin-fixed, paraffin embedded tissues (6 μm) were stained with the hematoxylin-eosin technique for a light microscopic examination of the tissue morphology. The immune infiltrate was characterized by immunohistochemical staining as described earlier [17]. Briefly, the sections were preincubated with 3% H2O2 in 0.01% sodium azide to inhibit endogenous peroxidase activity. The sections were pretreated, when required, with enzymes or heat treatment to expose the antigenic epitopes followed by blocking with normal goat serum. Primary mouse monoclonal antibodies to different leukocyte surface antigens or to mycobacterial antigens, cross-reactive lipoarabinomannan (LAM), and M leprae-specific PGL-1 were applied to the specimen followed by incubation with biotinylated rabbit antimouse immunoglobulin (Ig) and subsequently horse radish peroxidase (HRP)-labeled streptavidin. The color reaction was developed using 3 amino-9 ethyl carbazole as a substrate. The sections were counterstained with hematoxylin. The percentage of the various cellular populations in the lesions was determined by light microscopy in 3 randomly selected fields of sequential sections. The antibodies used for the stainings were CD3 (Immunologic, the Netherlands), CD4 (Lab Vision Corp, UK), CD8, CD20, CD79, CD68 (Dako, Denmark); CD138 (IQ products, the Netherlands); LAM (clone F50-5), PGL-1 (clone DZ-1) (Dr. A. Kolk, the Netherlands); cutaneous lymphocyte antigen (CLA; clone HECA-452, Dr. A. Dijver, the Netherlands). Antigen retrieval for CD3, CD8, CD79, and CD138 antigens was carried out by microwaving the sections in 0.01 mol/L sodium citrate buffer, pH 6.0, for 10 minutes at 99°C; whereas for CD68 staining, the slides were treated with 0.25% pepsin in 0.01 mol/L HCl for 10 minutes at 37°C.

The double stainings were carried out to study the localization of T-cells in relation to the B-cells and to observe the extent of skin homing receptor CLA expression on B-cells and T-cells. The staining methods were similar to that used by Bos et al [18]. The immunostained sections were evaluated by 3 independent observers, including 1 experienced pathologist, and scored as indicated in Table 1.

2.5. Organotypic culture of the skin

The protocol was in accordance to that used by Pistoor et al [14]. Briefly, 5-mm full-thickness skin biopsies were placed on cellulose nitrate membrane filters (Millipore, Billerica, MA; 1.2-μm pore size) on a sterile stainless steel grid in a 25-mm disposable Petri dish. Five hundred
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Briefly, flat-bottomed ELISA plates (Nunc, Roskilde, Denmark) were coated with 50 μL of MLESA at a concentration of 10 μg/mL in 0.05 mol/L carbonate buffer, pH 9.6, and incubated at 37°C for 4 hours, then overnight at 4°C. Plates were preblocked with 1% bovine serum albumin (Sigma Chemical Co, St. Louis, MO, USA) followed by addition of 50 μL of 1:10 diluted culture fluid in duplicate for 2 hours at 37°C. The plates were probed with 50 μL of HRP-conjugated rabbit antihuman IgG (1:1000, Dako, Carpinteria, CA, USA) for 90 minutes at 37°C. The color reaction was developed with o-phenylene diamine dihydrochloride substrate solution (Sigma Chemical Co), stopped with 7% H₃PO₄, and the optical density was measured at 492 nm using a Titertek Multiplate Plus ELISA reader (Flow Laboratories, High Wycombe, UK).

2.7. Measurement of cytokines

Levels of a panel of cytokines, that is, interleukin 4 (IL-4), IL-6, IL-10, interferon γ (IFN-γ), and tumor necrosis factor (TNF-α) were measured in the sequential culture fluid of the organotypic cultures by specific sandwich ELISA systems described previously [19]. Briefly, flat-bottomed ELISA microtiter plates (Costar, Cambridge, MA, USA) were coated overnight with the specific mouse antihuman cytokine monoclonal capture antibody in 0.1 mol/L carbonate buffer (pH 9.6, U-Cytech, Utrecht, Netherlands). The plates were washed, preblocked with 3% bovine serum albumin, and incubated with the organotypic culture fluids or standard antigens in duplicate. The plates were washed and incubated with biotinylated detection antibody followed by HRP-labeled streptavidin (CLB, Amsterdam, The Netherlands). Tetramethyl benzidine (Sigma Chemical Co) was used as the color substrate, and the reaction was stopped with an equal volume of 1 mol/L H₂SO₄. The absorbance was read in a Bio-Rad microplate reader (Richmond, CA, USA) at 450 nm with a reference wavelength of 655 nm.

2.8. Statistical analysis

The Student t test was used to assess the statistical significance of differences in antibody and cytokine levels over the culture period.

3. Results

3.1. Characterization of lesional cells

A comparison of BL/LL and BT lesions with respect to distribution of cell types and antigens is shown in Table 1. Distribution of the infiltrating cells and M. leprae antigens PGL-1 and LAM in the lesions are described below.

3.1.1. Macrophages (CD68)

CD68 staining was intense as both membrane and cytoplasmic staining and widely distributed over the granuloma and in foamy macrophages in BL/LL patients. In BT

Fig. 3 M leprae-specific antibody secretion in organotypic culture fluids: (A) autodaved (●) and unautodaved (□) halves of a BL lesion biopsy—the antibody secretion from the unautodaved lesion peaked at 24 hours and declined subsequently, whereas no antibody secretion could be seen in the autodaved tissue indicating active secretion of antibodies; (B) lesion biopsies from BT lesion (□) and HC (●)—significantly higher antibody secretion was seen in culture fluids from BT patients as compared with HC.

microtiter plates of serum-free RPMI 1640 medium (GIBCO, Invitrogen, Carlsbad, CA, USA) was added underneath the grid, thus maintaining the tissue in an air-liquid interface during the culture period. The tissue was incubated at 37°C in a 5% carbon dioxide atmosphere. The culture fluid was collected every 24 hours from beneath the biopsy and replenished with fresh medium. Collected culture fluids were immediately frozen at −20°C until further analysis.

2.6. Measurement of anti-M leprae IgG antibodies by ELISA

The anti-M leprae IgG antibodies released into the culture fluid from the skin biopsies were measured by ELISA as described earlier with some modifications [15].
B-cells in leprosy

Fig. 4 Proinflammatory cytokines IFN-γ (A), TNF-α (B), and IL-6 (C) in organotypic culture supernatants of BT (●), BL/LL (○), leprosy lesions and skin from HC (●)–IFN-γ and TNF-α levels were higher in BT supernatants as compared with BL and HC at 24 hours and declined at subsequent time points. IL-6 levels were significantly higher in BT patients compared with HC and BL at 48 hours and declined subsequently, whereas HC showed increased secretion of IL-6 at 96 hours, which declined but still remained above levels seen in patients at the end of 120 hours.

granulomas, CD68 staining was diffused within epithelioid cells in the center of the granulomas (Table 1). Double staining of CD68+ cells for M. leprae antigens LAM and PGL-1 showed the antigens in association with the CD68+ cells, some of which were near the T-cells and B-cells (data not shown).

3.1.2. T-cells (CD3, CD4, and CD8)

Single stainings for CD3 showed intense positive staining in BT patients, with 70% to 80% of the infiltrating cells showing this marker, whereas the staining was moderate in the BL/LL patients. Double stainings for CD3/CD8 showed moderate numbers of double-positive cells in BL/LL patients and in BT patients (data not shown). However, a ratio of CD4 to CD8 cell numbers showed bias toward CD8 in BL/LL patients (0.6:1-4.3:1), whereas CD4 cells predominated in BT patients (1.25:1-2:1) as reported earlier [20].

3.1.3. B-cells (CD20, CD79, and CD138)

Single stainings of skin sections showed consistent presence of varied numbers of CD20-positive cells in BL/LL patients, whereas in BT patients, the presence was sporadic (Fig. 1A and E). Similarly, moderate positivity for CD79 and CD138 was seen in BL/LL patients, whereas it was only sporadically positive or negative in BT patients (Fig. 1B, F, and C, G). Presence of B-cells was seen both as single cells and as small clusters of cells both in and outside T-cell areas in BL/LL patients, and B-cells were found in contact with T-cells in BT patients (data not shown). Double staining for the skin homing CLA with B-cells (CD20/CD79/CD138) showed CLA-positive cells lying in close juxtaposition with B-cells in BT patients (Fig. 2A and C, inset). However, few B-cells were also positive for CLA (Fig. 2B and C, inset).

3.1.4. Mycobacterial antigens

Strong positive staining for mycobacterial LAM and M. leprae–specific PGL-1 was seen in BL/LL patients, whereas it was sporadically positive to negative in BT patients (Table 1). This indicated the presence of M. leprae antigens in active lesions despite absence of acid fast bacilli.

3.2. Antibody secretion of skin lesions is an active phenomenon

One biopsy from a BL was bisected with one of the halves being autoclaved. Both pieces were used for organotypic culture as described in Materials and methods. Although no antibody secretion was observed from the autoclaved biopsy, antibody secretion from the untreated biopsy followed a time-bound response (Fig. 3A), indicating that the antibody detected in the culture supernatant was a result of active secretion and not a result of serum contamination of the biopsy.

3.3. Kinetics of antibody production in organotypic culture supernatants

Anti-M. leprae antibodies (IgG) against MLSA were measured in organotypic skin culture supernatants from
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3.4. Kinetics of cytokine production in organotypic culture supernatants

3.4.1. Prolinflammatory cytokines IFN-γ, TNF-α, and IL-6

As depicted in Fig. 4A and B, respectively, peak levels of IFN-γ and TNF-α were detected in culture supernatants of BT lesions at 24 hours gradually declining to negligible levels by 120 hours. On the other hand, biopsies from BL/LL lesions showed significantly lower levels of IFN-γ and TNF-α, which were comparable to levels seen in HC. IL-6 secretion (Fig. 4C) in culture supernatants of BT lesions was maximal at 48 hours and dropped sharply at 72 hours of culture. The IL-6 secretion was lower in BL/LL lesions, although the difference was not statistically significant (P > 0.05). In HC, IL-6 secretion increased over time, peaking at 96 hours before dropping, although it still remained higher than in both groups of patients at the end of 120 hours.

3.4.2. IL-4 and IL-10

IL-4 levels were similar in both groups of patients and were lower than the levels in HC. However, the difference was not statistically significant (Fig. 5A). IL-10 was low in culture supernatants of both BT and BL biopsies, and no significant difference in the levels of secretion was observed between the 2 groups of patients (Fig. 5B). However, significantly higher levels of IL-10 were observed in the supernatants from HC at 48 hours of culture, declining gradually at later time points but remaining higher than in the patient groups at 120 hours.

4. Discussion

The present study focused on identifying functionally active B-cells in the cellular infiltrate in leprosy lesions by immunohistochemistry and organotypic tissue culture. Histopathological presence of plasma cells within skin lesions of patients with leprosy [21,22] and within biopsies of lepromin reaction lesions [9] has been sporadically reported in the literature. However, the presence and functional activity of these cells in the lesions has not been critically studied. We here demonstrate, using immunohistochemical analysis, the presence of B-cells indicated by markers CD20, CD79, and CD138, in skin from active lesions of patients with leprosy including skin smear negative BL and panchromatous BT leprosy patients. Among B-cell markers, the CD20 antigen is a 35- to 35-kDa phosphoprotein expressed on B-lymphocytes from the early pre-B to the late B stage, although its expression ceases when they differentiate into plasma cells [23]. Expression of CD79, the B-cell receptor, precedes Ig heavy-chain gene rearrangement and CD20 expression during B-cell ontogeny and disappears later than CD20 in the late (plasma cell) stage of B-cell differentiation [24]. CD138 is used often as a marker of plasma cells in tissues [25]. The positive scores for CD20, CD79, and CD138 were higher in BL/LL patients as compared with the BT patients. Cells with CD79 and CD138 staining were seen in moderate numbers in BL/LL and BT patients in serial sections, indicating presence of mature B-cells and plasma cells, respectively, within the lesions. Immunostaining of skin sections for immunglobulins (IgA, IgG, and IgM) were not performed because in our preliminary experiments, these stainings, especially in skin sections, resulted in high background, making it difficult to judge the specificity of the stainings. The presence of relatively more B-cells toward the lepromatous pole in this study is consistent with the higher antibody levels demonstrated in this form of leprosy [26]. Double stainings showed T-cells often lying in close juxtaposition to the
B-cells. Furthermore, immunostained serial sections of the biopsies showed most T-cells to be positive for the skin homing CLA (data not shown), whereas a few of the B-cells were also positive for CLA in double stainings. CLA- T-cells in skin inflammation as reported in literature [27] are compatible with the present finding. However, CLA-positive T-cells and, in particular, CLA positive, skin homing B-cells in leprosy have never been reported earlier.

Moreover, at a functional level, organotypic skin cultures of active BT lesions were shown to release anti-MLSA IgG antibodies into culture supernatant. In contrast to previous studies [11-13], the demonstration of anti-MLSA antibody secretion in in vitro organotypic skin culture in the present study was accompanied by the concomitant immunohistochemical demonstration of mature B-cells within tissue sections from the same lesion. Furthermore, the lesional biopsy was subjected to minimal processing before culture, and the experimental setup was so designed that the tissue was in contact with culture medium only at the air-liquid interface. Thus, the antibodies detected in the culture fluid suggest that this was a result of active and ongoing secretion of antibody by in situ B-cells within lesional tissue. In contrast, in the previous studies [11,12], the biopsies were minced and incubated in the medium for 48 hours before detection of antibodies by crossed immunoelectrophoresis, which did not necessarily reflect an ongoing antibody production. Moreover, no data on the status of different B-cell stages within these lesional biopsies were available.

Furthermore, the analysis of the cytokine profiles within culture fluids of the lesional organotypic cultures showed the in situ existence of a proinflammatory microenvironment, which was conducive to maturation of B-cells. Higher levels of cytokines IFN-γ, TNF-α, and IL-6 were observed in BT patients as compared with BL/LL or HC. IL-6 is cytokine produced by multiple cell types including macrophages, B-cells, and T-cells maintaining the granulomatous response by its proinflammatory role and may further be involved in an autocrine or paracrine manner in differentiation of B-cells to antibody-producing plasma cells [22,28].

In this regard, IL-6 levels in HC also increased at 48 hours and remained higher than in patients at 120 hours. IL-6 detection in the HC, although surprising, is compatible with its proinflammatory function and could probably be related to endemcity of various infectious diseases, including leprosy, in India from where the tissues were obtained. In comparison, no IL-6 could be detected in organotypic cultures of normal skin from healthy individuals from nonendemic areas, like the Netherlands (data not shown). Moreover, the high TNF-α levels in this study and reported previously in plasma cells and macrophages could also help in the maintenance of the granulomatous response [22].

With regard to the role of M. leprae-specific antibodies in relation to the pathology of BT lesions, the following speculations can be put forward. The locally secreted antibodies may form immune complexes with M. leprae antigens or with cross-reactive host molecules [29,30]. These complexes can then be internalized by antigen presenting cells through specific membrane receptors and be presented to CD4+ or CD8+ T-cells through major histocompatibility complex (MHC) class II or I, respectively [31,32], and maintain the BT granuloma. On the other hand, at the lepromatous pole, where high level of M. leprae antigens and specific antibodies are detected, the high concentrations of immune complexes may suppress specific T-cell responses to antigen as suggested by Iyagi et al [33]. An interesting observation reported previously suggested that the ratio of antigen to the antibody in the immune complexes may also influence T-cell activation [34]. Immune complexes close to equivalence or in moderate antibody excess provided optimal T-cell activation. In contrast, complexes in extreme antibody excess, although taken up efficiently by antigen presenting cells (macrophages) fail to activate T-cells, probably because antigen processing is disturbed by such antibody excess [34]. However, it should be noted that the implications for leprosy are, at this juncture, purely speculative.

Previous reports have suggested that B-cells might contribute to the development and maintenance of the granuloma [22] in diseases like cutaneous leishmaniasis [35] and other granulomatous conditions such as nonsarcoïd sarcoidosis [36]. In this respect, a study by Bisio et al [37], in a B-cell-deficient mouse model of tuberculosis, showed markedly less severe pulmonary granuloma formation, smaller lesions, little cellular infiltrate, and delayed dissemination of bacteria to spleen and liver despite having a bacterial burden comparable to wild-type mice in the lungs. It was suggested that B-cells played an important role in granuloma formation by influencing recruitment of other inflammatory cells such as macrophages and T-cells [37]. However, in relation to leprosy, paradoxically, TT leprosy shows a strong granulomatous response [1] despite having few B-cells within lesional biopsies. However, any of the previously mentioned speculations should be interpreted with caution in relation to leprosy until further functional studies on the role of B-cells are carried out. In this regard, it should be emphasized that in our experience, the isolation of antibody-secreting B-cells from skin granulomas was not possible, in contrast to T-cells. For this reason, functional studies on skin B-cells except by organotypic skin culture as described in the article will be a limiting factor.

In summary, this study demonstrated conclusively the presence of different functionally active B-cell stages within lesional patients with leprosy, including the BT region of the spectrum, which could secrete anti-M. leprae-specific antibodies. However, their role in leprosy pathology remains to be elucidated.

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References

Modulation of dendritic cell maturation by 
*M.leprae* and its association with 
leprosy spectral pathology

*Manuscript in preparation*
Modulation of dendritic cell maturation by *M.leprae* and its association with leprosy spectral pathology

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Summary

Activation of an appropriate cell mediated immune response is a crucial step in the control of most intracellular pathogens including mycobacteria. Professional antigen presenting cells (APC) like dendritic cells (DC) play a central role in orchestrating appropriate T cell responses to various infectious agents by differentially regulating the Th1/Th2 profile of immunity. DCs, on the other hand, are instructed by different pathogen or environment-derived signals which influence their maturation state and consequently the induction of the immune response. In this respect, leprosy, on account of its spectral pathology, provides a unique human model to study the development of differential immune responses to a single pathogen. The present report studied the ability of *M. leprae* and its sub-cellular fractions to influence the maturation state of monocyte-derived DCs (MoDC) from 11 healthy donors and 2 leprosy patients. *M. leprae* whole sonicate (MLS) induced maturation of MoDCs, as measured by CD83 expression and allogenic T-cell proliferation, to a similar extent as the previously reported for a standard maturation cocktail. Apart from MLS, the cell membrane fraction was most potent in inducing DC maturation. On the other hand, the 2 lepromatous leprosy (LL) patients showed defective differentiation of their monocytes to DC and reduced maturation in response to MLS. TNF-α was produced by the maturing DCs, which may play a role in an autocrine or paracrine manner in the maturation process. However, blocking of TNF-α showed only a partial reduction of maturation suggesting the activity of alternative mechanisms. Furthermore, CD40 ligation of MLS matured DCs resulted in a significantly increased production of IL-12. Moreover, blocking of Toll-like receptor (TLR)-2, thought to be important in the development of lepromatous leprosy, did not abrogate DC maturation by *M. leprae* suggesting redundancy in TLR binding of the *M. leprae* components. Importantly, pre-incubation of monocytes with MLS impaired their differentiation to DCs and their further maturation suggesting that *M. leprae* may actively subvert the differentiation and maturation of DCs and influence the development of the leprosy spectrum.
Introduction

Dendritic cells (DC) are professional antigen presenting cells (APC) that play a central role in orchestrating the appropriate immune responses to various infectious agents [1]. In addition to presenting antigens in the context of MHC molecules (Signal 1) and costimulatory signals (Signal 2), DCs also provide a polarizing signal (Signal 3) which differentially regulates the Th1/Th2 profile of immunity [2; 3; 4]. In their turn, DCs are thought to be guided to promote a Th1- or Th2 biased response by previously identified instructive mechanisms [5]. The modulation mechanism proposed that the Th1/Th2 inducing potential of DCs depend on different maturation inducing stimuli (including pathogens and pathogen derived antigens) and environments [4]. Thus, immune regulation by DCs, depends on their functional plasticity at the immature stage [6]. On the other hand, the observation of preferential induction of Th1 and Th2 responses by myeloid and plasmacytoid DCs respectively, suggested that selective interaction of the appropriate DC subtype with distinct classes of pathogens determines the Th1/Th2 profile of the immune response [5]. Importantly, activation of different surface receptors immature DCs or their precursors by signals derived from pathogens and the local microenvironment may generate distinct functional subsets influencing the immune response to infectious agents [7; 8; 9; 10; 11; 12].

Leprosy, a chronic mycobacterial disease, provides a unique human model to study the development of an immune response to intracellular pathogens on account of its spectral pathology [13]. The spectral manifestation is related to the differential immune response of the patient to Mycobacterium leprae (M.leprae), the causative agent. Polar tuberculoid leprosy (TT) is characterized by a strong M.leprae specific cell mediated immune response (CMI) [14], associated with a T-helper 1 (Th1) response with high interferon-γ (IFN-γ) production [15; 16]. Polar lepromatous leprosy (LL), on the other hand, shows an absence of M.leprae specific CMI and predominantly displays T-helper 2 (Th2) responses with high anti-M.leprae antibody levels [15; 16]. Between the polar extremes, are the borderline forms of leprosy including borderline lepromatous (BL), mid-borderline (BB) and borderline tuberculoid (BT) showing clinical and immunological characteristics intermediate to the polar forms. Further, the reactional states – erythema nodosum leprosum (ENL) and reversal reaction (RR) – occurring in some of the leprosy patients is also associated with an increase in CMI [17; 18]. Although the existence of differential immune responses in leprosy is widely acknowledged, what prompts the development of such varied responses to a single organism is at present not clear.

The presence of CD1+CD83+ mature DCs in tuberculoid leprosy lesions suggested a role for DCs in the protective immune response against leprosy [7; 19]. On the other hand, it was observed that integral M.leprae caused down-regulation of MHC class I and II on DCs in in vitro cultures. Expression of the maturation marker CD83 was induced only at
very high bacterial doses [20]. Furthermore, *M. leprae* was less efficient in DC-mediated induction of T cell responses as compared to *M. bovis* BCG or *M. avium*, [20]. This suggested that *M. leprae* actively downregulates DC maturation thereby blocking the development of an effective, specific CMI. However, sub-cellular fractions of *M. leprae* show differences in their ability to induce maturation of DCs and thus an effective T-cell mediated immunity. Among the different sub-cellular components of *M. leprae*, the cell membrane fraction was shown to upregulate MHC Class II and CD86 expression in DC [21]. Furthermore, it could induce strong IFN-γ production in CD4+ and CD8+ T cells and perforin production in *M. leprae* specific CD8+ cytotoxic T lymphocytes [21]. Apart from differences in the maturation state of DCs, it is also suggested that different DC subsets are associated with the different spectral forms of leprosy [7]. However, the relation of the maturation state or subsets of DCs to the development of the spectral pathology is poorly understood.

Since dendritic cells are central to antigen presentation and polarization of T-cell responses to infectious agents, we hypothesized that modulation of maturation of dendritic cells (DC), by *Mycobacterium leprae* components may influence T-cell responses thus determining the spectral pathology of leprosy.

### Materials and Methods

#### Media and Reagents

Iscove’s modified Dulbecco’s medium with 10% fetal calf serum (both from Gibco/Invitrogen) were used to generate DCs. The following factors were used to generate mature DCs: rhu granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4; rhuTNF–α, rhuIL-1β, and (all from Peprotech); rhuIL-6 (Strathmann Biotech, Hannover, Germany); lipopolysaccharide (Escherichia coli 011:B4, Sigma, St. Louis, MO); and PGE2 (Sigma). Flow cytometry analyses were performed with Beckman Coulter FacsCalibur, after labeling with CD86, CD3, CD14, CD1a, and isotype control monoclonal antibodies (all BD-PharMingen, San Jose, CA) and CD83 (Immunotech).

#### Generation of Dendritic Cells

Peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were isolated with Ficoll (Amersham Biosciences). Monocytes were isolated on density gradients, with Percoll (GE Healthcare Life Sciences; ref. 9), followed by plastic adherence. Isolated monocytes were cultured for 6 days in 24-well plates (Costar) at 5.10⁵ cells per well in rhu
GM-CSF (500 IU/mL) and IL-4 (250 IU/mL). The following optimal concentrations of the maturation factors were used: standard maturation cocktail - IL-1β (25 ng/mL); IL-6 (1000 IU/mL) TNF-α (50 ng/mL), PGE2 (10^4M); lipopolysaccharide (250 ng/mL), all added at day 6 until day 8. In some experiments, monocytes were incubated with MLS or LPS at the onset of 6 days culture with GM-CSF and IL-4. LPS was subsequently added at day 6 until day 8 to induce maturation of these “DCs”

**Antigen fractions**

*M. leprae* whole sonicate (MLS) and its subcellular fractions – cell wall (MLCwA), membrane (MLMA), cytosol (MLcyt) and PGL-I antigen were obtained from Drs J. Spencer and P.J. Brennan, Colorado State University, USA. BCG whole sonicate (BCGS) was obtained from

**Interleukin-12p70 Production**

Dendritic cells were harvested, washed, and plated in 96-well plates at 2.10^4 cells/well. To mimic the interaction with CD40L-expressing Th cells, CD40L-transfected J558 cells (a gift from Dr. E. de Jong, Dept of Cell Biology and Histology, Academic Medical Center, Amsterdam, that in previous studies proved equivalent to activated CD4^+ T cells and soluble CD40L [9]) were added at 5. 10^4 cells/well. Twenty-four-hour supernatants were analyzed by IL-12p70 ELISA (Pharmingen International).

**Assessment of cytokine production**

The levels of IL-10, IL-12 p70 and TNF-α produced by MLS-pulsed DCs were measured in 24-h culture supernatant using an enzyme immunoassay. The concentrations of IL-12 p70 and IL-10 were quantified using the enzyme assay kit Opt EIA Human IL-12 (p70) SET or Opt EIA Human IL-10 SET, respectively, (Pharmingen International). ELISA for TNF-α was performed using specific mAb pairs and standards obtained from Biosource Europe (Etten-Leur, Netherlands).

**Assessment of APC function of antigen-pulsed DCs**

The ability of MLS or its cellular fraction - matured DCs to stimulate allogenic T cells was assessed using a mixed DC–allogenic-T-cell reaction. DCs exposed to MLS, its
cellular fractions, LPS or the standard maturation cocktail for 48 h were washed extensively to and were used as stimulators. CD4+ T cells purified using immunomagnetic MACS beads coated with MAbs (Miltenyi Biotec) were used as a responder population. Responder cells (10^6 per well) were plated in 96-well round-bottom tissue culture plates, and DCs were added to give a DC/responder CD4+ T-cell ratio of 1:20, 1:40, or 1:80. The T-cell proliferation during the last 16 h of a 5-day culture was quantified by incubating the cells with 0.3 μCi of [3H]thymidine/well. The results were expressed as the mean difference in counts per minute obtained from triplicate cultures.

**Blocking of TNF-α and TLR-2**

In the TNF-α blocking experiments, neutralizing anti-TNF-a antibodies (Diaclone) were added at a concentration of 2μg/ml to the DC cultures along with the maturation factors and MLS from day 6 till day 8. TLR-2 on the DCs was blocked using the anti-TLR2 antibody (Clone TLR2.1, Ebiosciences) at a concentration of 10μg/2.10^6 cells/ml for 30 mins prior to addition of the maturation factors. DC maturation was measured at day 8 by FACS.
**Results**

*In vitro generation of monocyte derived dendritic cells (MoDC) from normal healthy individuals and their maturation in response to standard maturation cocktail and M.leprae sonicate*

Monocyte derived dendritic cells were generated from buffy coats of 11 normal healthy individuals using a modified protocol of Sallusto et al [22]. Although donor-to-donor variation was seen in the yield of immature DC (iDC), characterized by the expression of the CD1a marker, the average yield of CD1a+ cells was 60% (Fig 1B). However, expression of CD14, a monocyte marker, was negative in all the cells suggesting that the cells had differentiated from monocytes.

On day 6, the iDCs were exposed to standard maturation factors such as LPS or a standard maturation cocktail (IL-6 + TNF-α + PGE-2 + IL-1β) [23] or mycobacterial antigens, *M.leprae* sonicate (MLS) or BCG sonicate (BCG) for 48 hours. MLS strongly induced maturation of DC, comparable to that induced by the standard maturation cocktail and LPS, as measured by flow cytometry using the DC maturation marker CD83. Titration of MLS concentrations showed that a concentration of 10μg/ml was most potent in inducing DC maturation (data not shown). Hence this concentration was used for all further experiments. The functionality of the mature DC was assessed in a mixed-lymphocyte reaction by incubating them with allogenic CD4+ T cells. MLS induced proliferation of allogenic T-cells which was comparable to that induced by the standard cocktail and LPS (Fig 2A and B).
Cytokine profiles of the supernatants of DCs matured with MLS

Supernatants of DCs matured with MLS were assayed for DC derived cytokines by ELISA. The cytokines assayed included TNF-α, IL-10, TGF-β and IL-12. TNF-α levels in the supernatants reflected the maturation state of DCs as measured by the expression of the CD83 marker (Figs 3A and B). TGF-β, IL-10 and IL-12 were undetectable in the supernatants of MLS matured DC. However, ligation of the CD40 on the DCs by CD40L expressing J558 cells strong induction of IL-12 secretion was seen in the supernatants (Fig 3C).

Influence of TNF-α blocking on DC maturation

Levels of TNF-α in MLS-matured DC supernatants paralleled the extent of DC maturation suggesting autocrine or paracrine maturation. This hypothesis was tested by blocking TNF-α induced maturation. DCs were incubated with neutralizing antibodies to TNF-α during the course of maturation with MLS (Fig 3D). Anti-TNF-α antibodies were able to completely block maturation of DCs in response to LPS whereas maturation in response to MLS showed a trend towards partial blocking and failed to reach statistical significance. The results suggest the involvement of additional mechanisms together with TNF-α in DC maturation by MLS.
Fig 3. Cytokine secretion in the supernatants of matured DCs. A) DC maturation as measured by CD83 expression; B) TNF-α measured in the supernatants paralleled the maturation of the DCs; C) IL-12 is strongly induced in mature DCs on CD40 ligation. D) Blocking of DC maturation by anti-TNF-α antibodies. Although DC maturation in response to LPS was completely blocked, the response to MLS was only partially blocked. Average of 3 separate experiments.

Maturation of DCs by M.leprae fractions

In order to identify the M.leprae components which may be involved in the maturation of the DCs, preparations of different cellular fractions including the cell wall fraction (MLCwA), membrane fraction (MLMA), cytosolic fraction (MLCyt), the M.leprae phenolic glycolipid – I (PGL-I) were obtained from Dr. P.J.Brennan, Colorado State University, USA. The different M.leprae fractions were incubated with iDCs for 48 hrs to test their ability to mature DCs from healthy controls. All the fractions tested could induce DC maturation as measured by CD83 induction. Nevertheless, MLCwA and MLMA were found to be the more potent DC maturing fractions (Fig 4A). MLR with allogenic CD4+ T
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cells however showed that all the fractions tested were potent in inducing T-cell proliferation (Fig 4B). Combining DC maturation and T-cell proliferation data, MLMA was found to be the most efficient DC maturing fraction.

![Fig 4. Maturation of DCs by M.leprae fractions. A) MLCwA and MLMA fractions were most potent in induction of DC maturation; B) MLMA was as potent as MLS in inducing CD4+ T-cell proliferation in a MLR. Average of 3 different experiments.](image)

**Toll-like receptors in DC maturation by MLS**

Recently, Toll-like receptors (TLR) were shown to be important in the spectral pathology of leprosy [7]. A mutation in TLR2 is associated with susceptibility to the lepromatous form of leprosy [24] whereas TLR2 triggering has also been shown to be involved in killing of *M.tuberculosis* [25]. Involvement of TLR2 in recognition of MLS by DCs was tested by incubating DCs with anti-TLR2 blocking antibody (clone TLR2.1, Ebiosciences) for 30 mins before incubation with MLS. DC maturation by a positive control (Pam3CSK4) was blocked by 50% while with MLS an increase in DC maturation was noted (Fig 5). This suggests a possible redundancy in TLR usage by the DCs or the usage of other receptors in response to MLS.
Response of monocyte-derived DCs from lepromatous leprosy patients to MLS.

Monocyte-derived DCs were generated from 2 leprosy patients using the standard protocol described previously. Patient 1 had active neural leprosy classified as borderline lepromatous (BL) whereas Patient 2, was a lepromatous (LL) patient who had completed multi-drug treatment 10 years previously. Both the patients showed a complete impairment of differentiation to CD1a+ DCs (Fig 6A). Furthermore, Patient 1 showed reduced maturation of DCs in response to MLS as compared to Patient 2 (Fig 6B). This suggests that leprosy patients (especially lepromatous patients) have an inherent defect in the capacity of their monocytes to differentiate into DCs. Furthermore, active leprosy patients show anergy with respect to MLS induced maturation as compared to treated patients.

Fig 6. Response of DCs from lepromatous patients to MLS. A) Both patients showed an impaired differentiation of their monocytes to CD1a+ DCs, B) Patient 1 (active BL) showed reduced maturation of DCs in response to MLS as compared to the treated patient 2.
Influence of pre-incubation of monocytes with MLS on DC differentiation and maturation

The influence of *M. leprae* on DC differentiation was studied by pre-incubated monocytes with MLS before differentiation to DCs with GM-CSF and IL-4 according to the standard protocol. Pre-incubation with MLS but not BCGS or LPS blocked differentiation of monocytes to CD1a⁺ DCs (Fig 7A). Moreover, the *M. leprae* pre-incubated “DCs” showed an impaired maturation in response to LPS stimulation (Fig 7B). This suggests that the stage at which *M. leprae* encounters the cells of the immune system may be important in deciding the fate of DC differentiation and maturation and hence the course of the disease.

**Discussion**

The present study was undertaken to gain an insight into the modulation of dendritic cell maturation by *M. leprae* and its antigenic components which may play a role in the spectral pathology of leprosy. Previous studies suggested that activation of T-cell responses by DCs required unphysiological numbers of live or killed *M. leprae* [20; 21]. This led to the hypothesis that subcellular components, rather than whole bacteria, could be more potent in inducing a T-cell response and hence useful as vaccine candidates [21]. In the present study, *M. leprae* whole sonicate (MLS) was the most potent inducer of DC maturation, as shown by upregulation of the DC maturation marker CD83, as well as proliferation of allogenic CD4⁺ T cells in a mixed-lymphocyte reaction. In contrast, whole *M. leprae* were found to be much less potent in inducing proliferation of both CD4⁺ and CD8⁺ cells by DCs in previous studies [20; 21]. Similarly, heat-killed *M. leprae* were also found to be less potent in maturation of monocyte-derived DCs than *M. tuberculosis* or BCG [26]. In the present study, although
various *M. leprae* subcellular fractions, including the cell wall fraction (MLCwA), membrane fraction (MLMA), cytosolic fraction (MLCyt), the *M. leprae* phenolic glycolipid – I (PGL-I), were able to induce DC maturation and CD4+ T cell proliferation, they were all less effective as compared to MLS. Reflecting the trend seen in previous studies [21], MLMA was one of the most potent cellular fractions both in terms of CD83+ induction in DCs as well as in inducing proliferation of allogeneic T-cells. One difference between the two studies is that the former used autologous CD4+ T-cells whereas the present study used bulk CD4+ T-cells isolated from another donor. This may account for the lack of a highly significant difference in the extent of DC maturation induced by MLMA in comparison to the other fractions. Among the major constituents of the mycobacterial cell membrane are derivatives of phosphatidic acid such as phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and its mannosides, collectively called PIMs. They form the lipid base of lipoarabinomannan (LAM) and lipomannan which are important antigens of mycobacteria [27]. It would be interesting to study the ability of purified antigenic components such as LAM, and PIMs in the modulation of leprosy pathology.

TNF-α is an important cytokine in the differentiation of monocytes into DCs [2,5]. In the present study, the secretion of TNF-α paralleled DC maturation in response to MLS. This suggested an autocrine or paracrine effect of the TNF-α in DC maturation. In support of this observation, culturing immature DCs with anti-TNF-α antibodies resulted in a partial blocking of DC maturation in response to MLS although the results failed to reach statistical significance. However, this also suggested that TNF-α is not solely responsible for the observed DC maturation and that additional factors may be involved. On the other hand, the present results are in contrast to observations with heat-killed whole *M. leprae* in a previous study which, unlike *M. tuberculosis* or BCG, did not induce secretion of TNF-α in DCs [26]. It could be speculated that MLS used in the present study represents a mixture of *M. leprae* cellular fractions and as such presents significantly more immunostimulatory epitopes to the DCs in contrast to whole *M. leprae* resulting in an increased secretion of the cytokines. With respect to other cytokines like IL-10, IL-12 and TGF-β, MLS did not induce their secretion in DCs as also reported by Murray et al. [26]. IL-12 secretion was however increased several fold by ligation of CD40 on the DCs by CD40L expressing J558 cells. IL-12 is involved in the polarization of naïve T-cells to a T-helper 1 (Th1) type. The results suggest that *M. leprae* on its own is unable to induce spontaneous IL-12 secretion, but in the presence of CD40 co-stimulation, can act as a potent inducer of Th1 responses in healthy individuals.

Numerous studies *in vitro* and *in vivo* have shown that whole mycobacteria or mycobacterial components act as agonists for TLRs [28; 29; 30; 31]. Toll-like receptors (TLR) were shown to be important in the spectral pathology of leprosy [7]. The TLR2 polymorphism Arg677Trp was reportedly associated with susceptibility to the lepromatous
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form of leprosy with a frequency of 22% in the populations tested [24; 32] and was suggested to be important in the poor CMI associated with LL [33]. However, in the present study, blocking of TLR2 with an anti-TLR2 neutralizing antibody did not affect maturation of DCs suggesting redundancy in the stimulation of TLRs by \textit{M. leprae} components. On the other hand, the Malhotra et al [34] demonstrated that showed that the TLR2 Arg677Trp was not a true polymorphism of the TLR2 gene but was the result of a 93% homologous duplicated region of TLR2 exon 3 present approximately 23 kb upstream. Further studies are needed to elucidate the association of TLR2 with spectral leprosy. Furthermore, \textit{M. leprae} may also bind to other receptors on the DCs such as DC-SIGN, the mannose receptor or other combinations of TLRs with consequences for the development of immunity.

Interestingly, pre-incubation of monocytes with MLS subverted their differentiation to CD1a+ DCs and their subsequent maturation. Similar results were also reported previously for BCG and \textit{M. tuberculosis} suggesting \textit{M. leprae} may actively subvert DC differentiation thus modulating the immune response. This may in turn represent an important strategy of the bacterium to elude immune surveillance and persist in the host [35; 36]. On the other hand, two lepromatous leprosy patients studied showed an impaired differentiation of their monocytes into DCs suggesting an inherent defect in monocyte differentiation to DCs in these patients. Moreover, one of the patients who had active leprosy showed reduced maturation of DCs in response to MLS as compared to the other patient who was treated, suggesting that active disease may be associated with additional suppressive mechanisms which are at present not clear. However, since number of patients in the study is limited this data needs to be interpreted with caution. A follow-up study is being undertaken at present to address the effect of \textit{M. leprae} sonicate and antigenic fractions on differentiation and maturation to DCs of monocytes derived from LL and TT patients and healthy controls from endemic areas

In conclusion, the results of the present study suggest that \textit{M. leprae} and its antigenic fractions may have a modulating effect on differentiation and maturation of DCs, influencing, and probably even subverting, the development of the immune response in the host.

\textbf{Acknowledgements}

The Dutch Leprosy Foundation (NSL) and the Q.M. Gastmann-Wichers Stichting are gratefully acknowledged for the financial support for A.M. Iyer.
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Dendritic cells in leprosy

Summary and Concluding Remarks
The introduction of multi-drug therapy in 1981-82 and the WHO campaign to eliminate leprosy as a public health problem since 1991 has resulted in a considerable decline in the prevalence of leprosy globally. It is reported that more than 14 million leprosy patients were diagnosed and treated with MDT between 1985 and 2005 resulting in a decline of the countries with a prevalence rate of more than one per 10,000 population from 122 in 1985 to nine at the beginning of 2004 [1]. This led to a call for integration of the specialized leprosy units into the general health services and an approach using general health staff in leprosy detection and control activities [1]. However, a great deal of scepticism exists among experienced leprologists who fear that such a move has led to the involvement of less experienced and trained staff who are unable to distinguish between the various forms of leprosy on clinical grounds [2]. Moreover, disagreements still exist regarding the parameters used as benchmarks for control programmes such as prevalence, point-prevalence and incidence; the duration of and type of treatment for leprosy and reactions; the criteria used for diagnosis and classification of patients for treatment purposes [2]. In this context, reliable laboratory tests to aid early diagnosis of leprosy and reactions and/or to monitor efficacy of treatment are conspicuous by their absence [2; 3].

Measurement of antibodies to the *M. leprae* specific PGL-I antigen while being specific lacks sensitivity in detecting paucibacillary (PB) leprosy patients [4; 5]. Previous studies showed an association of Th2 cytokines IL-4, IL-5 and IL-10 with lepromatous leprosy whereas tuberculoid leprosy showed a predominance of Th1 cytokines IL-2, IFN-γ and TNF-α within lesional skin [6; 7]. Furthermore, both leprosy reactions, RR and ENL, are reportedly associated with changes in cytokine activity [7; 8] whereas, *M. leprae*-specific T-cell clones isolated from RR lesions showed a polarized Th1-like cytokine profile [9]. These results indicated the association of discrete cytokine profiles with the spectral forms of leprosy suggesting that identification of cytokine profiles associated with spectral leprosy and reactions might be useful in detection and monitoring the efficacy of treatment. However, later studies increasingly demonstrated the lack of a clear cut dichotomy in the cytokine profiles associated with the leprosy spectral forms and reactions [9; 10; 11; 12; 13].

A significant proportion of the leprosy patients, especially in the borderline region of the spectrum, develop leprosy reactions either sometime during the course of the disease or even after the completion of multi-drug therapy (MDT) [14; 15; 16; 17]. Both RR and ENL if undetected and not treated early can lead to irreversible nerve damage and, as a consequence, severe disabilities. The seventh WHO expert committee on leprosy stated that ‘the crucial elements in the management of leprosy reactions and thereby the prevention of disabilities are early diagnosis of reactions together with prompt and adequate treatment’ [18]. Corticosteroids are the drugs of choice in the treatment of RR and help in the recovery of nerve function after a reaction [2; 17]. Corticosteroids influence the cytokine milieu in
patients with RR causing a decrease in the pro-inflammatory cytokines IFN-γ and TNF-α. However, although standard treatment duration of 12 weeks with a gradually tapering dose of prednisolone is recommended by the WHO [18], this is a matter of considerable disagreement and debate among leprologists, several of whom present evidence supporting longer treatment durations [2]. In case of ENL, the treatments of choice include corticosteroids and thalidomide whereas clofazimine, a component of MDT, has also been shown to have a suppressive effect on ENL. However, thalidomide is restricted in many countries due to its known teratogenic effects [2]. Moreover, a problem associated with prolonged corticosteroid therapy, especially in ENL, is the induction of steroid dependence [19]. Due to the considerable doubts about the duration and type of treatment for reactions, a laboratory test for monitoring the disease activity is of considerable value. Such a test would aid decision making and would be an extremely useful tool for clinicians and leprosy control programmes.

A limitation of serum cytokine measurement in association with leprosy is that most studies measured one or few cytokines or cellular activation markers in association with the disease manifestation. It is difficult to obtain the broader picture of cytokine profile associated with the different leprosy spectral forms since the disease is immunologically complex. Moreover, many studies presented contradictory results with respect to the predominant cytokines associated with the leprosy spectral forms and with reactions, which may be related to the different assay conditions, samples and populations examined [20; 21; 22; 23]. Hence the studies detailed in chapters 2, 3 and 4 demonstrated the level of a broader panel of cytokines, cytokine receptors, soluble cellular activation products and anti-PGL-I antibodies (except chapter 3) concomitantly in the sera of the patient groups.

The attempt to address the often contradictory results of previous studies mentioned above forms the major part of this thesis which includes chapters 2, 3, 4, and 5. In addition, chapter 5 reported, for the first time, detection of the enzyme chitotriosidase, which is a measure of the activity of lipid-laden macrophages across the leprosy spectrum and reactions in relation to cytokines and other soluble cell activation products and anti-PGL-I antibody in the patient groups detailed in chapter 2.

Chapter 2 was a cross-sectional study of the profiles of serum cytokines a, the soluble IL-6 receptor (sIL-6R), soluble T cell (sCD27) and macrophage (neopterin) activation products and Mycobacterium leprae-specific anti-PGL-I IgM antibodies in relation to the leprosy spectrum and reactions. As with previous reports, a wide variability was observed in the cytokine levels in the sera of the different groups. Nevertheless, IL-6, TNF-α and neopterin levels were higher in patients as compared to healthy controls, suggestive of increased immune activity, although no difference was observed between reactional and non-reactional patients. Moreover, neopterin and PGL-I were elevated in multibacillary (MB) as compared to paucibacillary (PB) patients which is useful in the stratification of patients for...
treatment purposes. On the other hand, IFN-γ and sIL-6R were significantly increased in ENL as compared to non-ENL patients and declined on corticosteroid treatment which correlated with clinical improvement of patients suggesting their utility in monitoring therapy in ENL.

**Chapters 3 and 4** describe longitudinal studies where patients were followed up during the development of reversal reaction or the course of multi-drug therapy of leprosy respectively. **Chapter 3** followed up seven patients, classified as BL to LL leprosy and developing RR during the course of the disease, at diagnosis of leprosy, at onset of RR and at different time-points during and at the end of prednisolone treatment. Cytokines IL-4, IL-5, IFN-γ, TNF-α, cytokine receptors TNF-RI/II and the macrophage activation product neopterin were measured in the patient sera at these different time points. Six of the seven patients showed increased serum neopterin at onset of RR or within 1 month of onset which declined to levels observed at diagnosis of leprosy with prednisolone treatment, confirming observations of a previous retrospective study [24]. This paralleled clinical improvement of the patients. In contrast, no specific cytokine profile was associated with onset or subsidence of RR with corticosteroid treatment. The results suggest the utility of serum neopterin measurement as a marker for prognosis of corticosteroid treatment of reversal reaction in leprosy.

**Chapter 4** describes a prospective study following up twenty-five leprosy patients, including 15 MB and 10 PB, during the course of multi-drug treatment (MDT). *M. leprae* specific anti-PGL-I antibody, macrophage activation product neopterin and the acute phase protein, C-reactive protein (CRP), were measured in sera at diagnosis and after 2, 4, 6 and 12 months of MDT. As observed previously in Chapter 2, levels of anti-PGL-I antibodies and neopterin were elevated in MB as compared to PB patients and this correlated with the bacterial load (Bl) in slit-skin smears. On the other hand, CRP was elevated in ENL as compared to non-reactional patients. Only anti-PGL-I antibody levels were found to decline significantly during the course of MDT. This study reaffirmed the utility of anti-PGL-I antibody and neopterin in stratification of MB and PB patients with implications for therapy. Furthermore, increased CRP serum levels, together with IFN-γ and sIL-6R as described in chapter 2, could serve as potential markers for ENL.

Macrophage activation plays an important role in the control of *M. leprae* infection [25; 26; 27]. **Chapter 5** studied the relation of chitotriosidase, a macrophage activation associated product, with leprosy both in serum and *in situ* in lesional skin biopsies from patients. Importantly, chitotriosidase activity was measured in largely the same subsets of patients included in Chapter 2 allowing for comparisons with cytokine/cellular activation product levels reported previously. MB patients showed increased chitotriosidase activity in serum as compared to PB patients and healthy controls, which was similar to observations for neopterin in Chapter 2. Moreover, as with neopterin, no significant difference was observed
in chitotriosidase activity between non-ENL and ENL suggesting that the expression of these products could be independent of the reactional status of the patient. Furthermore, a positive correlation was observed between serum chitotriosidase activity and levels of neopterin. However, in contrast to neopterin, which correlated with macrophage derived cytokines IL-6 and TNF-α, albeit weakly, no correlation was seen between chitotriosidase activity and cytokines reported in Chapter 2. On the other hand, chitotriosidase declined on treatment of ENL with corticosteroids, similar to the cytokines TNF-α and IFN-γ, although, surprisingly, in contrast to neopterin. This strongly suggests that the regulation of chitotriosidase activity is different as compared to neopterin or cytokine response pathways although both chitotriosidase and neopterin are macrophage products. It may be speculated that elevated chitotriosidase in this context is a response to lipid accumulation within alternatively activated macrophages, as is seen in Gaucher disease [28], whereas neopterin levels are related to the IFN-γ related activation of macrophages [29].
Utility of the serum bio-markers studied in Chapters 2, 3 and 4 of this thesis in diagnosis and monitoring of leprosy and reactions

<table>
<thead>
<tr>
<th>Markers</th>
<th>Diagnosis of leprosy</th>
<th>Diagnosis of reactions</th>
<th>Stratification of MB/PB</th>
<th>Monitoring MDT</th>
<th>Monitoring Reactional treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGL-1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Neopterin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+ (RR)</td>
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<tr>
<td>Chitotriosidase</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>+ (ENL)</td>
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<tr>
<td>CRP</td>
<td>-</td>
<td>+ (ENL)</td>
<td>-</td>
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<td>IL-4</td>
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<td>IFN-γ</td>
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<td>+ (ENL)</td>
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<td>TNF-α</td>
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<tr>
<td>sIL-6R</td>
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<td>+ (ENL)</td>
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<td>ND</td>
<td>+ (ENL)</td>
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<tr>
<td>sCD27</td>
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<td>TNF-RI</td>
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<td>TNF-RII</td>
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**Key:**
+ : useful
- : not useful
ND : not done
Chapter 6 deals with an aspect of immunology which is often neglected in leprosy i.e. the presence of B-cells in lesional skin across the leprosy spectrum especially in tuberculoid patients. Immunity to intracellular infections is considered to be an almost exclusively T-cell mediated event. Although presence of plasma cells in LL and BL lesions has been sporadically reported the importance of their in situ presence related to the pathology of leprosy lesions has never been elucidated [30; 31]. The present study used immunohistochemistry to demonstrate the presence of B-cells in lesions of lepromatous leprosy, smear negative borderline lepromatous and BT leprosy. Furthermore, an organotypic skin culture using part of the same lesional leprosy skin showed an active secretion of \textit{M.leprae} specific antibodies in the culture supernatants suggesting involvement of the local plasma cells in antibody production. Analysis of the culture fluid also showed the presence of pro-inflammatory cytokines, IFN-\(\gamma\), TNF-\(\alpha\) and also IL-6, which plays a role in the differentiation of antibody-producing plasma cells [32]. Speculating on the role of the B-cells within lesions, they may be involved in local secretion of antibodies and maintenance of granulomas either by influencing T-cell activation or by inducing the recruitment of other inflammatory cells into the lesional sites, although further functional studies are essential to elucidate this.

Professional antigen presenting cells (APC) like dendritic cells (DC) play a central role in orchestrating appropriate immune responses to various infectious agents. In addition to presenting antigens in the context of MHC molecules (signal 1) and costimulatory signals (signal 2), DCs also provide a polarizing signal (signal 3) which differentially regulates the Th1/Th2 profile of immunity. DCs, on the other hand are guided to promote a Th1 or Th2 biased response depending on different maturation inducing stimuli (including pathogens and pathogen derived antigens) and environments [33]. Thus, immune regulation by DCs depends on their functional plasticity at the immature stage [34]. Another mechanism, based on the observation of preferential induction of Th1 and Th2 responses by myeloid and plasmacytoid DCs respectively, suggested that selective interaction of the appropriate DC subtype with distinct classes of pathogens determines the Th1/Th2 profile of the immune response [35]. Leprosy provides a unique human model to study the development of immune responses to pathogens on account of its spectral pathology [36] which is related to the differential immune response of the patient to \textit{M.leprae}, the causative agent. The working hypothesis of Chapter 7 was that \textit{M.leprae} interact with antigen presenting cells (APC) such as dendritic cells (DC), leading to their maturation and polarization to phenotypes which can induce Th1, Th2 T cell responses and/or regulatory T cell responses. Moreover, \textit{M.leprae} may also act on precursors of DCs like monocytes and prevent their differentiation to DCs and their maturation, as has been reported previously for \textit{M.tuberculosis} and \textit{M.bovis} BCG [37; 38]. The balance between the different responses may ultimately determine the outcome of the infection and the spectrum of leprosy. Results indicate that \textit{M.leprae} and its cellular fractions
can induce maturation of monocyte-derived DC generated by treatment with GM-CSF and IL-4 from normal healthy individuals. Apart from *M. leprae* whole sonicate (MLS), the cell wall and membrane fractions were most potent in inducing DC maturation. Supernatants of MLS matured DCs showed the presence of TNF-α paralleling the extent of DC maturation. Since TNF-α also plays a role in DC maturation, it was hypothesized that this cytokine on secretion by DCs may act on the same or neighbouring DCs in an autocrine or paracrine manner inducing their maturation. However, using blocking antibodies against TNF-α, only a partial blocking of DC maturation was achieved suggesting that additional mechanisms were involved in DC maturation. MLS-matured DC supernatants did not show detectable levels of the suppressive cytokines IL-10 and TGF-β or the Th1 polarizing cytokine IL-12. However, IL-12 production was induced in response to CD40 ligation by CD40L transduced J558 cells. CD40 ligation is known to be an important 3rd signal for complete maturation and polarization of DCs. The results seem to suggest that DCs from healthy individuals are inherently programmed to evoke a Th1 immune response to *M. leprae*. In contrast, 2 lepromatous leprosy patients studied showed an impaired differentiation of their monocytes into DCs suggesting a defect in these patients. Moreover, one of the patients who had active leprosy showed reduced maturation of DCs in response to MLS as compared to the other patient who was treated, suggesting suppressive mechanisms at play in the active disease. Toll-like receptors (TLR) are important pattern recognition receptors on DCs and TLR2 is reportedly important in the immune response against *M. leprae* [39; 40]. However, blocking of MLS binding to TLR2 using anti-TLR2 antibodies failed to block DC maturation. This suggests a role for other receptors such as other TLRs, mannose receptor, DC-SIGN or a combination of receptors such as TLR2/1 in the MLS induced maturation of DCs. Finally, the consequence of pre-incubation of monocytes with MLS before GM-CSF and IL-4 treatment for DC generation resulted in a reduced differentiation to DCs and reduced responsiveness to maturation using lipopolysaccharide, a standard DC maturing agent. This suggests that *M. leprae* actively subverts DC differentiation and maturation which may be crucial in determining the immune response and the development of the leprosy spectrum.

In conclusion, the present studies raise further questions regarding the present approach to the laboratory diagnosis and monitoring of leprosy patients and the understanding of leprosy pathology. Serum cytokines such as IFN-γ, TNF-α and macrophage activation product chitotriosidase are useful in monitoring treatment of ENL whereas neopterin is useful in RR. On the other hand, chitotriosidase, neopterin and PGL-I are important markers of multibacillary leprosy and may be useful in stratifying patients for MDT. However, the wide variability of the levels of these markers in the patient groups suggests that these laboratory assays can, at present, only be used as supportive evidence to diagnosis and monitoring by an experienced clinician. The presence of B-cells and plasma cells were demonstrated
within leprosy lesions, even in the BT region of the spectrum, as was the secretion of anti-\textit{M. leprae} antibodies by these cells within lesional skin. However, the functional implication of these cells in the lesions remains to be elucidated. Finally, although DCs could mature in response to \textit{M. leprae} infection, monocytes pre-exposed \textit{M. leprae} antigens prevented their differentiation to DCs and further maturation suggesting that \textit{M. leprae} might actively subvert the development of the immune response. This opens up newer avenues to study the modulation of DC function by \textit{M. leprae} and its influence on the overall immunity and spectral pathology of leprosy. Considering the enormous complexity of leprosy, the present thesis demonstrates that the understanding of leprosy pathology can only be achieved by undertaking a coordinated multidisciplinary approach.
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Summary and concluding remarks


Immunopathology of leprosy
Samenvatting
De introductie van multi-drug therapie (MDT) in 1981-82 en de campagne van de WHO, sinds 1991, om lepra als volksgezondheidsprobleem te elimineren, heeft geresulteerd in een aanzienlijke daling van de wereldwijd lepraprevalentie. Meer dan 14 miljoen lepra patiënten werden gediagnosticeerd en behandeld met MDT tussen 1985 en 2005. Deze behandeling had als resultaat dat het aantal landen met een lepraprevalentie van meer dan één lepra patiënt per 10000 personen afnam van 122 landen in 1985 tot 9 aan het begin van 2004, met als gevolg een behoefte aan integratie van de gespecialiseerde lepra programma’s in de algemene gezondheidszorg waarbij niet-specialisten betrokken zouden worden bij de opsporing en behandeling van lepra. Toch bestaat er veel scepticisme onder ervaren leprologen die vrezen dat door deze aanpak, de gezondheidswerkers onvoldoende ervaren en opgeleid zullen zijn om een onderscheid te kunnen maken tussen de diverse vormen van lepra. Verder bestaan er nog steeds meningsverschillen betreffende a) de parameters die als ijkpunten worden gebruikt zoals prevalentie, punt-prevalentie en incidentie; b) de duur van en het type van behandeling van lepra en lepra reacties – de zogenaamde reversal reaction (RR) en erythema nodosum leprosum (ENL); en, c) de criteria die voor diagnose en classificatie van patiënten voor behandelingsopleidingen worden gebruikt. Betrouwbare laboratoriumtesten voor vroege diagnose van lepra en lepra reacties zijn nog niet beschikbaar.

Meting van antilichamen tegen het *M. leprae* PGL-I antigeen is specifiek maar onvoldoende sensitief voor het diagnosticeren van paucibacillaire (PB) lepra patiënten. Eerdere studies toonden een associatie van T-helper (Th) 2 cytokinen IL-4, IL-5 en IL-10 met lepromateuze lepra (LL) aan, terwijl bij tuberculoïde lepra dominantie van de Th1 cytokinen IL-2, IFN-γ en TNF-α in de laesionale huid wordt aangetoond. De lepra reacties worden, zowel RR als ENL, geassocieerd met veranderingen in de cytokine-activiteit, terwijl, *M. leprae* -specifieke T-cell klonen, die uit de RR-laesies worden geïsoleerd, een naar Th1 gepolariseerd cytokine-patroon toonden. Deze resultaten laten het verband zien tussen afzonderlijke cytokine-patronen met de spectrale vormen van lepra en lepra reacties. Dit suggereert dat de identificatie van dergelijke cytokine-patronen bruikbaar kan zijn voor de detectie van lepra en lepra reacties en het volgen van het effect van de behandeling. Echter, recentere studies tonen dat een duidelijke dichotomie binnen de cytokine patronen van de verschillende vormen van lepra en de reacties niet aanwezig is.

Een belangrijke gedeelte van de leprapatiënten, vooral die met een borderline classificatie, ontwikkelt lepra reacties gedurende de ziekte of zelfs na voltooiing van MDT. Zowel RR als ENL, indien niet vroegtijdig ontdekt en behandeld, leiden tot onherstelbare zenuwbeschadiging met als gevolg ernstig invaliditeit. De zevende bijeenkomst van de lepradeskundigen van de WHO (1997) was van mening dat een vroege diagnose, en daardoor het voorkomen van beschadigingen, cruciaal is, voor een snelle en adequaat behandeling van de reactie. Bij de behandeling van een RR worden bij voorkeur corticosteroïden gebruikt.
Deze zijn belangrijk voor het herstel van zenuwfuncties na een reactie. Corticosteroiden beïnvloeden de cytokinen bij patiënten met een RR en geven een daling van pro-inflammatoire cytokinen zoals IFN-γ en TNF-α. Hoewel de standaardbehandelingsduur van 12 weken met een geleidelijk aan verminderende dosis prednisolone door de WHO wordt geadviseerd, leidt dit tot aanzienlijk meningsverschil en debat met de verschillende lepraspecialisten, die een langere behandeling voorstellen. Voor ENL is de aanbevolen behandeling corticosteroiden en thalidomide, terwijl clofazimine, een onderdeel van MDT ook een onderdrukkend effect op ENL heeft. Het gebruik van thalidomide voor de behandeling van ENL is in veel landen niet toegestaan vanwege de bekende teratogene bijwerkingen. Echter, een langdurige corticosteroidé therapie, vooral bij ENL, kan leiden tot het ontstaan van een steroidafhankelijkheid. Vanwege de aanzienlijke twijfels over de duur en het soort van de behandeling voor reacties, zou een laboratoriumtest voor het meten van ziekteactiviteit van groot belang zijn. Een dergelijke test zou bij de besluitvorming kunnen helpen en zou een uiterst nuttig hulpmiddel voor de werkers in het veld zijn.

Een beperkende omstandigheid voor het meten van lepra-gerelateerde cytokinen in serum is dat de meeste studies één of slechts enkele cytokinen of cellulaire activatie-parameters met betrekking tot de ziekteactiviteit hebben gemeten. Omdat de ziekte immunologisch zeer complex is, is het moeilijk om een duidelijk beeld van het cytokinepatroon verbonden aan de verschillende vormen van lepra te verkrijgen. Bovendien tonen veel studies tegenstrijdige resultaten met betrekking tot de belangrijkste cytokinen die verband houden met de verschillende vormen van lepra en leprareacties. Dit kan veroorzaakt zijn door verschillen in de onderzoeksopzet en verschillen in de onderzoeksbevolkingsgroepen. Vandaar dat in de studies in hoofdstukken 2, 3 en 4 een breder paneel van cytokinen, cytokinereceptoren, oplosbare cellulaire activeringsproducten en antilichamen anti-PGL-I (met uitzondering van hoofdstuk 3) aantonen in de sera van de verschillende lepra-patiëntengroepen in vergelijking met gezonde controles.

Het belangrijkste deel van dit proefschrift (hoofdstuk 2, 3, 4 en 5) richt zich op de vaak tegenstrijdige resultaten van voorgaande studies zoals hierboven vermeld. Bovendien wordt in hoofdstuk 5 voor de eerste keer, het enzym chitotriosidase bepaald. Dit is een maat voor de activiteit van lipide-geladen macrofagen binnen het lepraspectrum en de reacties in relatie tot cytokinen en andere oplosbare celactivatie-producten en anti-PGL-I antilichamen in de in hoofdstuk 2 beschreven patiëntengroepen.

**Hoofdstuk 2** betreft een studie van serum cytokinepatronen (IL-6, IFN-γ, TNF-α, IL-10), de oplosbare receptor van IL-6 (sIL-6R), de oplosbare T-cel (sCD27) en macrofaag (neopterine) activeringsproducten en de *M. leprae* specifieke anti-PGL-I IgM antilichamen in relatie tot de verschillende vormen van lepra. Zoals met vorige rapportages, werd een grote variatie binnen de cytokinespiegels in de sera van de verschillende groepen gezien. Niettemin
waren IL-6, TNF-α en de neopterinespiegels hoger in patiënten dan in gezonde controles, wat suggestief is voor een verhoogde immuun activiteit. Er werd echter geen verschil waargenomen tussen reactieve en niet-reactieve patiënten. Neopterine en PGL-I waren verhoogd in multibacillaire (MB) in vergelijking met de paucibacillaire (PB) leprapatiënten, wat nuttig kan zijn voor de indeling van patiënten voor behandeling. IFN-γ en sIL-6R waren beduidend verhoogd gedurende ENL in vergelijking met niet-ENL patiënten en waren verlaagd gedurende corticosteroidenbehandeling, wat duidelijk correleerde met de klinische verbetering van patiënten. Dit zou er op kunnen wijzen, dat deze parameters in de controle van de therapie van ENL van nut zouden kunnen zijn.

Hoofdstukken 3 en 4 beschrijven longitudinale studies waarbij de patiënten werden gevolgd respectievelijk gedurende het ontstaan van een RR of tijdens het doorlopen van de multi-drug therapie van lepra. Hoofdstuk 3 volgt zeven patiënten geclassificeerd als, BL of LL die een RR ontwikkelden tijdens het beloop van de ziekte. Cytokinen IL-4, IL-5, IFN-γ, TNF-α, de cytokine receptoren TNF-RI/II en het macrofaag-activeringsproduct neopterine werden op verschillende tijdstippen in de patiënten sera gemeten, op het moment van diagnose van lepra, bij het begin van de RR en op verschillende tijdstippen gedurende de prednisolonebehandeling en aan het eind van de behandeling. Zes van de zeven patiënten vertoonden verhoogde serumneopterine bij het begin van de RR of binnen één maand na het begin. Deze waarden daalden na prednisolonebehandeling tot waarden zoals bij de diagnose van de lepra waren bepaald. Dit volgde de klinische verbetering van de patiënten. Daarentegen, werd er geen specifiek cytokinepatroon gevonden dat gerelateerd was met het begin van de RR en de verbetering onder steroïdenbehandeling. Deze resultaten benadrukken het belang van het bepalen van serum-neopterine als ijkmiddel ter begeleiding van de corticosteroidbehandeling van RR in lepra.

Hoofdstuk 4 beschrijft een prospectieve studie die vijftwintig leprapatiënten (15 MB en 10 PB) gedurende de loop van hun MDT-behandeling volgt. Het M.leprae specifieke anti-PGL-1 antilichamen, het macrofaag activeringsproduct neopterine en het acute fase eiwit, C-reactieve proteïne (CRP) werden in serum bepaald op het moment van diagnose en na 2, 4, 6 en 12 maanden MDT. Zoals ook in hoofdstuk 2 gezien werd, waren de spiegels van de anti-PGL-1 antilichamen en neopterine verhoogd in MB- in vergelijking met PB patiënten. Dit correleerde met het aantal bacterieën (BI) in de huidsmeer. Daarentegen, was de CRP verhoogd in ENL in vergelijking met patiënten die niet in reactie waren. Alleen de anti-PGL-1 antilichaamspiegels gingen gedurende het gebruik van MDT significant omlaag. Deze studie bevestigde het nut van het bepalen van de anti-PGL-1 antilichaamspiegel en van neopterine in de stratificatie van MB- en PB- patiënten met implicaties voor therapie opnieuw. Daar komt bij dat de verhoogde CRP-spiegels, samen met IFN-γ en sIL-6R, zoals beschreven in hoofdstuk 2, zouden kunnen dienen als potentiële ijktesten voor ENL.
Samenvatting

Macrofaagactivatie speelt een belangrijke rol in de controle van een M.leprae infectie. **Hoofdstuk 5** bestudeert de relatie van chitotriosidase, een macrofaag activering gerelateerd enzym, met lepra zowel in het serum als in situ in de laesionale huidbiopsieën van patiënten. Het is van belang op te merken dat de chitotriosidaseactiviteit in grotendeels dezelfde groep patiënten werd gemeten die geïncludeerd zijn in hoofdstuk 2. Dit maakt het mogelijk deze te vergelijken met cytokinen/ cellulaire activatie productspiegels die eerder zijn bepaald. MB-patiënten toonden verhoogde chitotriosidase-activiteit in het serum en in vergelijking met PB-patiënten en gezonde controle-personen. Dit werd ook gezien voor neopterine in hoofdstuk 2. Net als bij neopterine, werd geen significant verschil gezien in chitotriosidaseactiviteit bij patiënten met en zonder ENL, wat suggereert dat de expressie van deze producten onafhankelijk zou kunnen zijn van de reactiestatus van de patiënt. Verder werd een positieve correlatie tussen de activiteit van serumchitotriosidase en de neopterinespiegels gezien. Echter, in tegenstelling tot neopterine, dat correleerde, hoewel zwaar, met de uit de macrofaag afkomstig cytokinen IL-6 en TNF-α, hoewel zwaarder, werd er geen correlatie gezien tussen chitotriosidaseactiviteit en cytokinen die besproken worden in hoofdstuk 2. Aan de andere kant nam de chitotriosidaseactiviteit tijdens behandeling van ENL met corticosteroïden af, net zoals de cytokinen TNF-α en IFN-γ, in tegenstelling tot neopterine. Deze bevinding geeft duidelijk aan dat de regulatie van chitotriosidaseactiviteit verschillend is van die van neopterine of van de cytokinen, hoewel zowel chitotriosidase als neopterine producten van de macrofaag zijn. Men zou kunnen speculeren dat een verhoging van de chitotriosidase binnen deze context een gevolg is van een accumulatie van lipiden in alternatief geactiveerde macrofagen, terwijl de neopterinespiegels gerelateerd zijn aan een IFN-γ-gerateerde activatie van macrofagen.

**Hoofdstuk 6** behandelt een aspect van immunologie dat vaak binnen de lepra wordt veronachtzaamd, namelijk de aanwezigheid van B-cellen in de aangetaste huid in alle vormen van lepra, met name ook in tuberculoïde, hoewel de afweer tegen intracellulaire besmettingen als een bijna uitsluitend T-cel gemedieerd proces wordt beschouwd. Ofschoon de aanwezigheid van plasmacellen in de laesies van LL- en BL-patiënten een enkele keer wordt gemeld, is hun aanwezigheid ter plaatse met betrekking tot de pathologie van lepra nooit nader uitgelegd. De gepresenteerde studie gebruikt immunohistochemie om de aanwezigheid van B-cellen in de laesies van LL- en BL-patiënten te tonen. Verder toonde een organotypische huidkweek van een deel van diezelfde aangetaste huid, een actieve afscheiding van M. leprae specifieke antilichamen in het kweekmedium (supernatant). Dit suggereert, dat lokale plasmacellen bij antilichaamproductie betrokken zijn. Onderzoek van het kweekmedium toonde de aanwezigheid van pro-inflammatoire cytokinen, IFN-γ, TNF-α en ook IL-6, die een rol spelen in de differentiatie van antilichaamproducerende plasmacellen. Speculerend naar de rol van de B-cellen binnen de laesies,
kunnen zij verantwoordelijk zijn voor lokale afscheiding van antilichamen en behoud van granulomas hetzij door de T-cel activering te beïnvloeden of door de rekrutering van andere ontstekingscellen in de laesies te bewerkstelligen. Echter, om dit nader uit te zoeken is verder onderzoek naar de functies van de cellen is essentieel.

Utility of the serum bio-markers studied in Chapters 2, 3 and 4 of this thesis in diagnosis and monitoring of leprosy and reactions

<table>
<thead>
<tr>
<th>Markers</th>
<th>Diagnosis of leprosy</th>
<th>Diagnosis of reactions</th>
<th>Stratification of MB/PB</th>
<th>Monitoring MDT</th>
<th>Monitoring Rectional treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGL-I</td>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>Neopterin</td>
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<td>+</td>
<td>-</td>
<td>+ (RR)</td>
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<tr>
<td>Chitotriosidase</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>+ (ENL)</td>
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<tr>
<td>CRP</td>
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<td>IL-4</td>
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<td>IL-10</td>
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<td>IFN-γ</td>
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<td>ND</td>
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<tr>
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<td>ND</td>
<td>+ (RR)</td>
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<tr>
<td>sIL-6R</td>
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<td>+ (ENL)</td>
<td>-</td>
<td>ND</td>
<td>+ (ENL)</td>
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<td>sCD27</td>
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<td>TNF-RII</td>
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<td>TNF-R1II</td>
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</table>

**Key:**
- : useful
- : not useful
ND : not done
Professionele antigeen-presenterende cellen (APC), zoals dendritische cellen spelen binnen de immuunreacties op diverse infectieuze agentia een centrale rol. Naast het presenteren van antigenen in samenhang met MHC moleculen (signaal 1) en costimulerende signalen (signaal 2) geven DC’s ook een polariserende prikkel (signaal 3), die het Th1/Th2 patroon van de immunitie reguleert. DC’s, worden gestimuleerd om een meer Th1- of een meer Th2-gerichte reactie te bevorderen, afhankelijk van de omgevingsfactoren en de verschillende stimuli die rijping induceren (met inbegrip van ziekteverwekkers en ziekteverwekkerafgeleide antigenen). Aldus hangt de immuunregulatie door DC’s af van hun functionele mogelijkheden in het onrijpe stadium. Een ander mechanisme, dat op de observatie van een preferentiële inductie van Th1- en Th2-reacties door respectievelijk myeloïd en plasmacytaire DC’s wordt gebaseerd, suggereert dat de selectieve interactie van het juiste DC-subtype met verschillende soorten ziekteverwekkers het Th1/Th2 patroon van de immunreactie bepaalt. De ziekte lepra is een uniek menselijk model om de totstandkoming van immunreacties op ziekteverwekkers te bestuderen, vooral vanwege de spectrale pathologie.

De vooronderstelling in Hoofdstuk 7 is dat *M. leprae* een interactie aangaat met antigeen-presenterende cellen zoals de DC’s, wat leidt tot hun rijping en polarisatie tot fenotypen die een Th1, Th2 en/of en regulatoire T-cel respons teweeg brengt. Ook kan *M. leprae* de voorlopers van DC’s zoals monocyten beïnvloeden en hun differentiatie tot DC’s en hun verdere rijping verhinderen, zoals eerder is beschreven voor *M. tuberculosis* en *M. bovis* BCG. De balans tussen de verschillende reactiepatronen kan het resultaat van de besmetting en het spectrum van lepra uiteindelijk bepalen. De resultaten wijzen er op, dat *M. leprae* en zijn cellulaire fracties een uittijding van de monocyte-afgeleide DC kunnen geven, die door behandeling met GM-CSF en IL-4 van normale gezonde individuen wordt geproduceerd. Naast het sonicatie de gehele leprabacterie (MLS), waren de celwand (MLCwA) en membraanfracties (MLMA) het meest potent in de inductie van DC rijping. Kweekmedia van MLS-gerijpte DC’s toonden de aanwezigheid van TNF-α in relatie tot de rijping van de DC’s. Omdat TNF-α ook een rol speelt in de rijping van DC’s, is de hypothese, dat dit cytokine bij productie door DC’s - dezelfde of naburige DC’s- op een autocrine dan wel paracrine wijze kan beïnvloeden en hun rijping in gang zetten. Omdat met TNF-α−blokkerende antilichamen, slechts een gedeeltelijke blokkade van de rijping werd bereikt, wordt verondersteld dat ook andere mechanismen betrokken zijn bij deze uitrijping. Kweekmedia van MLS-uitgerijpte DC toonden geen detecteerbare spiegels van de suppressieve cytokinen IL-10 en TGF-β noch van het Th1-polariserende cytokine IL-12. Er werd echter wel een productie van IL-12 geïnduceerd na CD40 ligatie door CD40L getransduceerde J558 cellen. CD40 ligatie blijkt een belangrijk 3e signaal te zijn voor volledige rijping en polarisatie van DC’s. Deze bevindingen lijken aan te tonen dat DC’s van gezonde individuën inherent geprogrammeerd zijn om een Th1 immuun reactie in antwoord op *M. leprae* op te roepen. In tegenstelling hiermee, toonden
twee lepromateuze leprapatiënten een verminderde differentiatie van hun monocyten naar DC’s, wat een deficiëntie bij deze patiënten suggereert. Bovendien toonde één van de patiënten, die actieve lepra had, een verminderde rijping van DC’s in antwoord op MLS in vergelijking met de andere patiënt die geen actief ziektebeeld had, hetgeen zou kunnen wijzen op een onderdrukkende mechanisme bij actieve ziekte. Toll-like receptoren (TLR) zijn belangrijke receptoren voor patroonerkenningspeiling op DC’s en van TLR2 wordt geschreven dat deze belangrijk is in de immuunreactie tegen *M. leprae*. Echter, het blokkeren van MLS binding aan TLR2 door anti-TLR2 antilichamen had niet tot gevolg dat de rijping van een DC tot stand kwam. Dit veronderstelt een rol voor andere receptoren zoals andere TLR’s, mannosereceptor, DC-SIGN of een combinatie van receptoren zoals TLR2/1 in de MLS geïnduceerd rijping van DC’s. Tot slot, resulteerde de pre-incubatie van monocyten met MLS vóór de generatie van DC in een verminderde differentiatie van DC’s en een verminderde gevoeligheid tot rijping, wanneer gebruik gemaakt werd van lipopolysaccharide, een standaard rijpingsproduct voor DC. Dit zou kunnen betekenen dat *M. leprae* de differentiatie en de rijping van DC actief beïnvloedt en vermindert. Dit kan belangrijke gevolgen hebben voor het ontwikkelen van de immuunreactie en van het lepraspectrum.

Samenvattend, de gepresenteerde onderzoeken werpen verdere vragen op met betrekking tot de huidige benadering van de laboratoriumdiagnose, het volgen van leprapatiënten en het begrijpen van de leprapathologie. Cytokinen in het serum, zoals IFN-γ, TNF-α en macrofaag activeringsproduct chitotriosidase, zijn nuttig in het volgen van de behandeling van ENL, terwijl neopterine van belang is voor RR. Anderzijds zijn chitotriosidase, neopterine en PGL-I belangrijke ijkwaarden voor multibacillaire lepra en kunnen gebruikt worden bij indelen van patiënten voor MDT. Echter, de grote variatie in de spiegels van deze ijkwaarden binnen de patiëntengroepen geeft aan dat deze laboratoriumbepaling, momenteel, slechts als ondersteunende gegevens door een ervarene gezondheidszorgwerker bij de diagnose en controle kunnen worden gebruikt. De aanwezigheid van B-cellen en de plasmacellen werd binnen de lepraafleesies zowel immunochemisch aangetoond, zelfs in het BT gebied van het spectrum, als door het aantonen van anti-*M. leprae* antilichamen geproduceerd door deze cellen binnen de laesionale huid. De functies van deze cellen in de lepraafleesies moet nog nader worden toegelicht. Hoewel DC’s door *M. leprae* tot rijping kunnen worden aangezet, verhinderen *M. leprae* antigenen de monocyten die er aan blootgesteld worden in hun differentiatie tot DC’s en verdere uitrijping, wat suggereert dat *M. leprae* de ontwikkeling van de immuunreactie actief zou kunnen ontwrichten. Dit stelt nieuwe wegen open om de modulatie van de functie van DC door *M. leprae* en de invloed op de immunitiet en de pathologie van lepra te bestuderen. De enorme complexiteit van de ziekte lepra in acht genomen, toont dit proefschrift aan dat begrip van pathologie van lepra alleen dan kan worden bereikt, wanneer een gecoördineerde multidisciplinaire benadering wordt gevolgd.
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My promotors Prof. Steven Pals and Prof. Pranab Kumar Das. Thank you Steven for agreeing to be my promoter and for your interest in my work. Pran, many thanks for your patience, support, ideas, friendship and for pushing me across the finish line. We have discussed, argued and fought over many things – an unforgettable experience for both of us, I am sure! But in the end it came out fine!

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Dr. Ben and Ike Naafs – Ben, it’s a privilege to have you on my thesis committee. Who could have imagined that a chance meeting in Bombay would result in home in Munnekeburen (by the way, most Dutch people that I know are not even able to pronounce the word, let alone know where the place is!). Ike, thanks for always making me feel welcome and teaching me some of the aspects of the Dutch culture and way of life and for going over the introduction, summary and the samenvatting with a fine comb.

The work presented in this thesis would never have been successfully completed without the help of the various collaborators involved. Drs. Linda Oskam and Annemiek Geluk deserve thanks for all their support, especially their suggestions and help with writing of the articles. Dr. Mochammad Hatta and his team in Indonesia are thanked for the patient material. I had the pleasure of working together with Romi Usman from Dr. Hatta’s lab who visited our lab for a short while. Thanks are also due to Elaine Silva and Dr. Fatima Moreno, both from Bauru, Brazil, for a successful collaboration, which culminated in an article. Another collaboration was with Drs. Utpal Sengupta and Keshar Mohanty of the Central Jalma Institute for Leprosy (CJIL), Agra, India – thank you Dr Sengupta and Dr. Mohanty for your support and help in completing the work and writing up the paper. Dr. Marco van Eijk – Marco, I must have frustrated you, taking so long over this article, but its submitted finally – thanks for your help and patience and hope to be able to continue this collaboration.

Dr. Esther de Jong and Toni van Capel deserve special mention for teaching me the culture of monocyte-derived dendritic cells and for advice on experiments and interpretation of results.

My deepest gratitude to those faceless collaborators who made the biggest contribution to this work – the patients. The one aspect which I missed most in this study, unlike my masters’ study in India, was the lack of contact with the patients and study subjects although this was partly due to the fact that the patient samples came from different continents. I do feel strongly that contact with patients makes the researcher aware of the real problems of the disease at the level of the individual and allows the development of a humane approach.
to tackling the problem.

I am grateful to the Koninklijke Nederlandse Akademie van Wetenschappen (KNAW), the Q.M. Gastmann-Wichers Stichting and the Nederlandse Stichting voor Leprabestrijding (NSL) for the financial support which kept me going. Thanks also to the department of pathology of the AMC for the lab facilities and support.

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Lucie Das – we have always had wonderful discussions about life and books. Sorry that I am so cynical about everything – but maybe there is still hope for me….

My family – my little sis, Kavi, you were quite “busy” all the time that I was “wasting” on this thesis – got married, had a wonderful little one and with a second one on the way. And you kept me quite busy travelling up and down for each occasion! Thanks for your love, my little one! Mohan, thanks for everything. Little Sri – yes, I will finally finish my exams in Amsterdam and come home. Amma – finally you can get relieved that my endless “studies” are getting over and that you can start pestering me about the “more important” things in life.

Finally, Appa – wish you were here.

Anand
Curriculum vitae

Anand Mahadevan Iyer was born in Palghat, Kerala, India on the 5th of June 1974. He completed his higher secondary education (equivalent to A-levels) with physics, chemistry and biology from the Mithibai College in Bombay. He went on to obtain his B.Sc with microbiology (main) and chemistry (subsidiary) in June 1994. This was followed by a protracted but educative stint of research for his M.Sc. at the Foundation for Medical Research (FMR) affiliated to the University of Bombay until 2002. The research involved studying mycobacterial secretory and cytoplasmic antigens for monitoring multidrug therapy and detection of relapses in leprosy patients. Between December 1999 and April 2000, he visited the lab of Dr. P.K. Das at the department of Pathology, Academic Medical Center (AMC-UvA), Amsterdam where he gained experience in the use of molecular biology and immunological techniques involved in mycobacterial research. Returning to India in April 2000, he continued working at the FMR on the epidemiology of multi-drug resistant tuberculosis in Bombay using spoligotyping and other molecular techniques. After completing his M.Sc, he started his Ph.D in September 2002 under the supervision of Dr. P.K. Das at the University of Amsterdam. Since April 2008 he has taken up a position with the neuropathology group at the AMC, working on the role of inflammatory pathways in epilepsy under Dr. E. Aronica.
List of publications


