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

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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.

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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 (BI) 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

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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].

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

Markers	Diagnosis of leprosy	Diagnosis of reactions	Stratification of MB/PB	Monitoring MDT	Monitoring Reactional treatment
PGL-I	+	-	+	-	-
Neopterin	-	-	+	-	+ (RR)
Chitotriosidase	-	-	+	ND	+ (ENL)
CRP	-	+ (ENL)	-	-	-
IL-4	-	-	-	-	-
IL-6	-	-	-	-	-
IL-10	-	-	-	-	-
IFN-γ	-	+ (ENL)	-	ND	+ (ENL)
TNF-α	-	-	-	ND	+ (RR)
sIL-6R	-	+ (ENL)	-	ND	+ (ENL)
sCD27	-	-	-	-	-
TNF-RI	-	-	-	-	-
TNF-RII	-	-	-	-	-

Key:

+	:	useful
-	:	not useful
ND	:	not done

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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 *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-γ, TNF-α 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 *M.leprae*, the causative agent. The working hypothesis of **Chapter 7** was that *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, *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 *M.tuberculosis* and *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 *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

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within leprosy lesions, even in the BT region of the spectrum, as was the secretion of anti-*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 *M.leprae* infection, monocytes pre-exposed *M.leprae* antigens prevented their differentiation to DCs and further maturation suggesting that *M.leprae* might actively subvert the development of the immune response. This opens up newer avenues to study the modulation of DC function by *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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