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

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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 \textit{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. \textit{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-\(
\alpha\) 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-\(
\alpha\) 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 \textit{M. leprae} suggesting redundancy in TLR binding of the \textit{M. leprae} components. Importantly, pre-incubation of monocytes with MLS impaired their differentiation to DCs and their further maturation suggesting that \textit{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
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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; *rhu*TNF–α, *rhu*IL-1β, and (all from Peprotech); *rhu*IL-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 (10⁻⁶M); 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 2.10⁴ 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. 10⁴ 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 MAb (Miltenyi Biotec) were used as a responder population. Responder cells (10^5 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-α 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).
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Fig 2. Maturation of DC by MLS. A) MLS and BCG were able to induce maturation of DCs; B) MLR of mature DCs with allogenic CD4+ T cells, MLS induced strong proliferation of the T cells which was comparable to that induced by the standard cocktail (Std).

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.
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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 \textit{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.
**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.

![Fig 7](image)

**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 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
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 *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, *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 *M. tuberculosis* suggesting *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 *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 *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.

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