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ADVANCES IN LEPROSY IMMUNOLOGY AND THE FIELD APPLICATION:

A GAP TO BRIDGE

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ABSTRACT

Advances concerning the hosts' immune response to *M. leprae* infection have focused on elucidating the immune patho-mechanism(s) involved with the hope that predictive diagnostic and prognostic parameters (biomarkers) for field use would emerge. However, improvements in our understanding of the immunological responses to this complex disease have to date somewhat failed to provide the effective and robust methods for improving its predictive diagnosis in the field situation, particularly in those patients suffering from paucibacillary disease. In this article we have attempted to review some of the advances both in the immunology and immunopathology of leprosy and also highlight the limited clusters of immune parameters which are now available. Most importantly, we point out the limitations that still prevail in the provision of effective biomarkers in the field situation for either: (i) the diagnosis of indeterminate disease, (ii) predictive diagnosis of individuals developing reactional states, (iii) monitoring efficacy of treatment or (iv) monitoring treatment of reactional states.

PREFACE

Leprosy is one of the oldest recorded diseases to affect mankind. Archeological studies have revealed evidence of leprosy in an Egyptian skeleton of the 2nd century BC and the earliest written records, dating back to 600 B.C., come from India.[1] Interestingly, in the American continent the disease seems to be more recent as was most likely imported from Europe.[2] The stigma about leprosy prevails to these days because the close relationship between patients and new cases and the fact that *M. leprae* is not observed in healthy individuals.[3]

Although the Koch's postulates established a causative link between the presence of a microorganism and the associated disease, *Mycobacterium leprae* continues to defy this postulate. The presence of *M. leprae* in leprosy lesions discovered by Hansen in 1873 has lead to the

concrete evidence that the disease is associated with an infectious agent but experimental studies have failed to demonstrate the full human disease in animal models.[3] Moreover, the characteristic of *M. leprae* of being non-cultivable *in vitro* added to the absence of experimental models, has proven to be a major hurdle in the elucidation of the pathology of leprosy.

Despite some contrary observations[4], it is a widely recognized that this unique spectral disease results from the complex manifestations of varied immune responses that occur in different individuals susceptible to the same organism (*M. leprae*). One of the remarkable features of the disease is that the majority of individuals exposed to *M. leprae* remain normal and healthy and <10% of infected individuals succumb to the full-blown disease.

Another unique characteristic of *M. leprae* is its affinity for the Schwann cells of peripheral nerves, probably either as an evasive mechanism by the bacteria to avoid host immunity or on account of it being a favorable microenvironment, supporting bacterial growth This facilitates the slow but sure progression of nerve impairment, until the immune system recognizes the bacteria and the subsequent inflammatory response destroys the nerve further, the major single cause of leprosy associated impairment.

On account of the long incubation period of *M. leprae* within a host, the population at large, particularly in endemic areas, must be kept under constant surveillance. A pressing need for the containment of the disease in this millennium, is establishing predictive diagnostic and prognostic biomarkers for the infection and its complications (reactional states).

A surge of research particularly concerning the hosts' immune response has focused on elucidating the immune pathomechanism(s) with the hope that predictive diagnostic and prognostic parameters (biomarkers) will emerge. Hence, the advances in leprosy immunology that we describe in the following paragraphs are rather the application of increasing immunological knowledge about the disease rather than the immunology of leprosy. This

exercise is also an attempt to elucidate the immunity of patients versus those "immune" individuals who have been exposed to infection but do not develop overt disease (asymptotic).

ADVANCES IN IMMUNOPATHOLOGY OF LEPROSY AND LIMITATION

1) Ridley & Jopling classification and the advancing immunological concept

The Ridley & Jopling classification of leprosy is based on the manifestation of varying proportion of cellular infiltrates in lesions of different patients. The dynamic changes in lesional cellular infiltrates in combination with the clinical appearance of the lesions gave rise to the spectral concept of the disease.[5] The lepromatous leprosy (LL) pole shows multiple, symmetrically distributed lesions, showing an infiltrate largely composed of macrophages with varied degrees of foamy changes and few, scattered lymphocytes and plasma cells[6]; bacilli are numerous within and outside macrophages and many aggregate to form *globi*.[7] Nerves may show some structural damage or enlargement but hardly any cellular infiltration or lymphocyte cuffing; the nerve destruction is gradual, slow and frequently unnoticed. In addition, Schwann cells, perineural cells, axons, intraneural macrophages of dermal nerves may also contain bacilli.

On the other hand, tuberculoid leprosy (TT) shows few lesions with well-defined margins in which the center is markedly hypoaesthetic and does 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.[6] Within the granulomas, small nerves may be destroyed beyond recognition and a thickened peripheral nerve is regularly palpable in the vicinity of a lesion.

In between the two polar leprosy types are the unstable borderline forms including borderline lepromatous (BL), mid-borderline (BB) and borderline tuberculoid (BT), showing

clinical and histopathological characteristics intermediate to the polar forms. BT patients present several anesthetic and granulomatous lesions with cellular pattern similar to that are seen in TT patients but may contain a few bacilli. In BB patients, lesions are intermediate in number and size between tuberculoid and lepromatous patients with moderate anesthesia and irregular form; they are composed of epithelioid cells and lymphocytes diffusely spread while giant cells are absent and a bacilloscopic index (BI) of 3 or 4+ is regularly found. BL patients usually present numerous lesions sometimes hypoesthetic in some parts composed of histiocytic cells that tend to evolve to epithelioid cells, lymphocytes are scanty and a BI of *M. leprae* may be seen.[5]

A significant proportion of the leprosy patients, especially borderline ones, develop leprosy reactions either during the course of the disease or even after the multi-drug therapy (MDT).[8-10] Reactions are thought to be immune exacerbations as can be postulated from the changes in the characteristic proportion of lymphocytes to histiocytes and interpreted due to the lymphocytic reactivities to *M. leprae* and its antigens.[11] Principally two types of reactions are seen: i) type 1 or reversal reaction (RR) localized to dermal patch and neighboring nerves showing acute increase in both matured and blast lymphocytes most likely being *M. leprae* specific indicating an increase specific cell mediated immunity accompanied by excessive release of Th1 cytokines in the tissue; ii) type 2 reaction or erythema nodosum leprosum (ENL) whose histopathology appears to be complex involving immune complex deposition in the vessel walls[12] and later in the tissues[13], besides the fluctuation of T cell immunity also plays important role. The main features of reactions are summarized in BOX 1 and 2.

2) Immunopathology for further refinement of spectral pathology of leprosy

Rees and coworkers using an experimental model of leprosy showed that the elimination of *M. leprae* within macrophages is mainly T cell-mediated.[14] With this background, renewed

studies of the *in situ* characterization of the cellular infiltrates in leprosy lesions were undertaken. The availability of monoclonal antibodies aided the characterization of different cell populations and also immunohistochemical detection of cytokines/chemokines, enzymes and bacterial antigens within the tissues. These have served as confirmatory biomarkers of disease and as indicators of the spectral form of leprosy. For example, LL/BL lesions exhibit a characteristic infiltrate involving significant numbers of macrophages, B/plasma cells and scattered T cells, predominantly of the CD8^{+} subtype (Photomicrograph 1), in contrast to TT/BT lesions which exhibit primarily T cell infiltrates, mostly CD4^{+}, with little involvement of plasma cells, macrophages or CD8+ and regulatory T cells (Treg). Additionally, monoclonal antibodies against *M. leprae* antigens helped to optimize the differential diagnosis of leprosy[15,16] even on formalin fixed tissues.[17] With respect to the leprosy spectrum, LL/BL lesions exhibit strong expression of mycobacterial lipoarabinomannan (LAM) and the *M leprae*-specific phenolic glycolipid (PGL)-I antigens whereas TT/BT lesions exhibit much weaker staining. Importantly, the *in situ* detection of *M .leprae* antigens appears to be a confirmatory diagnosis of leprosy lesions even in the absence of bacilli. In addition, the differing expression pattern of these two antigens also identifies the reactional lesions as compared to the non reactional lesions.[16]

Further characterization also revealed that LL/BL skin lesions exhibit decreased numbers of CD1a^{+} Langerhans cells in the epidermis as compared to BT/TT lesions.[18] In this context, it is worth mentioning that the expression of a secretory antigenic epitope recognized by the monoclonal antibody 3A8 on CD1a epidermal LC clearly identifies RR lesions.[19]

In addition to immunohistopatholgy, molecular technology like polymerase chain reaction (PCR) for measuring mRNA of cytokines within the granuloma of the lesions from leprosy patients across the spectrum showed an improved classification of leprosy pathology.[20,21]

Moreover, techniques like T cell cloning or flow cytometry using CD markers help to isolate T cell subsets from blood or lesions to characterize their cytokine profile and association with leprosy spectrum and reactional episodes.[20,22,23] Taken together with the cumulative results of immunopathological analysis, as reported in literature, the Ridley Jopling's original classification of leprosy and reaction can now be modified as depicted in the Figure 1.

It should be, however, emphasized that such approach will need at least the facilities of modestly equipped laboratory.

A BRIEF OUTLINE ON ADVANCES OF IMMUNOLOGY

The preceding discussion demonstrates that a basic understanding of immunology is important in interpreting the pathology of leprosy and reactions. Briefly, the immune response is characterized by the innate immune response providing the first line of defense against pathogens and the acquired or adaptive immune response which is the antigen-specific arm.

1) Innate arm of immune response

Apart from mechanical and chemical barriers, the innate immune response consists of cells such as the polymorphonuclear leucocytes (PMN) and the monocyte/macrophage lineage which use pattern recognition receptors (PRR) to recognize pathogen-associated molecular patterns (PAMPs), evolutionarily conserved and widely distributed among different classes of pathogens. Among the important PRRs are the Toll-like (TLR), NOD-like (NLR) and C-type lectin (CLR) receptor families. Engagement of PRR by PAMPS results in activation of specific signal transducing systems within the host cells resulting in the release of pro- and anti-inflammatory mediators. At the same time, release of chemokines help to recruit lymphocytes and PMNs in order to sequester and eliminate the organism via inflammation and by releasing

enzymes, free radicals and other cytokines.[24] Another important component of innate immunity is complement activation, which is also implicated recently in the pathology of leprosy spectrum and reactions.[25] The presently recognized paradigm of innate immunity applicable to leprosy is summarized in Figure 2. For complete immunologic description the readers are also advised to refer to an article by Nath *et al*. in the previous issue.

2) Acquired arm of immune response

The acquired immune response starts with dendritic cells (DCs), potent antigen presenting cells (APC), which function as a bridge between innate and acquired arms of the immunity. DCs migrate from the site of infection and present antigen to naïve T-cells within the regional lymph node. Depending on their degree of maturation and signals (in terms of co stimulation and cytokines), DCs can stimulate naïve T cells to differentiate into distinct effector subpopulations.

CD4+ T cells are the dominant players in both the induction and effector phases of the immune response. On antigen engagement by their T cell receptors (TCRs), they can differentiate into (i) T helper 1 (Th1), secreting interleukin-2 (IL-2) and interferon-gamma (IFN-γ) and resulting in macrophage activation; (ii) T helper 2 (Th2) cells, secreting interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-13 (IL-13) which stimulates the production of antibodies and inhibits macrophage activation[26]; or (iii) into T helper 17 cells (Th17) that produces interleukin-17 (IL-17) and interleukin-22 (IL-22) and are involved in inflammation and autoimmunity.[27] Further the activities of resulting repertoire of antigen specific Th1 and Th2 cells appear to be under the control of another subset set of CD4+CD25+ FOXP3+, interleucina-10 (IL-10) or transforming growth factor beta (TGFβ)+ T cells known as Treg.[28,29]

Usually antigen specific T cells are recruited together with macrophages and DC in a lesion and form different types of granuloma which are modulated by different cytokines during the evolution of the disease, giving rise to a spectral disease as seen in leprosy. The basic mechanism of granuloma formation and its modulation is summarized in Figure 3.

THE EVOLUTION OF DIAGNOSTIC TOOLS FOR LEPROSY

1) Cell-mediated immunity and skin reactivity in leprosy – Mitsuda test

An important tool in measuring specific cell-mediated immunity in leprosy is lepromin, a crude preparation of inactivated *M. leprae* homogenates, also known as Mitsuda reagent. The Mitsuda reaction is a robust skin test, which can discriminate between LL and TT patients. The reagent, prepared from highly bacilliferous LL lesions or from *M. leprae* infected armadillo tissue, is injected subcutaneously being examined 28 days later for signs of a DTH reaction. To classify a patient the use of this skin test should be recommended as a primary, "stand alone" mode of definitive diagnosis of suspected paucibacillary (PB) leprosy cases and to make the diagnosis of PB leprosy highly likely in patients with slit-skin smear negativity and indeterminate histology but with a suspect cellular infiltrate and/or granuloma. A negative Mitsuda test can also aid in the confirmation of multibacillary (MB) disease where the patient presents with acid fast bacillus positive lesions and enlarged nerves. Some major disadvantages are its relative insensitivity, subjectivity of interpretation and lengthy response kinetics. There are also concerns about its contamination with animal material from which it was extracted, and batch variability in activity. However, in Brazil, lepromin reagent is routinely produced at the Institute of Lauro de Souza, Bauru, São Paulo. Lepromin and *in vitro* lymphoproliferative assay using patient PBMC (LpA) in presence of *M. leprae* antigens can thus aid in classification of patients in the leprosy spectrum.

2) Antibody titers in leprosy

Although population presents circulating anti-mycobacteria antibodies due to environmental exposure to different Mycobacteria species, the search for a *M. leprae*-specific dominant antigen resulted in the demonstration of the species-specific phenolic glycolipid (PGL)-I[30] and paved the way for serological assays for detection of anti-PGL-I antibodies in the sera of patients. Anti-PGL-I IgM antibody is regarded to be highly specific and useful for leprosy diagnostic as well as valuable in monitoring the contacts that might be at risk of developing the disease.[31-35] While the advantage of the use of PGL-I serology is its relatively high specificity, up to 90% in various studies[36-38], the major drawback is its lack of sensitivity in the detection of PB patients[36-39] and also in assessing the clinical status of those patients.[40]

The use of anti PGL-I serology has been simplified by the development of a rapid immunochromatographic flow test (ML-Flow test) as a simple *dipstick assay* even using whole blood samples. The ML flow test has been shown to be comparable to the ELISA in its sensitivity, being able to detect >90% of MB patients and 40% of PB patients, with background seropositivity in endemic controls at around 10% (31). Moreover, the dipstick test was reported to be applicable in the field, identifying multibacillary patients without the need for a slit-skin smear test. However, the use of these tests have gradually declined due mainly to cost.[41] It is our contention that such a simple test, a spin off product of advances in immunology is a useful tool that should be implemented in endemic regions for the control of transmission, on the understanding that it is the best assay we have, excluding good clinical assessment.

3) Cross reacting antigens and recombinant *M. leprae* antigens

The differential host responses observed to immunologically cross-reactive antigenic components of mycobacteria (mycobacterial-ImCRAC) among individuals exposed to different mycobacteria has long been recognized[42-43]. Therefore several investigators have focused efforts on the development of diagnostic antibody assays for leprosy based on such mycobacterial cross-reacting antigens, i.e. a "bar code" recognition system.[44]

The antigen 85 complex (Ag 85) is known to be a dominant antigen in the immune response to all mycobacterial species.[45,46] Despite the cross-reactivity, which restricts its utility as a specific marker in the diagnosis of leprosy, seropositivity for anti-Ag 85 components have been observed in 50-100% of lepromatous and 0-38% of tuberculoid leprosy patients.[47-49] Interestingly, the use of combination of native gel purified Ag 85 components and the mycobacterial heat shock protein 65kDa (hsp65) in an ELISA serology have been reported to be capable of differentiating LL from TT forms of the disease.[44,50]

Further studies revealed that the sera of >90% of LL/BL patients and > 85% of TT/BT leprosy patients reacted strongly to mycobacterial (29/33kDa doublet) and Hsp 65 (64-65kDa singlet) antigen fractions in Western blot assays (WB) combined with ELISA. The ImCRAC signature on WB by leprosy patient sera against whole mycobacterial antigen was found to be disease-specific.[44,47] Moreover the WB could be used to discriminate between TT, BT, BL or LL forms of leprosy. These candidate antigens have subsequently been purified from SDS-electrophoretic gels and used in an ELISA format, but unfortunately the ELISA could neither identify reactional patients nor monitor patient antibody responses to MDT since these antibodies appear to have a long half-life. The full potential of these surrogate markers merits further investigation for uses in combination with anti-PGL-I (IgM) and another assays using 35kDa.[51]

Most leprosy patients and contacts, but not TB patients, showed T cell proliferation or IgG response to the 35kDa protein of *M. leprae*[52,53] demonstrating its specificity. In both ELISA and dipstick assays, a relatively high sensitivity was obtained for both MB (70-100%) and PB (40-60%) of leprosy patients.[54-56] However, anti-PGL-I assays were found to be more specific[56] at least in MB and, in cases of PB its applicability appeared to be controversial. Another approach using Ag 85 detection of the antigen in the sera of leprosy patients was investigated[57]. A ratio of Ag85 to circulating 65kDa levels was then proposed as being indicative of the presence of viable *M. leprae* in patients, based on a similar approach adopted in TB diagnosis.[58] However, the low sensitivity for Ag 85 in the sera of untreated MB patients limited it potential use for monitoring leprosy patients.[57]

Various investigators have also attempted to use recombinant biotechnology to produce different antigens from the Ag 85 complex and to test these recombinant proteins for measuring both leprosy-associated antibody and T cell responses. However, T cell responses were not found to be useful to leprosy diagnostic or prognostic, whereas high antibody levels, to the different peptides or to the Ag 85 complex, were associated with MB but not with PB disease or reactional states. Therefore the use of the Ag 85 complex has not provided any major advance on other available leprosy associated dominant antigens particularly PGL-I.

4) Further advances for discovering *M. leprae* specific antigens

With the sequencing of the *M. leprae* genome[59] new recombinant antigens with no known homologues emerged to provide potential improvements in the diagnostic tool box. Five of these antigens were subsequently shown to be able to recognize individuals exposed to *M. leprae* as assessed by IFN-γ release assay (IGRA). Their sensitivity was reported to be high, being able to detect 71% of healthy contacts not identified by PGL-I IgM serology. These reports indicated that

these antigens could be potential candidates for the diagnosis of leprosy.[60] After, other proteins and peptides were tested for assessing immune response against *M. leprae* in order to develop adjuvant tool to evaluate exposure to leprosy or disease.[61-68]

Although IgG antibody response to several of these proteins showed geographic variation, two proteins named ML0405 and ML2331 were largely recognized by serum of patients. Based on this, a chimeric fusion protein termed LID-1 (leprosy IDRI diagnostic 1) was constructed from overlapping sequences of these proteins.[69] Positive titers of antibodies against LID-1 protein were found in 87 to 92% MB and 7 to 48% PB patients in different populations.[61,69,70] Of special interest was that some individuals presented high titers of antibodies against LID-1 one year before the appearance of clinical symptoms of leprosy[69] suggesting a role of this protein in the monitoring of contacts. Interestingly, LID-1 can also be used in a cell-based IGRA assay to determine the cell-mediated immune status as in case of the "Quantiferon" assay for TB.[71] However, when the antibody assay using LID-1 and PGL-I in parallel was carried out the results were more or less similar (Unpublished data). Consequently, a new study brings nine more new hypothetical unknown proteins with potential to leprosy diagnostic based seroreactivity.[72] However, the potential of these proteins should be evaluated in a larger casuistic and so the advances in the pursuit of new antigens using high throughput technology (HTPT) goes on but the gap between the fruits of these studies and field application remain as wide as before.

5) Special focus on the complex pathology of leprosy reactions

The preceding discussion demonstrates that laboratory-based immune tests concentrated on the diagnosis of leprosy. However, one of the major complications of leprosy is the associated reactional states, which occurs in a significant proportion of the patients. As a conservative

estimate, the incidence of reactions during the evolution of the disease could be in the range of 20-30% of patients on treatment, which varies from country to country.

The diagnosis of ENL lesions has been problematic for clinicians. Nath and coworkers addressed the question as to what causes a spontaneous development of an antigen-specific T cell response, during ENL in lepromatous patients who at diagnosis were Mitsuda LpA negative. Using a recombinant antigen LSR2 and its peptides, they reported that cryptic epitopes in the bacillus get exposed and recognized by the LL T cells only in ENL.[73] More importantly, they showed that during ENL and prior to ENL the T cells recognized the sequences RGD, GVTY and NAA and these differ from the sequences of LSR2 recognized by the T cells of LL patients without ENL.[74-76] Therefore, LSR 2 and its peptides can be used in an IGRA assay for ENL diagnosis. Unfortunately, these observations were not confirmed by other investigators yet.

OTHER SEROLOGICAL AND *IN SITU* PARAMETERS: CYTOKINES, SOLUBLE CELL ACTIVATION MARKERS AND CHEMOKINES

The generation and maintenance of immune cytokines and chemokines, which mediate multiple immunologic and non-immunological functions, 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. These results in a complex fine-tuned regulatory network of cytokines which often determines the clinical course of the infection and the outcome.

1) Cytokine cascades and roles in leprosy pathology and immunity

With respect to leprosy, research has focused on the association of differential cytokine profiles with the spectral pathology.[20, 77, 78] However, results from such studies have been varied

and conflicting and in retrospect it is difficult to associate distinct cytokine patterns with different spectral forms of leprosy or its reactions.[22,23,79-81]

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[82,83] suggesting activation of the immune cells by *M. leprae* antigens in all leprosy patients. IFN-γ and TNF (tumor necrosis factor) were elevated in TT as compared to LL patients with a significant negative correlation with BI[82] Besides, upon *in vitro* stimulation with *M. leprae* or its antigens a vast majority of the T cells recruited in tuberculoid leprosy are CD4+ with Th1 phenotype producing IFN-γ, IL-2 and TNF but little or no IL-4, IL-5 and IL-6.[77, 84, 85] Furthermore, i*n vivo* analyses evidenced mRNA for IFN-γ, IL-2, lymphotoxin (LT), TNF and Granulocyte-macrophage colony-stimulating factor (GM-CSF).[20,78, 86] LL patients, on the other hand, showed higher serum levels of IL-10 and interleukin-1 beta (IL-1β) as compared to TT patients.[82] *In vivo* studies have demonstrated predominance of IL-4, IL-5 and IL-10 in LL lesions previously[20] and also a positive correlation between IL-10 levels and BI (82). In our studies[21] using immunohistochemistry and PCR in the same tissue specimen, we were not able to establish any clear cut cytokine profile specific for the spectral type of leprosy, as was found before.[20] Monitoring IFN-γ and IL-4 is valuable in evaluating the efficacy of the treatment and in the clinical management of reactions, since both cytokines declines during treatment.[21]

Consequently, several studies have been carried out to assess the validity of measuring serum cytokines for diagnosing and monitoring the leprosy spectrum and reactions. These 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.[87,88] Moubasher *et al.*[82] observed that while all leprosy patients showed elevated levels of IL-1β and

TNF as compared to healthy controls, some degree of differential expression was noted with IFN-γ and TNF being elevated in TT sera whereas the opposite response was seen with respect to IL-10 and IL-1β respectively. RR patients showed elevated levels of IFN-γ, IL-2R and IL-1β as compared to non-reactional patients liable to such reaction.[82, 89-91] Whereas in ENL patients, in addition to the above mentioned cytokines, IL-10 levels were also elevated.[82, 84, 92] Moreover, patients who developed reactions had significantly higher IL-1β levels as compared to those who did not, suggesting a prognostic value of IL-1β measurement in serum in predicting reactions.[93] In this context, our studies showed that cytokine profiling is useful in the diagnosis and monitoring of RR (TNF and IFN-γ) and IL-6/IL-IL-6 R for ENL.[83, 87]

However it should be noted that the *in situ* analysis of cellular interaction and the cytokine/chemokine expression needs specialized laboratory facilities.

2) Other soluble factors related to cellular activation

Besides T cell cytokines, other indicators of cellular activation have been used as markers for CMI activity. The presence of neopterin that belongs to the class of pteridines in body fluids is suggested to be evidence for the activation of the CMI response, since its production is stimulated by IFN-γ (94). Elevated levels of serum neopterin were previously reported in 75% of leprosy patients including lepromatous (LL –BL) patients[83] and in particular in reactions[87,95] and could distinguish MB (LL-BL) from PB (BT) leprosy.[83,87] It is paradoxical that the elevated neopterin, associated with increased IFN-γ production, is associated with lepromatous forms of leprosy suggesting that CMI response may not be completely defective in these patients.

Human phagocyte-specific chitotriosidase, an endoglucosaminidase belonging to family 18 of glycosylhydrolases, is an important component of the innate immune response.[96,97] Elevated serum chitotriosidase activity has been reported in malaria[98], sarcoidosis[99] and

tuberculous pleural effusions.[100] In leprosy, chitotriosidase activity in serum was significantly elevated in LL/BL patients as compared to BT patients and healthy controls.[101] ENL sera showed increased chitotriosidase activity as compared to healthy controls which declined after corticosteroid treatment. Moreover chitotriosidase activity correlated with levels of neopterin.

Acute phase proteins (APP), which are systemic markers of inflammation, have been evaluated in leprosy and reactions. The most frequently assessed APPs 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.[102-106] With respect to SAA there are contradictory results with elevated levels reported in LL as compared to TT patients[102], whereas other studies did not show a significant difference.[105,106] 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 biomarkers.[102,103,105,106]

3) Chemokines in cell migration and tissue immunity in leprosy

Chemokines are potent chemoattractants of leukocyte and play an important role in migration of effector cells. However, not much is known about the chemokine profiles in leprosy patients. Some of the early studies showed intense IP-10 expression by keratinocytes in TT lesions; LL lesions did not express IP-10 constitutively, however, administration of PPD or IFN-γ into these lesions resulted in a strong induction of IP-10 expression, suggesting a differential expression of IP-10 across the leprosy spectrum associated with IFN-γ expression (107). Kirkaldy *et al.*[108] studied the expression of the chemokines MCP-1 (CCL2), RANTES and IL-8 (CXCL8) in leprosy lesions by *in-situ* hybridization (ISH). Although all chemokines were elevated, no differences in the level of expression were noted across the spectrum. However, MCP-1 and

RANTES were elevated in reversal reactions suggesting a role for these chemokines in migration and activation of the monocytes and T cells in these lesions.[108]

In subsequent studies, MCP-1 and IL-8 were found to be elevated in serum in LL patients.[109,110] Hasan *et al.*[111] demonstrated elevated levels of MCP-1 in sera of LL patients as compared to healthy controls (EC) or pulmonary tuberculosis patients probably related to the dissemination of the disease. In contrast, RANTES levels were lower in the LL patients as compared to EC or tuberculosis patients suggestive of a shift away from the Th1 phenotype of these patients.[111] Mendonça *et al.*[112] reported elevated levels of only CCL3 (MIP-1α) 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.

An important limitation of the use of cytokines, chemokines, acute phase proteins and cellular activation markers as biomarkers is that they reflect the general inflammatory response and would be expected to change in all immune-mediated conditions, thus they lack disease specificity. Hence such markers need to be combined with other indicators such as the anti-PGL-I titers, which are more disease specific, in addition to clinical and neurophysiological observations to obtain a more accurate and global view of the progression of the disease. However, these parameters may be of limited utility in monitoring treatment efficacy especially in reactional cases thus, probably, reducing the consequences of incomplete treatment.

4) Other innate and acquired immunity related molecules

i) Complement

The activation of complement results in the assembly of the membrane-attack complex (MAC), forming pores on the surface of the target cell and its eventual death. Recently, we

observed that measurement of MAC complex in serum of leprosy patients could be of value as a prognostic tool[25], since the preliminary studies showed that serum level of C9 and MAC are significantly elevated in reactional patients than those of no reactional individuals. However, but this pilot data needs confirmation.

ii) Metabolomic and nutritional origins

Since the exact mechanisms involved in disease susceptibility, onset and progression are presently unclear, recent research has focused on other aspects, including nutritional, genetic and metabolomic aspects associated with the disease.

Fatty acids

Various studies have examined lipid metabolism in leprosy, but there has been limited work using whole metabolite profiles to distinguish the clinical forms of leprosy. Some of the fatty acids are known, to have anti-inflammatory and others pro-inflammatory properties, and, could be potential markers for susceptibility and pathogenesis of the disease. In this regard, Al-Mubarak *et al.*[113] reported higher levels of polyunsaturated fatty acids in lepromatous leprosy. A new study[114] revealed the metabonomic profile in leprosy patients showing an increase in the levels of omega-6 and omega-3 polyunsaturated fatty acids (PUFA) metabolites with anti-inflammatory and pro-resolving roles in serum and skin during *M. leprae* infection, mainly in lepromatous patients, with normalization after multi-drug. The lipid profile observed suggests the development of host tolerance to the pathogen as a strategy to avoid tissue damaged.

Essential amino acid like tryptophan metabolizing enzyme

A recent study[115] reported the probable association of indoleamine 2, 3-dioxygenase (IDO) expression in LL macrophages with immunosuppression in lepromatous leprosy, using immunohistochemistry and serology. The authors concluded that IDO may be involved in the immunosuppressed status of LL patients in a pathogen-specific manner and may serve as an additional marker for the diagnosis and prognosis of the disease.

Vitamin D and its metabolic enzymes

It has long been appreciated that Vitamin D may influence the innate immunity against intracellular bacteria. Besides, the vitamin D-dependent induction of antimicrobial peptides in keratinocytes also provides a mechanism for host defense in skin.[116] In leprosy, Montoya *et al.*[117] suggests that in TT patients infection with *M. leprae* triggers a protective vitamin D–mediated antimycobacterial innate immune response characterized by the generation of the antimicrobial peptides cathelicidin and DEFB-4. Conversely, in lepromatous leprosy it lacks activation of this Vitamin D mediated pathways and consequently occurs reduced expression of these defensins. It requires further investigation to determine whether any of these mediators can be utilized as biomarkers in the diagnosis of leprosy.

The alpha1-acid glycoprotein

Recent comparative analysis of the serum proteome of leprosy patients, highlighted the differential expression of the isoforms of the acute-phase protein alpha 1-acid glycoprotein (AGP).[118] The same group reported that changes in serum levels of AGP and the differential expression of its isoforms can be used in the diagnosis and monitoring of ENL reactions, serving as a putative biomarker for ENL although the robustness of this association needs to be

established. Unfortunately, until to date, it remains unclear to what extent this marker is robust predictor and a marker for diagnosing ENL and monitoring the efficacy of the treatment.

Nitric Oxide Synthase

Nitric oxide generation is controlled by nitric oxide synthases which are regulated by IFN-γ, an important cytokine for leprosy immunity. The expression of nitric oxide synthases {inducible (i-NOS), endothelial specific (e-NOS) and neuronal specific (n-NOS) expressions was studied in leprosy spectrum and reactions, with the expectation of a better management of reactional patients. Although it has been reported in the literature that i-NOS expression could be used as a diagnostic marker for leprosy spectrum and reactions[119] our own data could not confirm the findings as reported in literature.

CONCLUDING REMARKS

The preceding discussion shows that the application of immunological knowledge has aided the understanding of the complex pathology of leprosy. In the process, some molecular parameters which may be used as biomarkers of the disease have been identified. Indeed the list of such biomarkers is still growing whereas the robustness of these markers is still lacking.

If we reflect back, it may be concluded that: 1) there is no "gold standard" for the diagnosis of leprosy *per se* and for the prediction of onset of reaction. The disease is still best diagnosed on the basis of clinical symptoms, slit-skin smear analysis and simple tissue histology. However, these criteria can be supported by the use of the PGL-I antibody assay and the Mitsuda test, which can be performed in the field situation. In the problematic "indeterminate" cases, molecular techniques such as: (i) immunohistochemistry, (ii) PCR and (iii) *in situ* hybridization

may prove useful in the detection of *M. leprae* specific antigens and genes. However these techniques can only be carried out in a well-equipped laboratory serving as referral centers. 2) There are still no "gold standard" biomarkers that could serve to predict leprosy reactions in although some insight has been gained. ENL is associated with high circulating levels of aAGP1, IL-6, IL-6R. The LSR2 specific T cell response and RR is associated increased levels of neopterin, TNF, CXCL10 and IFN-γ. These assay parameters again require reference laboratory facilities, although some commercial kits can be developed for determining a cluster of parameters in the field, but at a great cost. 3) Importantly considerable progress has been made in identifying candidate biomarkers for monitoring treatment efficacy in leprosy patients. Thus, further investigations of observed differences in the kinetics of anti PGL-I, LID-1 and LSR2 responses between those who respond or do not respond to treatment are merited. 4) Progress has also been made in identifying disease associated inflammatory biomarkers which may prove useful for monitoring treatment of reactional states. These include; aAGP-1, IL-6 and IL-6R for ENL and neopterin, CXCL10 , IFN-γ ,TNF for reversal reactions.

We therefore still have a long way to go in solving all the immunodiagnostic deficiencies associated with leprosy before we can look forward to assigning this disease to the history books. Good biomarkers can help clinicians in the diagnosis and monitoring of patients, at a time of declining numbers of clinical experts who previously could successfully diagnose and treat leprosy and its complications based on clinical symptoms and histopathological assessment.

Some of the diagnostic problems with leprosy can be overcome by optimizing the collection of samples in the field and their transport to reference laboratories while investing efforts to develop simpler and more robust diagnostic/monitoring tests. Should we follow this path, it is hoped that disease transmission will be minimized by optimal determination of the end

point of treatment. In order to achieve such goals, the Public Health Authorities have to rethink their strategies for application of the evolving immunological markers for the management of patients suffering from leprosy. The other alternative is that the researchers will go on in the pursuit of high technology oriented approaches with contradictory results and with minimal benefit for the patients and clinicians. Sadly, it is our contention that the gap between advances in immunology and their field application remains a bridge too far at present but there is promise that the gap can be narrowed by using the available robust tools at hand.

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Box 1: Reverse Reaction (RR)

- More common in BB and BL patients as compared to BT patients.

- Acute inflammation of skin and/or nerves: nerve function impairment.

- Erythematous swelling of existing lesions, appearance of new lesions; onset or worsening of neuritis.

- Lesions usually present increased infiltrate of lymphocytes, epitheloid cells, giant cells, oedema and a decrease in bacterial load and the immune response is characteristic of a delayed-type hypersensitivity (DTH).

- Cell mediated immune process characterized by an increase in lymphoproliferative response of lymphocytes to *M. leprae* as well as pro-inflammatory cytokines such as IL-1, IL-2, IL-12, IFN-γ and TNF

- The specific role of T cells in RR is unknown.

Box 2: Erythema Nodosum Leprosum (ENL)

- Affects 20% of LL and 10% of BL patients. High bacterial load and greater infiltration of lesions as important risk factors.

- Painful and tender red papules or nodules which may be accompanied by fever, joint pain, oedema of the hands, feet, and face, proteinuria and malaise. Neuritis is usually milder than with RR.

- Immune complex mediated disease with some degree of CMI. Histologically characterized by neutrophils followed by increased number of lymphocytes, plasma cells and histiocytes. Vasculitis appears to be a major pathological event along with interstitial oedema and necrotizing changes

- Recruitment of immune cells into the lesional sites and their activation is largely effected through the various soluble molecules such as cytokines, chemokines and immune complexes.

- The antigen specific function of T cell is ill defined.
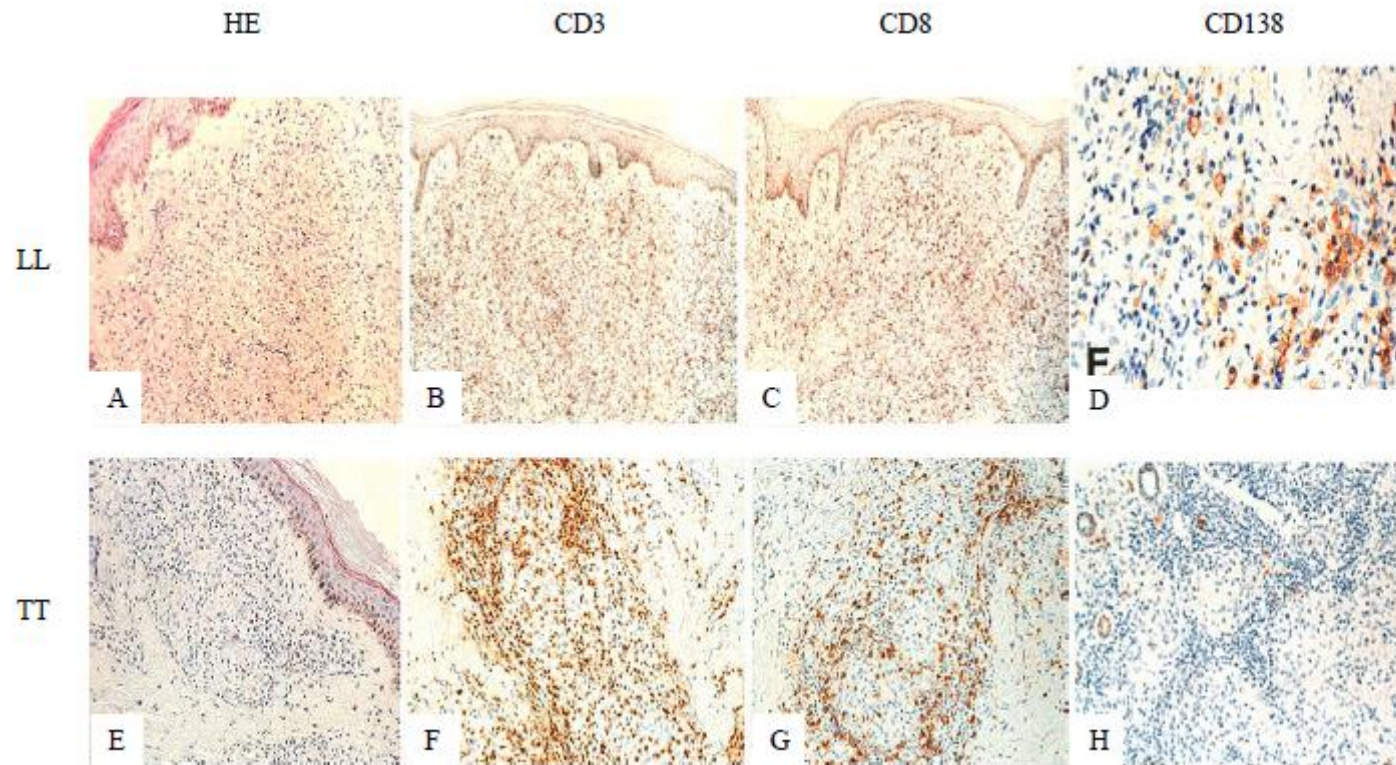
Photomicrography 1: Characterization of cellular infiltrate in leprosy lesions. HE: Hematoxilin Eosin. Magnification: 100x (except 1D: 400x)

TT	BT	BB	BL	LL

Reaction Type 1/RRR

Reaction Type 2/ENL

T-helper (Th) 1 *M. leprae* specific

- Cytokines: IL-2, IFN-γ, TNF, IL-10+/-

- Cell mediated immune response

- Activate macrophages

- Important against intracellular organisms

T-helper (Th) 2 *M. leprae* specific

- Cytokines: IL-4, IL-5, IL-13, IL-10+/-, IL-6-

- Antibody response

- Not very useful against intracellular organisms

Figure 1: Summarized Immunohistopathology of Leprosy Spectrum
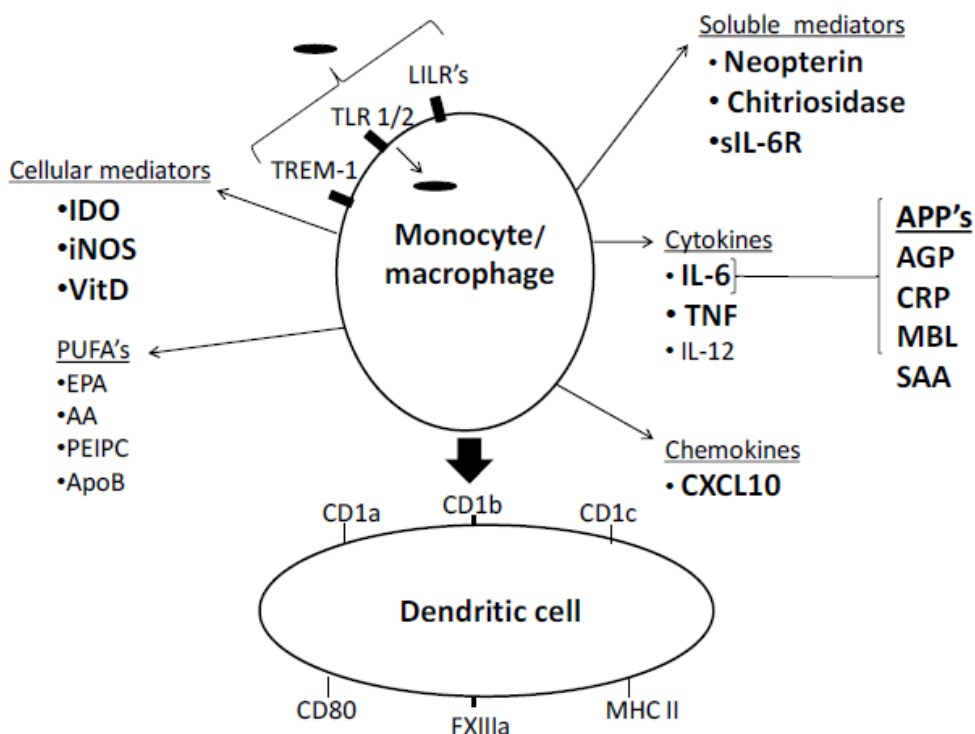
Figure 2: Biomarkers of Innate Response to *M. leprae* Infection. Abreviations: (AA: Arachidonic Acid; AGP: Alpha 1 Acid Glycoprotein; ApoB: apolipoprotein B; APP: Acute phase protein; EPA: eicosapentaenoic acid; CRP: C reactive protein; IDO: indoleamine 2 3-dioxygenase; IL-6: Interleukin-6; IL-12: Interleukin-12; iNOS: Inducible nitric oxide synthase; LILR's: leukocyte immunoglobulin-like receptors; MBL: mannose binding protein; PEIPC (1-palmitoyl-2-(5,6-epoxyisoprostane E2)-snglycero-3-phosphoryl choline); PUFA's: Polyunsaturated Fatty Acids; SAA: Serum amyloid A; sIL-6R: soluble interleukin-6 receptor; sTREM-1: soluble triggering receptor expressed on myeloid cells 1; TLR: Toll like receptor; TNF-α: Tumor necrosis factors alfa; TREM-1: Triggering receptor expressed on myeloid cells 1; VitD: Vitamin D. Molecules of particular interest are highlighted.
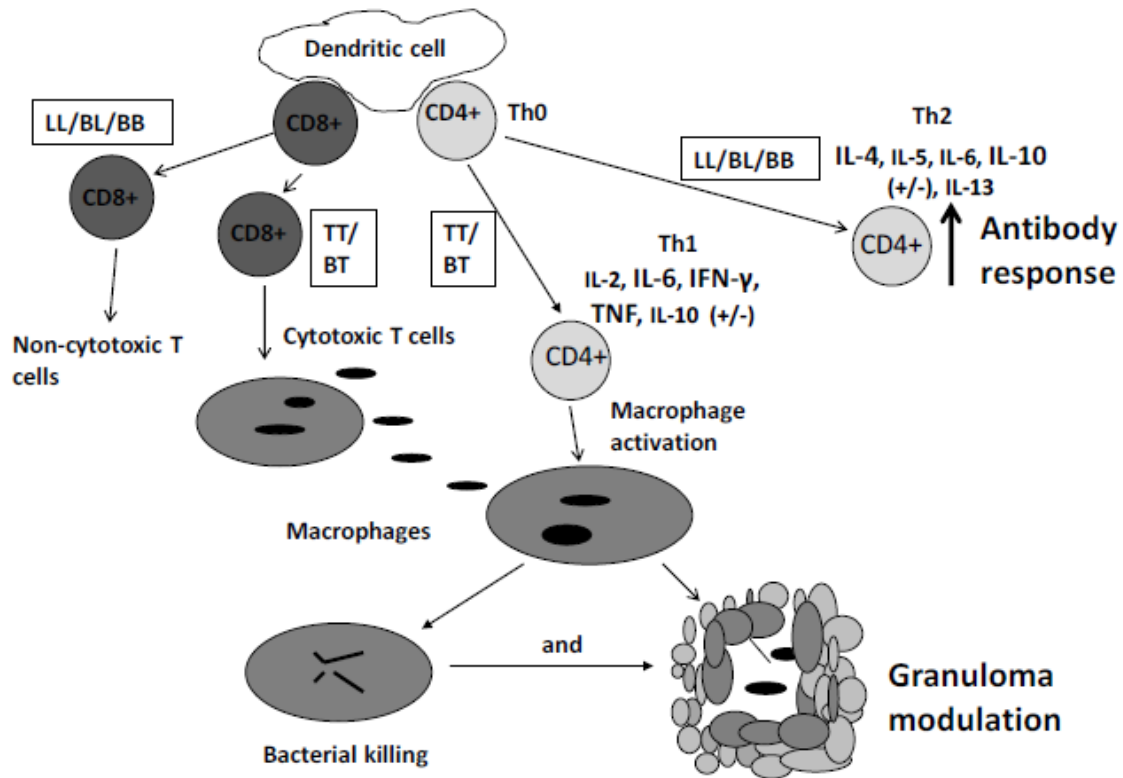
Figure 3: Acquired Immune responses to Leprosy. The key players are highlighted.