Leprosy

Leprosy is a chronic mycobacterial infection which elicits an extraordinary range of cellular immune responses in humans. This infection and its accompanying immunologic events initiate a peripheral neuropathy, which is the hallmark of this debilitating disease (Scollard et al., 2006).

Epidemiology of leprosy

Leprosy was recognized in the ancient civilizations of China, Egypt and India and the first mention of leprosy can be dated back to 600 BC. Throughout history, the afflicted have often been ostracized by their communities and family (WHO, 2010). Leprosy was once prevalent in most parts of the world but it vanished in several areas long before the development of an effective treatment. However, at the end of the 20th century, leprosy was endemic in many developing countries, particularly affecting the poorest segments of these societies. Every year more than half a million people were diagnosed as leprosy patients, while millions suffered from the manifestations of the disease (Visschedijk et al., 2000).

Global scenario

The estimated number of leprosy cases in the world during the 1980’s was between 10 and 12 million (Sugita, 1995). A high prevalence of 21.1 cases per 10,000 was recorded and 122 countries were considered endemic for leprosy. In 1991, the WHO adopted the goal of “elimination of leprosy as a public health problem by the year 2000” and thus, during the 1990s it launched a bold, ambitious leprosy elimination campaign. Elimination was defined as a reduction in the prevalence of leprosy patients receiving antimicrobial therapy to less than 1 per 10,000 populations. The multidrug regimens implemented was very effective in treating individual patients as well as in leprosy control programmes. By the end of 1999, the global burden had decreased to 7,53,000 and 107 out of the 122 countries had reached the elimination target. In 2002, WHO reported that there were 5,97,000 registered cases and 7,19,000 new detected cases during 2000, resulting in a global prevalence of registered leprosy
patients of just below 1 per 10,000 (WHO, 2010). However, a prevalence of more than 1 per 10,000 was still recorded in 15 endemic countries, mainly in Asia, Africa and South America. Nearly 83% of the registered cases resided in only six countries: India, Brazil, Burma, Indonesia, Madagascar and Nepal (Britton and Lockwood, 2004).

According to the WHO report which was prepared from the official reports received from 121 countries and territories, there were 2,13,036 registered prevalent cases at the beginning of 2009 and 2,49,007 detected cases in 2008. And, the report claims that leprosy has been eliminated from 119 of 122 countries with a prevalence of less than 1 per 10,000 as shown in figure R1. Some areas of Angola, Brazil, the Central African Republic, the Democratic Republic of Congo, India, Madagascar, Mozambique, Nepal and the United Republic of Tanzania still recorded a prevalence of 2 or more per 10,000. Over the past 20 years, more than 14 million leprosy patients have been cured, and about 4 million since 2000 (WHO, 2010).

**Indian scenario**

In the early 1990’s, 64% of all leprosy cases worldwide was accounted for in India alone, with a prevalence of 3.8 per 10,000 (Britton and Lockwood, 2004). By the year 2000, 107 countries had achieved the elimination target but India was in one of the six countries which still had a high percentage of registered cases. However, at the end of 2006, the number of cases registered stood at 82,801 with 1,39,252 new detected cases and a national prevalence of 0.84 per 10,000 (WHO, 2007). A comparison of the prevalence of leprosy in India over the past years is shown in figure R2 and the state wise contribution towards leprosy is displayed in figure R3. Hence, with a prevalence of less than 1 per 10,000, India has reached the WHO leprosy elimination target in 22 of the 29 states and 4 of the 6 Union Territories. According to the WHO report, pockets of high endemicity still remains in some areas of India (WHO, 2010).

**Clinical aspects of leprosy**

The clinical manifestations of leprosy can be grouped into three categories, depending on the mechanism involved namely outcome due to bacterial proliferation, features due to the immunologic responses of the host to the leprosy bacilli and features due to
Figure R1: Global burden of leprosy as of the beginning of 2009 (WHO, 2010).
Figure R2: Comparison of the prevalence of leprosy in India during the period 1981 to 2007 (Joshi, 2008).
Figure R3: Statewise contribution of new leprosy cases during the year 2006 (Joshi, 2008).
the peripheral neuritis caused by the first two processes (Hastings et al., 1988). In endemic areas, the subclinical transmission of *M. leprae* is believed to be widespread with transient infection of the nose (van Beers et al., 1996) and as many as 200 individuals become infected with *M. leprae* for each overt case (Hastings et al., 1988). However, most individuals with subclinical infection do not develop clinical disease and majority of them are capable of resisting infection with *M. leprae* effectively. In those who develop the disease, leprosy predominantly affects the skin, peripheral nerves and mucous membranes (Britton and Lockwood, 2004).

Leprosy manifests as skin lesion(s), skin anesthesia(s) and enlarged peripheral nerve(s) (Hastings et al., 1988). The disease always involves peripheral nerves, almost always the skin and frequently the mucous membranes. The ability of the host to develop a cellular immune response against *M. leprae* greatly determines the range of clinical and histopathological manifestations seen in leprosy (Scollard et al., 2006). Although the concept of “immunological spectrum” had come into existence by early 1960’s, it was Ridley and Jopling who proposed a practical classification that enabled a high degree of global uniformity in clinical practice (Ridley and Jopling, 1966).

Leprosy is a disease that presents as a spectrum and the Ridley-Jopling classification system uses strict clinical and histopathological features along with the bacteriological index to place each patient on the spectrum (Walker and Lockwood, 2007). The different states in the spectrum correlate with the activity of the host immune response to the pathogen as shown in figure R4. At one extreme, patients exhibit a high degree of cell mediated immunity with delayed hypersensitivity. They generally have one or few large well demarcated lesion with central hypopigmentation and erythematous anaesthetic lesions, often raised margins or occasionally scaly plaques (Sasaki et al., 2001;Scollard et al., 2006). Biopsies from these patients reveal well-developed granulomatous inflammation with rare acid-fast bacilli in the tissues. These patients are categorized as polar tuberculoid (TT). At the other extreme, patients display no resistance to *M. leprae* and they present widespread, poorly demarcated, raised or nodular lesions on all parts of the body. Occasionally, the disease is diffuse without distinct lesions. Biopsies reveal sheets of foamy macrophages in the dermis loaded with very large numbers of bacilli and microcolonies called globi. This anergic form of the disease is termed as polar
Infection with *M. leprae*

- **Subclinical Infection**
  - No disease
- **Spontaneous cure**

---

**Indeterminate group**

<table>
<thead>
<tr>
<th>TT</th>
<th>BT</th>
<th>BB</th>
<th>BL</th>
<th>LL</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2, IFN-γ</td>
<td>Tissue expression of cytokines</td>
<td>IL-4, IL-10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Antibodies against *M. leprae* antigens**
- **Cell-mediated immunity**
- **Skin lesions**
- **Bacterial Index**
- **Nerve injury**

**REVERSAL REACTION**

**ENL**

Figure R4: Disease spectrum of leprosy. IL, interleukin; IFN, interferon; ENL, erythema nodosum leprosum.
lepromatous (LL) (Sasaki et al., 2001). Majority of the patients fall into a broad borderline category between these two polar forms and are subdivided into borderline tuberculoid (BT), mid-borderline (BB) and borderline lepromatous (BL). In the borderline forms, there is a progressive reduction in the cellular immune response that is accompanied by frequent skin and nerve lesions, higher bacillary load and increasing antibody levels (figure R4). The polar forms of the disease are clinically stable while patients with borderline disease have a tendency to upgrade to a more lepromatous form (Britton, 1993). Very early lesions may present as non specific perineural infiltrates with rare acid fast bacilli, but the infiltrates are not sufficient to classify them. These are hence called indeterminate which has a variable course (Scollard et al., 2006). Three-fourth of these cases are cured spontaneously, some remain as indeterminate over long periods and some develop one of the clinical form of the disease (Hastings et al., 1988).

The Ridley-Jopling classification was on the basis of dermatological, neurological and histopathological findings (Ridley and Jopling, 1966). It not only aided in certain therapeutic trials and various projects but also in understanding the diverse manifestations of leprosy (Britton, 1993). However, this classification system is not practical in countries where there are not sufficient medical resources. Therefore, WHO established a more simplified classification system with just two categories – paucibacillary (PB) and multibacillary (MB). PB leprosy is defined as five or fewer skin lesions with no bacilli in skin smears, and MB leprosy cases as six or more lesions and may be positive for the bacilli. The correlation between the two classification systems were made as follow: indeterminate, TT and part of BT can be considered equivalent to PB leprosy and part of BT, BB, BL and LL correspond to MB leprosy (Sasaki et al., 2001).

**Classification systems followed in India**

The implementation of MDT necessitates the division of patients into two groups PB and MB. India harbors some unique clinical forms of the disease, such as neuritic leprosy, which are difficult to categorize in the field for treatment purposes. The formulation of a system that encompasses the clinical criteria along with the immunological and histomorphological expression and still be easily applied by the paramedical workers in field for effective treatment is the biggest challenge. Hence,
leprosy workers tend to revert to systems of classifying the disease to which they were earlier more familiar and table R1 summarizes some of the classification system in practice in India (Bhattacharya and Sehgal, 1999).

**Leprosy reactions**

Reactions in leprosy are the acute inflammatory complications that occur during the course of the disease in about 30-50% of all leprosy patients. These manifestations of leprosy are due to the immunologic response of the host to bacterial antigens. They are basically of two types.

**Type 1 or reversal reactions** (RR) are a consequence of spontaneous enhancement of cellular immunity and delayed hypersensitivity to *M. leprae* antigens. It affects patients in the borderline portion of the spectrum. Type 1 reaction can be seen as an acute inflammation in skin lesions (figure R5A) or nerves or both (Walker and Lockwood, 2007). They are characterized by edema and erythema of preexisting lesions often with progressive neuritis, resulting in sensory and motor neuropathy. The lesions may ulcerate in severe reaction cases. Type 1 reactions usually develop gradually, and their natural course may last for many weeks. The overall disease classification has a tendency to upgrade (Hastings *et al*., 1988; Scollard *et al*., 2006).

**Type 2 or erythema nodosum leprosum** (ENL) is a manifestation of an Arthus type of hypersensitivity reaction and occurs in multibacillary patients most often in BL and LL patients (Hastings *et al*., 1988). They generally occur in patients with poor cellular immunity to *M. leprae*, abundant bacilli in cutaneous and peripheral nerve lesions, and a strong antibody response. They are characterized by a sudden onset of crops of tender, erythematous skin nodules (figure R5B) along with fever, malaise and some degree of neuritis with sensory and motor neuropathy. Any tissue containing antigens of the leprosy bacillus can develop type 2 reactions and hence, the lesions of ENL are not confined to the skin but can affect the eye, joints, nasal mucosa etc (Hastings *et al*., 1988). The nodules may develop on the face, extremities or trunk without predilection for existing lesions. In severe form of this reaction, some of the cutaneous lesions may ulcerate. The natural course of type 2 reactions is usually one to two weeks, but multiple recurrences may occur over several months in many patients (Scollard *et al*., 2006). ENL reactions may also produce uveitis, neuritis,
<table>
<thead>
<tr>
<th>S. No</th>
<th>Classification system</th>
<th>Basis</th>
<th>Disease forms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Madrid Classification (1953)</td>
<td>Morphological /clinical</td>
<td>Lepromatous (L)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Tuberculoid (T)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Indeterminate (I)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Borderline (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tuberculoid (T)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Maculo-anaesthetic (MA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Polyneuritic (P)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Borderline (B)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Indeterminate (I)</td>
</tr>
<tr>
<td>3.</td>
<td>Ridley and Jopling Classification (1966)</td>
<td>Immunological</td>
<td>Lepromatous (LL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Borderline lepromatous (BL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mid-borderline (BB)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Borderline tuberculoid (BT)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tuberculoid (TT)</td>
</tr>
<tr>
<td>4.</td>
<td>WHO Classification (1994)</td>
<td>Clinical</td>
<td>Multibacillary (MB)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Paucibacillary (PB)</td>
</tr>
</tbody>
</table>

Table R1: Classification systems for leprosy adopted in India (Bhattacharya and Sehgal, 1999).
Figure R5: Leprosy Reactions. A. Type 1 reaction - Reversal Reaction; B. Type 2 reaction – Erythema Nodusum Leprosum (Adopted from Walker and Lockwood (2007))
Treatment of leprosy

Chemotherapy

The first-line drugs used for the treatment against leprosy include rifampicin, clofazimine and dapsone. All the patients receive WHO recommended multidrug combination with monthly supervision as shown in table R2A. As for the reactions, the reversal reactions peak at the first two months of treatment and can continue to even 12 months. In the case of ENL, it can start during the first or second year of antimicrobial therapy and relapses can occur over several years. The drugs used for treating these reactions are given in table R2B (Britton and Lockwood, 2004).

*Mycobacterium leprae*, the causative agent of leprosy

Taxonomy

Taxonomically, *M. leprae* belongs to the genus *Mycobacterium*, which is a single genus of the family *Mycobacteriaceae*, in the order Actinomycetales (Rastogi *et al*., 2001). Most mycobacteria have a high G+C content in their DNA in the range of 60 to 67 mol%. Interestingly, *M. leprae* has a lower G+C content of only 56% when compared to other mycobacteria (Imaeda *et al*., 1982). However, based on the 16S rRNA sequence, *M. leprae* was correctly positioned in the slow growing mycobacteria group (Smida *et al*., 1988; Cox *et al*., 1991; Kempsell *et al*., 1992).

Morphology

*M. leprae* is a non-motile, non-spore-forming, microaerophilic, acid-fast staining bacterium (figure R6A). It usually forms slightly curved or straight rods which measures 0.3-0.5 µm in width and 4.0-7.0 µm in length (Sasaki *et al*., 2001). Like other mycobacteria, *M. leprae* also tends to cluster as shown in figure R6B. The internal features of *M. leprae* as observed in an ultrathin section of the bacilli under transmission electron microscope (Scollard *et al*., 2006) are shown in figure R6C. The most important component of the cell of an intracellular pathogen like *M. leprae* is its cell wall as it constitutes the key interface between the pathogen and the host.
### Table R2A: Modified WHO-recommended Multi Drug Therapy (MDT) regimens

<table>
<thead>
<tr>
<th>Type of leprosy</th>
<th>Drug treatment</th>
<th>Duration of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monthly supervised</td>
<td>Daily, self administered</td>
</tr>
<tr>
<td>Paucibacillary</td>
<td>Rifampicin, 600 mg</td>
<td>Dapsone, 100 mg</td>
</tr>
<tr>
<td>Multibacillary</td>
<td>Rifampicin, 600 mg</td>
<td>Clofazimine, 50 mg</td>
</tr>
<tr>
<td></td>
<td>Clofazimine, 300 mg</td>
<td>Dapsone, 100 mg</td>
</tr>
<tr>
<td>Paucibacillary single lesion</td>
<td>Rifampicin, 600 mg</td>
<td>Ofloxacin, 400 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Minocycline, 100 mg</td>
</tr>
</tbody>
</table>

### Table R2B: Drugs used for treating reactions in leprosy

<table>
<thead>
<tr>
<th>S. No</th>
<th>Reaction type</th>
<th>Drug treatment</th>
<th>Duration of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Type 1 reaction (RR)</td>
<td>Prednisolone, 40-60 mg daily</td>
<td>3-4 months for BT patients</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>(Decreased by 5 mg every 2-4 weeks)</em></td>
<td>6 months for BB patients</td>
</tr>
<tr>
<td>2</td>
<td>Type 2 reaction (ENL)</td>
<td>Clofazimine, 300 mg daily</td>
<td>Several months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thalidomide, 400 mg daily</td>
<td></td>
</tr>
</tbody>
</table>
Figure R6: Cellular morphology of *M. leprae*. A. The acid-fast *M. leprae* (red rods) is seen in large numbers in the skin tissue of a lepromatous leprosy patient (Sugita, 1995). B. Scanning electron microscope image of a suspension of *M. leprae* derived from the foot pad of a nude-mouse (Scollard et al., 2006). C. Cross section of the bacilli as seen in transmission electron microscope (Scollard et al., 2006).
A great deal of information has been gained about the nature of the mycobacterial cell wall through biochemical and genetic manipulation of other cultivable mycobacterial species. Although similar approaches were not possible with *M. leprae*, the basic chemical studies have concluded that the cell wall of *M. leprae* is a highly complex structure. It is a covalently linked peptidoglycan-arabinogalactan-mycolic acid complex similar in composition to all mycobacterial cell wall (Draper, 1976; Daffe *et al.*, 1993; Vissa and Brennan, 2001). A schematic model of the cell envelope is illustrated in figure R7. The cell wall core is made of peptidoglycan, in which chains of alternating N-acetylglucosamine and N-glycolylmuramate are linked by peptide cross bridges. An arabinogalactan linker unit connects this peptidoglycan to the galactan layer, which is in turn linked to three branched chains of arabinan. These three layers of the cell wall core form an electron dense zone around the bacillus. Mycolic acids are important components of the cell wall of all mycobacterial species. In the cell wall, the inner leaflet of pseudolipid bilayer is formed by these mycolic acids linked to the termini of arabinan chains. The outer leaflet is composed of a rich array of intercalating mycolic acids of trehalose monomycolates and mycoserosic acids (PGLs), forming the electron-transparent zone. It has been postulated that a capsule like region is formed with many of these molecules along with phosphtidylinositol mannosides and phospholipids that are released from the cell wall after synthesis (Hirata, 1985; Draper *et al.*, 1987).

**Growth**

*M. leprae* has not been cultured in artificial media till date but it can be maintained in axenic cultures for a few weeks in a supposedly stable metabolic state (Truman and Krahenbuhl, 2001). It can remain viable for several days, *ex vivo* also. *M. leprae* is an obligate intracellular pathogen which multiplies very slowly with a long generation time of ~11 days (Ridley, 1988; Cole *et al.*, 2001). The optimal growth temperature of these bacilli is 32 °C and hence it prefers the cooler areas of the human body such as skin and ears (Eiglmeier *et al.*, 2001). *M. leprae* can be propagated in animal models including the armadillo (Truman, 2005) and normal, athymic and gene knockout mice (Krahenbuhl and Adams, 2000). These systems have provided with sufficient amounts of the bacilli for genetic, metabolic and antigenic studies of the pathogen.
Figure R7: Schematic representation of organization of the cell envelope of *M. leprae*. PI, phosphatidylinositol mannosides; LM, lipomannan; LAM, lipoarabinomannan; TMM, trehalose monomycolates; PDIMs, mycocerosic acids of phthiocerol dimycocerosate.
Genome

Determining the complete genome sequence of a strain of *M. leprae* was one of the highest priorities for leprosy research and control/eradication programmes during the 1990s. The strain that was used for sequencing was isolated from a patient from Tamil Nadu, India and is referred to as TN strain. To produce sufficient bacilli to extract DNA for library construction, this *M. leprae* was passaged in the armadillo (Eiglmeyer et al., 2001). The complete genome of the leprosy bacilli was sequenced and a detailed comparison of the genome and proteome sequences was carried out with that of *M. tuberculosis*. This comparative genomic analysis was found to be a powerful approach in predicting and unraveling the biochemistry, physiology and genetics of *M. leprae*. The complete genome sequence along with the principal findings of this analysis was presented in the landmark publication by Cole and his group in 2001 (Cole et al., 2001).

The complete genome sequence of the TN strain of *M. leprae* is 3,268,203 bases long with an average G+C content of 57.8%. The genome is a single circular chromosome and no plasmids existed. Based on the bioinformatics analysis, 1604 genes that encode proteins and 50 genes that encode stable RNA molecules were predicted. From the detailed pairwise comparisons of the genome and proteome sequences of *M. leprae* with that of *M. tuberculosis* (Cole et al., 1998), it is evident that only 49.5% of the *M. leprae* genome harbored protein-coding genes, whereas 27.0% were recognizable pseudogenes. The remaining 23.5% of the genome probably corresponds to regulatory sequences or even gene remnants mutated beyond recognition since they did not appear to be coding. Of the 1604 potentially active genes, 1439 are common to both pathogens. Although, orthologues were not detected for 165 genes in *M. tuberculosis*, functions could be attributed to 29 of these genes. Many of the 136 residual coding sequences were shorter than an average gene and did not show similarity to any known genes. Since these sequences were present in regions of low gene density, they probably represent pseudogenes (Cole et al., 2001).

Reductive evolution and massive gene decay

Reductive evolution is defined as the process by which large-scale loss of gene function arises. This phenomenon has been observed in other human pathogens like
Rickettsia and Chlamydia spp. which suggests that genes become inactivated once their functions are no longer required in their highly specialized niches. One hypothesis, known as Muller’s ratchet, suggests that the stochastic loss of genetic material during reductive evolution results in decreased fitness and little genetic variability (Eiglmeier et al., 2001). Earlier to the availability of M. leprae genome sequence, the most extensive genome degradation was reported for the typhus agent, Rickettsia prowazekii with 24.0% of non-coding DNA in its genome (Andersson et al., 1998). However, the massive gene decay and considerable reduction in the genome size observed in M. leprae is the highest reported till date. Comparative proteome analysis have indicated that the pseudogenes are translationally inert since only 391 soluble proteins were detected in M. leprae in comparison to ~1800 proteins in M. tuberculosis. The extent of gene reduction and genome decay is shown in figure R8 as well as in table R3, in comparison with the M. tuberculous genome (Vissa and Brennan, 2001). Despite this genome reduction, the G+C content of M. leprae genes (60.1%) is still higher than that of the pseudogenes (56.5%), and remainder of the genome (54.5%). Thus, since diverging from the last common mycobacterial ancestor, the leprosy bacillus may have lost more than 2000 genes (Cole et al., 2001).

In the course of reductive evolution, M. leprae has retained fewer numbers of genes in almost every category, particularly the insertion sequences (IS) and the acidic, glycine-rich families of proteins (PE and PPE proteins). Repressors, activators, oxidoreductases and oxygenases are also affected. One interesting observation from the comparative analysis of M. leprae and M. tuberculous genomes was that many genes are present in multiple copies in M. tuberculous genome whereas this redundancy is lost in M. leprae. Most of the paralogues in M. tuberculous are pseudogenes in M. leprae. For example, in contrast to the 167 genes in the PE and PPE families in M. tuberculous, only 9 intact PE and PPE genes are present in M. leprae along with 30 pseudogenes and no intact members of the PE-PGRS subfamily. Similarly, the mce operon which is implicated in the mycobacterial invasion process is present in just one copy in M. leprae as against four copies in M. tuberculous (Cole et al., 2001). Thus, M. leprae seems to have preserved only those genes that are required for transmission, establishment and survival in the host,
Figure R8: Massive gene decay in *M. leprae* genome. Percentage of the total potential reading frames assigned to major cellular functions are shown for *M. leprae* and *M. tuberculosis*.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Category of genes</th>
<th>Number of genes in each category</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Small molecule metabolism</td>
<td>464</td>
</tr>
<tr>
<td>2.</td>
<td>Macromolecule metabolism</td>
<td>394</td>
</tr>
<tr>
<td>3.</td>
<td>Cell processes</td>
<td>102</td>
</tr>
<tr>
<td>4.</td>
<td>Other functions</td>
<td>94</td>
</tr>
<tr>
<td>5.</td>
<td>Unclassified functions</td>
<td>501</td>
</tr>
<tr>
<td>6.</td>
<td>Pseudogenes</td>
<td>1116</td>
</tr>
</tbody>
</table>

Table R3: Extent of gene reduction in the *M. leprae* genome. For each category of genes, the number of putative functional gene in *M. leprae* (after eliminating the pseudogenes) is given and the corresponding numbers for *M. tuberculosis*. 
and has lost those genes that can be compensated for by the host-dependent parasitic lifestyle (Vissa and Brennan, 2001).

**Metabolism**

*M. leprae* cannot be cultured like any other bacterial species in a synthetic medium and this led to the notion that this bacterium lacks certain biosynthetic pathways. Sequencing and annotation of its genome has thrown light on its metabolic capabilities. *M. leprae* can generate energy by oxidizing glucose to pyruvate through the Embden-Meyerhof-Parnas pathway but not by utilizing acetate as the carbon source. Genome and biochemical studies suggest that the energy production in *M. leprae* is dependent mainly upon lipid degradation and glyoxylate shunt. Lipid metabolism is prominent in the biochemical repertoire. The pathogen has the full complement of genes for β-oxidation but far less genes for lipolysis when compared to *M. tuberculosis*. Far fewer enzymes namely the oxidoreductases, oxygenases and short chain alcohol dehydrogenases are present for the degradative pathways for carbon and nitrogenous compounds. Thus, it appears that *M. leprae* can grow only in a restricted or probably a specialized combination of carbon sources. Thus, from the metabolic clues obtained from the genome sequence, it is apparent that catabolism in *M. leprae* is severely limited. In addition, the bacilli have lost the anaerobic and microaerophilic electron transfer systems. The presence of an incomplete aerobic respiratory chain indicates that ATP generation from the oxidation of NADH is also not possible. On the other hand, the anabolic capabilities of *M. leprae* appear relatively intact. Complete pathways for the synthesis of purines, pyrimidines and most amino acids, nucleosides, nucleotides and most vitamins and cofactors were predicted. Thus, the retention of intact anabolic systems suggests that there might be limited availability of these metabolites or inadequate transport mechanism in the intracellular niche of *M. leprae* (Cole et al., 2001).

**Pathogenesis**

Iron metabolism is considered to play a central role in virulence, and a mechanism to scavenge iron is essential for microbial pathogenicity (Pessolani et al., 1994). *M. leprae* has many genes for haem and iron dependent proteins and uses the iron regulatory stems, ideR and furB. But the mbt operon that encodes the non-ribosomal
peptide synthase which is required for the production of iron-scavenging siderophores- mycobactin and exochelin, is absent and only a part of the iron uptake system seems to be functional in *M. leprae*. The laminin-binding protein is considered to play a significant role in the interaction of *M. leprae* with the Schwann cells and the gene for this important virulence factor was detected in the genome. Three of the four *mce* operons present in *M. tuberculosis* encoding putative invasion factors have been inactivated or lost in *M. leprae*. In contrast, many proteins namely the eukaryotic-like uridine phosphorylase, adenylate cyclase and the transport systems (ABC-transporter for sugars and Nramp1-like protein) present in *M. leprae* do not have homologs in *M. tuberculosis*. The leprosy bacillus survives within the host macrophages but its defense against the toxic radicals seems to be severely degenerated. Although it has retained the superoxide dismutase, *sodA* and *sodC*, it has no catalase-peroxidase and fewer peroxidoxins, epoxide hydrolases to combat the reactive oxygen species (Cole *et al.*, 2001).

Transcriptome

Transcriptional analysis of *M. leprae* genes gives a clue on the identity and nature of genes which are expressed at various stages of infection. This data complements the data generated from the proteome analysis of *M. leprae* and provides a complete picture on the events occurring during an infection (Scollard *et al.*, 2006). With less than 50% coding capacity along with a large number of pseudogenes, the analysis of the remaining genes also helps define the minimal gene set necessary for *in vivo* survival of this pathogen as well as genes potentially required for infection and pathogenesis of leprosy. In order to identify the genes transcribed during an infection, Williams *et al.* (2004) had analyzed the gene transcripts of *M. leprae* grown in athymic nude mice using RT-PCR and cross species DNA microarray technique. Transcripts were detected for 221 open reading frames, which included genes involved in DNA replication, cell division, SecA-dependent protein secretion, energy production, intermediary metabolism, iron transport and storage and genes associated with virulence. These results suggest that *M. leprae* actively catabolizes fatty acids for energy, produces a large number of secretory proteins, utilizes the full array of sigma factors available, and produces several proteins involved in iron transport, storage and regulation. The transcript levels of 9 of these genes (*aceA, esat-6, fbpA, relA, sigA, ...*
sigE, soda, aphC and mce1A) were compared with the levels in M. leprae derived from lesions of multibacillary leprosy patients as well as from infected nude mouse foot pad tissue using quantitative real-time RT-PCR. This comparison revealed that the transcript levels for all but one of these genes (esat-6) were comparable and this supports the continued use of the foot pad infection as a model for M. leprae gene expression profiling. Identifying genes associated with growth and survival during infection thus contributes to a more comprehensive understanding of the ability of M. leprae to cause disease (Williams et al., 2004).

Transcript analysis using real time PCR was carried out in our lab that enabled the study of the differential expression of mce operon genes of M. leprae across the leprosy spectrum. The mce operon consists of a set of 8 genes namely yrbE1A, yrbE1B, mce1A, mce1B, mce1C, mce1D, mce1E and mce1F. This study revealed the upregulation of mce1C and mce1E and the downregulation of mce1D across the leprosy spectrum. The transcript level of mce1A was found to be the lowest in comparison to the other mce genes. These results also suggest that mce1C may be an active virulent gene in the mce operon which helps in establishment of infection (Santhosh et al., 2005; Lini Ph.D., Thesis, 2008).

Bacterial pseudogenes are inactivated and presumably non-functional genes that can accumulate in the genomes of bacterial species, especially in those undergoing processes such as niche selection or host specialization. M. leprae with 1116 pseudogenes has less than 50% coding capacity of a 3.3 Mb genome. The overall pseudogene transcriptional profiling of M. leprae in the nu/nu mouse foot pad granulomatous tissue was done by global DNA array and RT-PCR analyses (Williams et al., 2009). This study demonstrated that M. leprae possesses the highest rate of bacterial pseudogene transcription documented to date. A large number of these degenerated ORFs which may no longer code for functional proteins were found to be expressed. The results of this study strongly suggested that even though a large number of pseudogenes present in M. leprae are transcriptionally active, majority of these transcripts are not translated. The presence of such ‘translational silencing’ mechanisms ensures that the valuable metabolites and energy are not wasted to produce proteins that would not benefit M. leprae in cellular survival or growth. Yet another study has also revealed the expression of pseudogenes in M. leprae upon
infection, supporting the fact that these are not just ‘decayed’ genes but may have a functional role (Suzuki et al., 2006).

**Proteome**

A proteome represents the functional complement of any genome and includes all the proteins that are expressed by a given organism under defined conditions. Understanding the basis of growth, virulence, and immunogenicity of *M. leprae* is essential for developing strategies towards improved treatment and control of leprosy. And, proteomics approach has greatly contributed towards this understanding (Scollard et al., 2006). Earlier analysis of *M. leprae* was dependent on the use of *E. coli* to demonstrate the expression (Jacobs et al., 1986) or promoter activity (Sela and Clark-Curtiss, 1991) of *M. leprae* genes. Majority of these analyses greatly relied on the traditional subcellular fractionation of the purified bacilli, raising antibodies against these fractions and genomic library screening using these immunologic reagents. Initially, based on this approach, five proteins were identified by Young et al. (1985) which were shown to be immunogenic. Subsequently, Sathish et al. (1990) were able to identify at least 22 new antigenic determinants of *M. leprae*. Over time, the combined efforts of many different laboratories led to the molecular identification of a large number of *M. leprae* protein antigens (Thole et al., 1995).

A major advancement in the knowledge of *M. leprae* proteins was achieved with the development of two dimensional maps for the different subcellular compartments of host-derived *M. leprae* (Marques et al., 1998). A total of 391 proteins were resolved of which 8 proteins were identified based on their reactivity to a panel of monoclonal antibodies. Further identification of new proteins was done through microsequencing of proteins in the cell wall fraction. This study further contributed to the already existing knowledge on the composition of *in vivo* expressed proteins of *M. leprae*. After the genome sequence of *M. leprae* was published, Marques et al. (2004) reported the first application of proteomics to a host-derived *Mycobacterium*. This study was undertaken to identify the proteins present in the soluble/cytosol and membrane subcellular fractions obtained from armadillo derived *M. leprae*. Proteins from each fraction were separated by two dimensional gel electrophoresis and identified by mass spectrometry. A total of 147 proteins were identified which were
the products of 44 different genes. Of these, 28 were newly identified proteins. Additional protein information was obtained with the use of chromatography and N-terminal sequencing.

Yet another high throughput proteomic approach resulted in the identification of 218 new *M. leprae* proteins from the cytosol, membrane and cell wall fractions. Although several proteins were identified in more than one subcellular fraction, the majority were unique to one. This study contributed to the understanding of the *in vivo* composition and physiology of the mycobacterial cell envelope, a compartment known to play a major role in bacterial pathogenesis (Marques *et al*., 2008). More recently, 1046 proteins have been identified from the total extract of *M. leprae* derived from armadillo using a Gel-LC-MS/MS approach (de Souza *et al*., 2009).

A relatively small gene-set and smaller proteome has made *M. leprae* an important model for conceptualizing the minimal gene set needed for obligate intracellular parasitism. The proteome analysis of *M. leprae* during different phases of growth and infection will provide insights into the mechanisms of virulence. In addition, identifying proteins expressed during early infection could give rise to better diagnostic reagents and potentially a vaccine to improve leprosy management (Scollard *et al*., 2006).

**Host response to *M. leprae***

The outcome of *M. leprae* infection and its manifestations greatly depends on the response of the host to the pathogen (Modlin, 1994). A number of factors are known to influence this host response which in turn determines the susceptibility or resistance of an individual to *M. leprae* infection (Scollard *et al*., 2006).

**Role of genetic Factors**

Genetic factors of the host play an important role in the susceptibility to leprosy. A large number of studies have indicated that different genes have influence in the human immune response to *M. leprae*. These genetic factors decide the overall susceptibility or resistance to the infection and also determine the degree of specific
cellular immunity and delayed hypersensitivity in the affected individual (Scollard et al., 2006).

Certain HLA phenotypes are associated with leprosy because the T-cell recognition of a foreign peptide is linked with host HLAs. HLA-linked genes have been associated with the clinical pattern of the disease (Serjeantson, 1983; Ottenhoff and de Vries, 1987). HLA-DR2 and HLA-DR3 alleles have been associated with TT leprosy while HLA-DQ1 conferred susceptibility to BL/LL leprosy (de Vries, 1991). In addition to the HLA region, the NRAMP1 gene (Abel et al., 1998), a series of microsatellite markers on chromosome 10p13 (Siddiqui et al., 2001), TAP2 (Transporter associated with antigen processing) gene (Rajalingam et al., 1997), Tumor necrosis factor alpha gene (Roy et al., 1997; Santos et al., 2002), Toll like receptors (TLRs) (Kang et al., 2002; Bochud et al., 2003; Krutzik et al., 2003; Kang et al., 2004), human vitamin D receptor gene (Roy et al., 1999; Hayes et al., 2003) and PARK2/PACRG (Mira et al., 2004) have all been implicated in the susceptibility to leprosy.

**Immune response to *M. leprae***

**Innate Immunity**

An effective immune response combined with the low virulence of leprosy bacillus determines the resistance to the development of the clinical disease. The innate immune response plays an important role in the development of an adaptive T-cell response. Cells of the innate immune system are equipped with germ line encoded pattern recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs) that are shared among groups of pathogens (Modlin, 2010).

**Toll-like receptors (TLRs)**

Mammalian Toll-like receptors are crucial for the recognition of microbial pathogens by macrophages and dendritic cells during innate immunity. This recognition affects the cytokine profile, which in turn determines the development of a Th-1 or Th-2 response. Strong expressions of TLR1 and TLR2 receptors have been reported in the monocytes and dendritic cells (DCs) isolated from the lesions of TT patients when compared to LL patients (Krutzik et al., 2003). TLR2 responses have been shown to be triggered by the 19 kDa and 33 kDa lipoproteins as well as by the major membrane
protein-II of *M. leprae*, which resulted in the production of cytokines such as TNF-α and IL-12 (Yamashita *et al.*, 2004; Maeda *et al.*, 2005). The IL-12 further mediates the instructive role of the innate immune response to determine the adaptive Th-1 cytokine response. The activation of TLR2-TLR1 heterodimers by killed *M. leprae* confirmed the presence of triacylated lipoproteins in *M. leprae* (Krutzik *et al.*, 2003). In addition, activation of TLR2/1 resulted in the upregulation of the receptor ligand pairs, GM-CSF and its receptors leading to the expansion of DC as well as IL-15 and its receptor component leading to macrophage differentiation (Krutzik *et al.*, 2005). Thus, these studies provide evidences that regulated expression and activation of TLRs at the site of disease contribute to the host defense against microbial pathogens (Modlin, 2010).

**Vitamin D**

When a pathogen invades the human host, a key function of the innate immune system is to trigger the anti-microbial activity and TLR2/1 mediated vitamin D pathway is one such pathway which is involved in this function. TLR induced IL-15 is required for the upregulation and activation of the vitamin D receptor (VDR) as well as the induction of 25-hydroxyvitamin D3-1α-hydroxylase, which converts the 25D into the active 1, 25D form of vitamin D3 (Krutzik *et al.*, 2008). The active form of vitamin D3 in turn strongly upregulates the gene expression of the antimicrobial peptide, cathelicidin (Wang *et al.*, 2004; Martineau *et al.*, 2007; Liu *et al.*, 2006; Liu *et al.*, 2007; Krutzik *et al.*, 2008). The simultaneous activation of VDR by IL-1β induces the antimicrobial DEFB4 (Liu *et al.*, 2009). The induction of vitamin D antimicrobial pathway may contribute to the disease outcome in leprosy and several studies have demonstrated the association of the polymorphisms in the VDR with the type of the disease (Roy *et al.*, 1999; Goulart *et al.*, 2006). This is further supported by the observation that the key components of the vitamin D antimicrobial pathway were differentially expressed in the TT and LL lesions (Prado-Montes de Oca *et al.*, 2009).

**Macrophages**

Macrophages, a key cell of the mammalian immune system, has a pivotal role in the pathogenesis of leprosy and these cells may participate in the infectious process as effector (phagocytes), antigen presenting or potent suppressor cells (Moura *et al.*, 2007).
They also serve as the primary host cells for *M. leprae* and in the absence of an effective immune response, *M. leprae* can multiply in the macrophages to over 100 organisms per cell (Hagge et al., 2004). Macrophages can be stimulated by whole *M. leprae* and/or its cell wall components can stimulate macrophages to release cytokines including TNF-α, *in vitro* (Suzuki et al., 1993).

Macrophages in different forms of leprosy are distinct. In tuberculoid leprosy lesions, the macrophage is thought to be activated, resembling epithelial cells and are hence called epithelioid cells. In lepromatous lesions, the macrophage harbors numerous bacilli and is called Virchow cells or lepra cells or foam cells due to its characteristic foamy appearance (Abulafia and Vignale, 1999; Modlin, 2010). Although there are histologic differences in the macrophages from TT and LL forms, the frequency of macrophages expressing CD209 in all forms of disease is similar (Krutzik et al., 2005). CD209 is a C-type lectin with carbohydrate recognition domains (CRD) that allows the recognition of pathogens containing mannose-rich glycoconjugates. A broad range of pathogens including *M. leprae* are phagocytosed through CD209 mediated binding (Barreiro et al., 2006). CD209 expressing macrophage has been detected in both the tuberculoid and lepromatous leprosy signifying the role of macrophages in phagocytosing *M. leprae* (Krutzik et al., 2005). Moreover, there seems to be a differential regulation of the macrophage functional programs in the leprosy spectrum. TT lesions showed high expression of IL-15 that induced vitamin D pathway while LL lesions had IL-10 that triggered the phagocytic pathway. In the clinically progressive lepromatous forms, the macrophage mediated phagocytosis pathway is prominent. In the tuberculoid forms as well as in patients undergoing reversal reactions from the MB to the self-limited form, the vitamin D dependent antimicrobial pathway is predominant (Montoya et al., 2009). Thus, the mechanism of activation of the phagocytic and antimicrobial pathway of the macrophages is central to the understanding of innate immune response against microbial pathogens (Modlin, 2010).

**Dendritic cells**

An effective host defense against intracellular pathogens involves the ability of the innate immune system to instruct the adaptive T-cell response. The dendritic cells are the professional antigen presenting cells that carry out this instructive role (MartIn-
Fontecha et al., 2003) and are highly efficient in the activation of T-cell responses to provide cell mediated immunity against the pathogen (Banchereau and Steinman, 1998). In the absence of an adaptive immune response, DCs may be the first to encounter the bacilli at the site of invasion such as nasal mucosa or skin abrasions. Uptake of *M. leprae* by the DCs in turn results in the local production of cytokines and chemokines that regulate the inflammation and also determines the ensuing course of the adaptive cell mediated immunity into a Th-1 or Th-2 response. DCs have been found to be very effective presenters of *M. leprae* antigen (Mittal et al., 1989; Santos et al., 2001). In addition, Langerhans cells, a subset of DCs that initiate immune responses in the skin, have been found to be present in increased numbers in TT lesions but not in LL lesions suggesting the active infiltration of these cells and their potential role in these sites (Gimenez et al., 1989). The mannose receptors (CD206) and DC-SIGN on the DCs also seems to play a role in the uptake and survival of virulent mycobacteria (Schlesinger, 1993; van Kooyk and Geijtenbeek, 2003). Lepromatous leprosy lesions are generally characterized by a marked deficit in DCs, (Sieling et al., 1999; Miranda et al., 2007; Simoes Quaresma et al., 2009) which is considered as a potential mechanism for the reduced cell mediated immune responses in these lesions (Ridley, 1964). This is further substantiated by the data obtained from various studies which suggest that *M. leprae* disrupts the differentiation of DCs and their antigen-presenting capacity (Krutzik et al., 2005; Lee et al., 2007; Murray et al., 2007; Santos et al., 2007).

**Adaptive immunity**

It has been estimated that >95% of the infected individuals are resistant to leprosy. And, in case of individuals with clinical leprosy, cell-mediated immunity plays an important role. In the infected persons, cell-mediated immunity exhibits both protective and destructive effects. The protective effects of this adaptive immunity in paucibacillary leprosy patients involve controlling the multiplication of *M. leprae*. At the same time, the cell mediated immunity results in a granulomatous inflammation and tissue damage which many a times leads to disfigurement and disability. The lack of CMI in LL patients is due to immunological anergy which is specific for the antigens of *M. leprae* (Scollard et al., 2006).
**T lymphocytes**

Cells of the T-cell lineage play an essential role in the resistance to *M. leprae*. Studies by Modlin *et al.* (1988) have revealed that T-cells of the CD4+ subset predominate in tuberculoid lesions, whereas CD8+ cells predominate in lepromatous lesions. In TT lesions, the CD4+ cells were distributed throughout the lesion and the cells of the T helper/memory phenotype outnumbered the naïve phenotype. On the other hand, numerous CD8+ cells were present primarily at the periphery of the lesions and these cytotoxic T-cells may play a role in mediating the macrophage localization, activation and maturation that leads to restriction or elimination of the pathogen. LL lesions exhibited a CD4+:CD8+ ratio of 0.6:1 with the CD4+ cells primarily belonging to the naïve phenotype and the CD8+ cells of suppressor subset. The CD8+ T-cells distributed throughout the lesion were proposed to downregulate macrophage activation and suppress cell-mediated immunity (Modlin *et al.*, 1983; Modlin *et al.*, 1988).

A strong correlation between CD1 expression and cell-mediated immunity in leprosy has also been reported (Rosat *et al.*, 1999). CD1 molecules present lipid and glycolipid antigens of mycobacteria to CD1-restricted T-cells. Based on *in vitro* and *in vivo* studies, the CD1 system of mycobacterial lipid antigen presentation has been implicated in the immunity to *M. leprae*. CD1b presents lipoarabinomannan from *M. leprae* and T-cells activated by LAM produced IFN-γ which were cytolytic (Sieling *et al.*, 1995). In leprosy patients, few CD1+ cells were found in LL lesions whereas there was a strong upregulation of CD1+ cells in the granulomatous lesions of patients with TT leprosy or reversal reaction (Rosat *et al.*, 1999; Sieling *et al.*, 1999).

Cytolysis of *M. leprae* infected macrophages or Schwann cells have been suggested to contribute to the protection against leprosy (Chiplunkar *et al.*, 1986). And, the CD8+ and CD4+ cells can function as class I and class II-restricted cytotoxic T-cells, respectively, which are capable of lysing *M. leprae* infected macrophages (Kaleab *et al.*, 1990). Cytotoxic T-cells bring about this lysis by the release of perforin and cytotoxic granules namely, granzyme B and granulysin. The distribution of granulysin and perforins has been correlated with the polar forms of the disease and are observed more frequently in TT skin lesions than LL lesions. Lysis of *M. leprae*-infected macrophage complements the attempts of the macrophage already involved
in the killing of intracellular \textit{M. leprae} and hence, contributes to the protection in leprosy. In addition, the cytotoxicity of natural killer cells and their more active IL-2 stimulated lymphokine-activated killer cells are also directed against \textit{M. leprae} infected macrophages (Chiplunkar \textit{et al.}, 1990) and Schwann cells (Steinhoff \textit{et al.}, 1991).

**Th-1/Th-2 dichotomy**

CD4$^+$ T-cells can be divided into Th-1 and Th-2 subsets based on the pattern of cytokines released (Mosmann and Coffman, 1989). The T helper type 1 (Th-1) subset is characterized by predominant IL-2 and IFN-$\gamma$ and it preferentially elicits cell-mediated immunity. On the other hand, Th-2 cells which produce IL-4, IL-5 and IL-10 augment humoral immunity. A number of studies have focused on the cytokine profiles of \textit{M. leprae} reactive T-cells and several major studies on the local immune responses in leprosy skin lesions have been published (Arnoldi \textit{et al.}, 1990; Flad \textit{et al.}, 1990; Mutis \textit{et al.}, 1993; Sieling \textit{et al.}, 1994; Yamamura \textit{et al.}, 1991; Shabaana \textit{et al.}, 2001). These studies have revealed the predominance of IL-2, TNF-$\alpha$ and IFN-$\gamma$ transcripts in tuberculoid lesions and IL-4 and IFN-$\gamma$ in lepromatous ones, reflecting the Th-1 and Th-2 patterns, respectively. A Th-1 like pattern of cytokine gene expression have been demonstrated in the early lesions which were histologically consistent with TT or BT disease (Stefani \textit{et al.}, 2003). Circulating leukocytes and T-cell clones from tuberculoid patients when stimulated by \textit{M. leprae, in vitro}, have also been found to produce a Th-1 cytokine pattern while those from lepromatous patients produced Th-2 cytokine pattern under similar stimulation (Misra \textit{et al.}, 1995). Thus, studies of cytokine gene expression in leprosy lesions have given a more detailed description of the immunological parameters of the polar types of leprosy (Scollard \textit{et al.}, 2006).

**Host-pathogen interactions**

**Interaction of \textit{M. leprae} with macrophages**

\textit{M. leprae} is an obligate intracellular pathogen that is capable of prolific growth in the macrophages (Hagge \textit{et al.}, 2004). The bacilli are readily apparent within tissue macrophages in lepromatous leprosy patients whereas it is rarely seen in tuberculoid
patients. The presence of enormous number of intracellular *M. leprae* within the macrophages in the granulomas of LL patients and infected nude mice indicates that *M. leprae* overcomes the microbicidal capacity of normal macrophages (Sibley *et al.*, 1987).

**Resistance of *M. leprae* to bactericidal killing by macrophages**

The phagocytic cells play a crucial role in the clearance of an infection by an invading microorganism. The bactericidal mechanisms in the phagocytic cells include the effects of toxic oxygen derivatives such as superoxide anion (O$_2^-$), hydroxyl radical (OH*) and/or hydrogen peroxide (H$_2$O$_2$) derived from the respiratory burst (Babior, 1978) as well as degradation by lysosomal enzymes (Dhople, 1983). However, *M. leprae* upon ingestion by cells of the monocye/macrophage series not only resists destruction but also proliferates within these cells (Holzer *et al.*, 1986). The mechanisms by which *M. leprae* survives and replicates inside the macrophages are being unraveled and has been an intense area of research. An illustration on some of the strategies employed by *M. leprae* for survival in macrophages is shown in figure R9 and is discussed below.

*M. leprae* has been shown to be ingested by phagocytic cells, *in vivo* (Mor, 1983) and *in vitro* (Mahadevan and Antia, 1980). Upon phagocytosis by macrophages, *M. leprae* was observed in a membrane bound cytoplasmic vacuoles in the cultured macrophages. An electron transparent zone (ETZ) characteristically surrounds the *M. leprae* in the macrophages (Sibley *et al.*, 1987) and it has been shown to be formed within one hour of phagocytosis of the bacilli (Ryter *et al.*, 1984). The ETZ has also been observed in *M. leprae* extracted and purified from experimentally infected armadillo or nude mice (Rastogi and Hellio, 1990). The intracellular growth of pathogenic mycobacteria has been linked to the presence of this electron transparent zone, which surrounds the phagocytosed bacteria and prevents the diffusion of lysosomal enzymes in infected macrophages (Ryter *et al.*, 1984; Rastogi and Hellio, 1990). Mycobacterial cell wall components such as mycoside C (Draper and Rees, 1970) and PGL-1 (Hunter and Brennan, 1981) have been implicated in ETZ formation and the subsequent protection of bacteria from the host defense mechanisms.
Figure R9: Schematic representation on the different survival strategies employed by *M. leprae* in the macrophages. a Schlesinger and Horwitz, 1991; b Sibley *et al.*, 1987; Ryter *et al.*, 1984; c Chan *et al.*, 1989; Thangaraj *et al.*, 1990; d Frehel and Rastogi, 1987; Sibley and Krahenbuhl, 1987; e van der Wel *et al.*, 2007.
Upon phagocytosis of microorganism by macrophages, a respiratory burst ensues in which there is an increased consumption of oxygen for the production of superoxide and this reaction is catalyzed by NADPH oxidase. Other reactive oxygen intermediates like hydrogen peroxide, hydroxyl radical and singlet oxygen, are subsequently generated. These toxic oxygen products are important antimicrobial defense mechanism of the phagocytes (Forman and Torres, 2002) and these macrophage derived oxygen products can kill intracellular pathogens such as *M. leprae*. However, studies have shown that human PMNs, monocytes and murine peritoneal macrophages does not respond to viable or irradiated *M. leprae* with a significant metabolic burst (Holzer *et al.*, 1986). It has been demonstrated that the complement receptors – CR1 and CR3 on monocytes and CR1, CR3 and CR4 on monocyte derived macrophages and fragments of complement C3 on the bacterial surface mediate phagocytosis of *M. leprae* (Schlesinger and Horwitz, 1990). Uptake of *M. leprae* into the monocyte is selectively mediated by the complement receptors, CR1 and CR3, which bind to the complement components activated on the surface of *M. leprae* by PGL-I (Schlesinger and Horwitz, 1991). Significantly, phagocytosis by this route is not associated with activation of the bactericidal oxidative mechanisms. This lack of stimulation of the respiratory burst could possibly be due to the downregulation of superoxide generation by PGL-I (Chan *et al.*, 1989). Further, *M. leprae* possesses superoxide dismutase that is capable of resisting oxidative stress (Thangaraj *et al.*, 1990). And, the expression of *sodA* and *sodC* in *M. leprae* during infection as revealed through transcriptome analysis further supports the role of this enzyme (Williams *et al.*, 2004). Thus, the leprosy bacilli appear to be well equipped to handle the antimicrobial reactive oxygen intermediates in the host macrophages.

Yet another crucial survival strategy of *M. leprae* in the macrophages is the inhibition of phagosome and lysosome fusion. In normal macrophages, majority of phagosomes containing freshly isolated viable *M. leprae* resisted fusion with secondary lysosomes, while this trend was reversed with γ-irradiated *M. leprae* (Frehel and Rastogi, 1987; Sibley and Krahenbuhl, 1987). However, in activated macrophages, phagosomes containing viable *M. leprae* cells, underwent extensive fusion with lysosomes and the bacilli were often morphologically damaged, suggesting enhanced digestion by the activated macrophages (Sibley and Krahenbuhl, 1987). The surface components of *M. leprae* has been implicated in this inhibition of phagosome-
lysosome fusion (Frehel and Rastogi, 1987). It has been shown that *M. leprae* avoids this fusion during the early hours of infection (Frehel and Rastogi, 1987; Sibley *et al.*, 1987) and subsequently moves into the cytosol (van der Wel *et al.*, 2007). And, it is the cytoplasmic matrix of the host cells that serves as the natural site of multiplication of *M. leprae* (Mor, 1983; van der Wel *et al.*, 2007).

Studies from *M. leprae* infected athymic nu/nu mice have shown that the macrophages in the footpad granulomas are phenotypically similar to the peritoneal macrophages. They harbored enormous numbers of *M. leprae* and were refractory to activation by IFN-γ for microbicidal activity. In addition, in the *M. leprae* engorged granulomas macrophages, there was neither IFN-γ induced augmentation of class II MHC expression nor PMA-induced respiratory burst (Sibley *et al.*, 1987; Sibley and Krahenbuhl, 1988). Thus, these results provide evidence that *M. leprae* is capable of modulating the effector functions of the macrophages to facilitate its survival in these cells.

**Interaction of *M. leprae* with Schwann cells**

**Adhesion to Schwann cells**

*M. leprae* primarily invades Schwann cells (SCs) in the peripheral nerves and this invasion leads to nerve damage resulting in deformities and disabilities which are the hallmarks of leprosy (Skinsnes, 1971; Job, 1989). The Schwann cell is a part of the Schwann cell-axon complex which may or may not be myelinated and this unit is surrounded by a layer of basal lamina (Barker, 2006). *M. leprae* probably enters via the vascular endothelium by mechanisms not yet determined and once inside the endoneurial compartment, they adhere to the SCs. Rambukkana *et al.* (1997) postulated that the first step of infection of the SCs is the specific interaction of *M. leprae* with basal lamina of the Schwann cell unit which is shown in figure R10. They have shown that, for *M. leprae* seeking the Schwann cell niche, the globular (G) domain of the α2 chain of laminin-2 is the initial specific target (Rambukkana *et al.*, 1997). Subsequent to this interaction, *M. leprae* is taken up by the Schwann cell which is dependent on α-dystroglycan, which is the receptor for laminin within the cell membrane, and other intracellular components (Rambukkana *et al.*, 1998). The tissue distribution of the laminin α2 chain is limited to SCs, striated muscle and the
Figure R10: Specific interaction of *M. leprae* with laminin-2 of the basal lamina in the Schwann cell unit. # represents PGL-I or LBP21 of *M. leprae*; DG, dystroglycan; SC, Schwann cell.
placenta and these are the tissues (Leivo and Engvall, 1988) which are the natural sites of *M. leprae* infection in human.

Amongst the bacterial factors involved in the Schwann cell-*M. leprae* interaction, phenolic glycolipid-I (PGL-I) particularly its terminal triglyceride binds specifically to laminin-2 in the basal lamina of the SC-axon units (Ng *et al.*, 2000). And, this bacterial cell wall component has been implicated in the induction of demyelination of nerve cells (Rambukkana *et al.*, 2002). In addition to PGL-I, another specific bacterial adhesin, LBP21 potentiates the interaction of *M. leprae* with the SCs, which also binds laminin-2 on peripheral nerves (Shimoji *et al.*, 1999). Hence, these studies indicate that there is more than one adhesin responsible for the initial attachment of *M. leprae* to the SC.

**M. leprae uptake and trafficking in the Schwann cell**

After *M. leprae* adheres to the SC surface, it is slowly ingested which has been demonstrated using primary denervated rat SC cultures (Hagge *et al.*, 2002). The ST 8814 Schwannoma cell line has also been shown to readily phagocytose both viable and irradiated *M. leprae* and this internalization event is dependent upon several protein kinases in the host cell (Alves *et al.*, 2004). After internalization, the SC appeared to be incapable of destroying this intracellular pathogen when cultures were maintained at 33 °C (Truman and Krahenbuhl, 2001). Acidification of vesicles containing irradiated *M. leprae* proceeded normally whereas it was minimal when live *M. leprae* was used, suggesting that viable *M. leprae* interferes with normal endocytic maturation in the SCs (Alves *et al.*, 2004). Thus, *M. leprae* appears to persist and grow within SCs in human nerves.

**Effects of *M. leprae* infection on Schwann cells**

Schwann cells apparently provide an environment favorable for the persistence and proliferation of *M. leprae*. The bacillus not only multiplies within SCs in human nerves, but also seems to increase the proliferation of non-myelinated SCs. *M. leprae* appears to be capable of subverting the signaling of the nervous system by activating the Erk1/2 pathway in order to propagate its cellular niche for colonization and long term survival (Tapinos and Rambukkana, 2005). Yet another study showed that *M. leprae* can modulate the immune response of the host via specific signaling
pathways (Pereira et al., 2005). In contrast to the proliferation of SC, binding of *M. leprae* derived lipoprotein to SCs through TLRs has been shown to result in apoptosis. The apoptosis of Schwann cells provides a mechanism by which activation of the innate immune response contributes to nerve injury in leprosy (Oliveira et al., 2003). In addition, there are reports which suggest that in the absence of immune cells, rapid demyelination occurs following the adherence of *M. leprae* to SCs (Rambukkana et al., 2002).

**Immune response to *M. leprae* infected SC**

Ultimately, *M. leprae* infected SCs are subjected to the effects of host immune response. Isolated human SC cultures are capable of processing and presenting *M. leprae* antigens to CD4+ T-cells and are efficiently killed by these activated T-cells (Spierings et al., 2000). This combined with other unknown mechanisms lead to the functional impairment and destruction of SCs in the infected nerves. Much of the nerve injuries result from the immunologically driven inflammation. This is supported by the fact that impairment of the nerve function occurs more rapidly and more severely in patients with a strong cellular immune response, as in tuberculoid disease. In lepromatous patients, minimal immunological response to *M. leprae* exists and hence, nerves may be heavily infected with only mild to moderate impairment of the nerve function. However, in all forms of leprosy, selected peripheral nerves undergo demyelination and many such nerves will become completely nonfunctional, leaving the patient with an insensate, paralyzed hand or foot (Rambukkana, 2004).

**Immunodominant antigens of *M. leprae***

The identification and characterization of the critical antigens of *M. leprae* is a prerequisite for deciphering the role of individual molecules in the virulence of *M. leprae* as well as in understanding the complex immune response to this intracellular pathogen. This is also important for establishing their potential use as diagnostic reagents or vaccine components. The inability to grow *M. leprae*, *in vitro* combined with the relatively complex biochemical structure of this bacterium had severely hampered the initial characterization of antigens. However, after the availability of animal models, particularly the armadillo, significant amounts of *M. leprae* were available for these characterization studies. As a result, a number of
immunodominant antigens have been identified and characterized over the last couple of decades. These antigens primarily belong to two important categories, namely the carbohydrate/lipid antigens and protein antigens.

**Carbohydrate and lipid immunodominant antigens**

Mycobacteria are characterized by the presence of cell wall with a complex and unique structure particularly rich in lipids which constitutes 30-60% of dry weight of the cell (Daffe and Draper, 1998). The lipid nature of this cell wall confers the bacilli resistance to adverse conditions like drying, chemical disinfectants and therapeutic agents. Pathogenic mycobacteria such as *M. tuberculosis*, *M. leprae* and *M. ulcerans* have evolved multiple strategies to establish residence in their hosts and provoke long term infections. And, there is mounting evidence that these unique lipids constituting their envelopes strategically located at the host-pathogen interface, contribute to their escape from immune surveillance (Guenin-Mace *et al.*, 2009).

Multiple factors contribute to the virulence of any pathogen and the complex carbohydrates and lipids represent one general class of virulence factors (Chan *et al.*, 1989).

**Phenolic Glycolipid-I**

The lipid rich cell wall of mycobacteria is dominated by a wide variety of species and type specific glycolipids (Chan *et al.*, 1989). A spectacular advance in the study of *M. leprae* was made by Hunter and Brennan (1981) with the identification of a peculiar chemical species of antigen which appeared unique for *M. leprae*, the phenolic glycolipid-I (PGL-I). The most striking feature of this glycolipid is that it constitutes 2% of the dry weight of the bacillus and it is also secreted in large quantities in the infected tissue (2.2 mg/g). PGL-I displays all hallmarks of a species-specific antigen.

PGL-I is basically an oligoglycosylphenolic phthiocerol diester. It has a phenolic phthiocerol “core” which is a branched glycolic chain. The two hydroxyl groups of the phthiocerol are esterified by methyl-branched fatty acids, mycoserosates. The phenol group of this core structure is linked glycosidically to a specific trisaccharide moiety and the three sugars of this are 3-O-methyl rhamnose, 2,3-di-O-methyl rhamnose and 3,6-di-O-methyl glucose (Hunter *et al.*, 1982). The combination of
sugars in the PGL-I is unique and the presence of 3, 6-di-O-methyl glucose in nature has not been reported (Hunter and Brennan, 1981).

PGL-I has some biological significance in the survival of *M. leprae* in the host. It is a major component of the ultrastructurally observed ETZ surrounding the bacillus in infected cells and tissue (Hunter and Brennan, 1981). It exists in high concentrations in vacuoles of parasitized macrophages and has been shown to scavenge the reactive oxygen species and prevent the microbial death within the phagosomes (Neill and Klebanoff, 1988; Chan et al., 1989). Phagocytosis of pathogens through complement receptors does not trigger an appreciable oxidative burst and thus provide safer passage of the pathogens into the host cells. It has been shown that PGL-I through its terminal trisaccharide can deposit significant C3 on its surface. Thus, the complement receptors on mononuclear phagocytes, complement component C3 and PGL-I comprise a three component receptor-ligand-acceptor molecule system for mediating phagocytosis of *M. leprae* (Schlesinger and Horwitz, 1991). PGL-I also binds specifically to the native laminin-2 in the basal lamina of the Schwann cell-axon unit and is involved in *M. leprae* invasion of Schwann cells through the basal lamina by a laminin-2 dependent pathway (Ng et al., 2000).

Immunological properties of PGL-I has been demonstrated through its reactivity with rabbit anti-sera to *M. leprae* and also with sera from lepromatous patients (Hunter et al., 1982). PGL-I is found in abundance in the tissues and serum of LL patients and the terminal trisaccharide is the target of species specific IgM antibody response which is maximal at the lepromatous pole of the disease (Britton, 1993). While PGL-I was detected in the urine of LL patients, antibodies to PGL-I has been detected in the CSF of leprosy patients (Patil et al., 1995). PGL-I has also been suggested to be a major factor in inducing the Th-2 type cytokine response observed in LL patients (Sharma et al., 1998). Anti-PGL-I assay using *M. leprae* particle agglutination (MLPA) has been shown to be a sensitive and specific diagnostic tool and also provides a sensitive tool for evaluating treatment efficacy during MDT (Kampirapap and Singtham, 1996; Kampirapap, 1999; Zenha et al., 2009).
Lipoarabinomannan

Lipoarabinomannan (LAM) is one of the several cell surface organic molecules that fortify mycobacterial species against external attack. It is a major cell wall associated glycolipid consisting of repeated saccharide units of arabinose and mannose linked to a phosphatidyl moiety. LAM is attached to the cytoplasmic membrane through a phospholipid moiety which has palmitate and 10-methyl octadecanoateas as the major acyl groups (Chan et al., 1991). It is considered to be the mycobacterial equivalent of bacterial LPS (Guenin-Mace et al., 2009) and is a potent virulence factor. LAM and LM (lipomannan) are the first prokaryotic versions of the biologically important phosphatidyl inositol glycans (Chatterjee et al., 1992).

LAM is composed of three domains – polysaccharide backbone, an MPI anchor and the capping motifs (Chatterjee and Khoo, 1998). Three types of LAM which differ by their capping structure has been identified: the mannose capped LAM (manLAM), phospho-myoinositol capped LAM (PILAM) and non-capped LAM (araLAM). LAM from *M. tuberculosis* and attenuated *M. bovis* BCG strains are mannose capped and the extent of capping varies between 40-70%. The non-reducing termini of LAM from *M. leprae* were also found to be capped with mannose but at a significantly lower level, with only one per molecule (Khoo et al., 1995).

CD1b molecule has evolved unique biochemical properties that enable the binding of lipid containing antigens from intracellular pathogens (Ernst et al., 1998). The macrophage mannose receptor is responsible for the uptake of LAM (Prigozy et al., 1997). CD1b presents the LAM to the αβ-TCR bearing lymphocytes (Sieling et al., 1995) and this presentation pathway in monocyte derived antigen presenting cells has been characterized (Prigozy et al., 1997).

LAM which is produced in prodigious amounts and secreted by the leprosy bacilli is highly immunogenic in patients with leprosy (Chan et al., 1991). It is the predominant, highly pervasive cross-reactive antigen within *M. leprae*. More than 30 monoclonal antibodies raised against *M. leprae*, and previously classified as reactive with carbohydrate, was shown to be directed against LAM (Gaylord et al., 1987). LAM is a major immunogen that is involved in the pathogenesis of leprosy (Dhandayuthapani et al., 1992). It effectively inhibits the effector functions of
macrophages by scavenging toxic oxygen free radicals, by inhibiting protein kinase C activity and by restricting transcriptional activation of the IFN-γ inducible genes (Chan et al., 1991). LAM purified from the cell walls of M. leprae is a potent inhibitor of IFN-γ mediated activation of macrophages (Sibley et al., 1990) and induces virtually no TNF-α production (Adams et al., 1993). This has been attributed as the possible reason for the defective macrophage function observed in lepromatous granulomas and thus constitutes an important aspect of pathogenesis in leprosy (Sibley et al., 1990).

**Protein antigens**

The characterization of protein antigens was carried out initially by immunological methods and subsequently by molecular cloning or chemical purification (Britton et al., 1985; Thole et al., 1995). The genes encoding the protein antigens was first isolated from a genomic library in λgt11 in 1985 using mouse monoclonal antibodies raised against M. leprae to screen the library (Young et al., 1985). Later on, a number of protein antigens of M. leprae have been isolated and their role in immune response to this pathogen has been studied (Thole et al., 1995). A representative list of protein antigens of M. leprae is shown in table R4.

**Cell wall proteins**

A range of important cell wall and membrane proteins have been identified through biochemical studies (Hunter et al., 1990). Many of these cell wall proteins were found to stimulate T-cell clones that were isolated from skin lesions and peripheral blood of TT leprosy patients (Mehra et al., 1989; Roche et al., 1992a). One example of such an important cell wall antigen is the major membrane protein II (MMP-II), an immunodominant antigen capable of activating T-cells through TLR2 (Makino et al., 2005; Maeda et al., 2005). Many more proteins are listed in table R4.

**Secreted antigens**

A separate class of mycobacterial proteins known as the secreted antigens were identified by their release into culture filtrates from viable mycobacteria. These may be amongst the first mycobacterial proteins encountered by the immune system and are considered to be important in eliciting an early protective immune response.
<table>
<thead>
<tr>
<th>S. No</th>
<th>Protein size</th>
<th>Sanger ID</th>
<th>Identity &amp; Function</th>
<th>Cell location</th>
<th>Immunological properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T-cell&lt;sup&gt;S&lt;/sup&gt; Ab&lt;sup&gt;∧&lt;/sup&gt; PR&lt;sup&gt;∧&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>6 kDa</td>
<td>ML0049c</td>
<td>ESAT-6</td>
<td>Cell wall</td>
<td>R M+, P+</td>
<td>Spencer et al., 2002; Parkash et al., 2007</td>
</tr>
<tr>
<td>2.</td>
<td>10 kDa</td>
<td>ML0050c</td>
<td>CFP-10</td>
<td>Secreted</td>
<td>R P+</td>
<td>Geluk et al., 2004; Parkash et al., 2006</td>
</tr>
<tr>
<td>3.</td>
<td>10 kDa</td>
<td>ML1053</td>
<td>PE-family protein</td>
<td>Cytosol, membrane associated</td>
<td>P+</td>
<td>Araoz et al., 2006</td>
</tr>
<tr>
<td>4.</td>
<td>10 kDa</td>
<td>ML0380</td>
<td>GroES, Heat shock protein</td>
<td>Cytosol, cell wall associated</td>
<td>I M+</td>
<td>Rivoire et al., 1994; Thole et al., 1995; Chua-Intra et al., 1998</td>
</tr>
<tr>
<td>5.</td>
<td>11 kDa</td>
<td>ML0410</td>
<td>PE-family protein</td>
<td>Membrane associated</td>
<td>P+</td>
<td>Araoz et al., 2006</td>
</tr>
<tr>
<td>6.</td>
<td>11 kDa</td>
<td>ML1055</td>
<td>EoxK1, ESAT-6 like protein</td>
<td>-</td>
<td>P+</td>
<td>Araoz et al., 2006</td>
</tr>
<tr>
<td>7.</td>
<td>12 kDa</td>
<td>ML0879c</td>
<td>-</td>
<td>Cytosol</td>
<td>M+</td>
<td>Lai et al., 1991; Sela et al., 1991; Thole et al., 1995</td>
</tr>
<tr>
<td>8.</td>
<td>15 kDa</td>
<td>ML0234</td>
<td>-</td>
<td>Cytosol</td>
<td>P+ +</td>
<td>Mustafa et al., 1986; Dockrell et al., 1989</td>
</tr>
<tr>
<td>9.</td>
<td>18 kDa</td>
<td>ML1795</td>
<td>sHsp18, Small heat shock protein</td>
<td>Cytosol, cell membrane</td>
<td>R P+ +</td>
<td>Pessolani et al., 1994; Thole et al., 1995; Maeda et al., 2005; Makino et al., 2005</td>
</tr>
<tr>
<td>10.</td>
<td>18 kDa</td>
<td>ML2038c</td>
<td>Bacterioferritin, MMP-II, BfrA</td>
<td>Membrane associated</td>
<td>R P+</td>
<td>Araoz et al., 2006</td>
</tr>
<tr>
<td>11.</td>
<td>23 kDa</td>
<td>ML2498</td>
<td>possible enoyl-CoA hydratase</td>
<td>Membrane associated</td>
<td>R P+</td>
<td>Araoz et al., 2006</td>
</tr>
<tr>
<td>12.</td>
<td>25 kDa</td>
<td>ML2055c</td>
<td>43L, related to MPT32</td>
<td>Secreted</td>
<td>R +</td>
<td>Wieles et al., 1994; Thole et al., 1995</td>
</tr>
<tr>
<td>13.</td>
<td>26 kDa</td>
<td>ML0308</td>
<td>Unknown</td>
<td>Cytosol, membrane associated, cell wall</td>
<td>R P+</td>
<td>Araoz et al., 2006</td>
</tr>
<tr>
<td>14.</td>
<td>27 kDa</td>
<td>ML0098</td>
<td>Homologous to MPT51 of M.tuberculosis</td>
<td>Secreted</td>
<td>R +</td>
<td>Thole et al., 1995; Harboe and Wiker, 1999</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>15.</td>
<td>28 kDa</td>
<td>ML0072c</td>
<td>Mn-SOD, Superoxide dismutase</td>
<td>M+</td>
<td>+</td>
<td>Young et al., 1985; Thangaraj et al., 1989; Thangaraj et al., 1990; Gelber et al., 1994</td>
</tr>
<tr>
<td>16.</td>
<td>28 kDa</td>
<td>ML0091c</td>
<td>Potential iron regulated protein</td>
<td>P+</td>
<td>+</td>
<td>Cherayil and Young, 1988; Young et al., 1988; Thole et al., 1995</td>
</tr>
<tr>
<td>17.</td>
<td>30/31A kDa</td>
<td>ML0097</td>
<td>FbpA, member of Ag85 complex family of fibronectin-binding proteins</td>
<td>Cell-wall associated, secreted</td>
<td>R</td>
<td>P+</td>
</tr>
<tr>
<td>18.</td>
<td>30/31B kDa</td>
<td>ML2028</td>
<td>FbpB, Mycolyl transferase</td>
<td>Cell-wall associated, secreted</td>
<td>I</td>
<td>P+</td>
</tr>
<tr>
<td>19.</td>
<td>30/31C kDa</td>
<td>ML2655</td>
<td>FbpC, Member of multigene Ag85 complex family of fibronectin-binding proteins</td>
<td>Cell-wall associated, secreted</td>
<td>R</td>
<td>Hermans et al., 1995; Silbaq et al., 1998</td>
</tr>
<tr>
<td>20.</td>
<td>34 kDa</td>
<td>ML0922</td>
<td>-</td>
<td>Cytosol</td>
<td>M+, P+</td>
<td>Thole et al., 1995; Winter et al., 1995; Triccas et al., 1996; Britton et al., 2000</td>
</tr>
<tr>
<td>21.</td>
<td>35 kDa</td>
<td>ML0841</td>
<td>-</td>
<td>Membrane associated</td>
<td>I</td>
<td>P+</td>
</tr>
<tr>
<td>22.</td>
<td>35 kDa</td>
<td>ML2177</td>
<td>Probable uridine phosphorylase</td>
<td>Unknown</td>
<td>R</td>
<td>P+</td>
</tr>
<tr>
<td>23.</td>
<td>36 kDa</td>
<td>ML2395c</td>
<td>-</td>
<td>Cytosol</td>
<td>P+, M+</td>
<td>+</td>
</tr>
<tr>
<td>No.</td>
<td>Molecular Weight (kDa)</td>
<td>Protein Accession</td>
<td>Protein Description</td>
<td>Subcellular Location</td>
<td>Recognition by</td>
<td>Other Information</td>
</tr>
<tr>
<td>-----</td>
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<td>----------------------</td>
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</tr>
<tr>
<td>24.</td>
<td>45 kDa</td>
<td>ML0411</td>
<td>serine rich antigen</td>
<td>Cytosol, membrane associated</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>25.</td>
<td>46 kDa</td>
<td>ML1036c</td>
<td>Possibly involved in small molecule transport</td>
<td>Membrane associated</td>
<td>P+</td>
<td></td>
</tr>
<tr>
<td>26.</td>
<td>49 kDa</td>
<td>ML2703</td>
<td>Bifunctional thioredoxin-thioredoxin reductase protein</td>
<td>Cytosol</td>
<td>P+</td>
<td>+</td>
</tr>
<tr>
<td>27.</td>
<td>53 kDa</td>
<td>ML1553</td>
<td>ProS, prolyl tRNA synthetase</td>
<td>Membrane associated, cytosol</td>
<td>R</td>
<td>P+</td>
</tr>
<tr>
<td>28.</td>
<td>64 kDa</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>M+</td>
<td></td>
</tr>
<tr>
<td>29.</td>
<td>65 kDa</td>
<td>ML0381</td>
<td>GroEL1, Heat shock protein</td>
<td>Cytosol, cell wall associated</td>
<td>I</td>
<td>M+</td>
</tr>
<tr>
<td>30.</td>
<td>65 kDa</td>
<td>ML0317</td>
<td>GroEL2, Heat shock protein</td>
<td>Cytosol, cell wall associated</td>
<td>I</td>
<td>M+</td>
</tr>
<tr>
<td>31.</td>
<td>70 kDa</td>
<td>ML2496c</td>
<td>HSP70, Heat shock protein</td>
<td>Cytosol</td>
<td>I</td>
<td>P+</td>
</tr>
</tbody>
</table>

Table R4: Representative list of immunodominant protein antigens of *M. leprae*.

S, Protein antigen is recognized by T-cells (R) or the antigen induces a strong T-cell response in patients and healthy contacts (I); ^, Protein antigen is recognized by monoclonal antibody (M+) or antibodies in patients (P+); #, Induces proliferative responses (PR) in patients and healthy contacts.
**Stress proteins**

Young and co-workers (1985) was the first to identify five immunodominant antigens of *M. leprae* corresponding to molecular masses 65 kDa, 36 kDa, 28 KDa, 18 kDa and 12 kDa. These proteins were shown to have multiple antibody epitopes and some of these epitopes were *M. leprae* specific. Interestingly, four of these proteins were shown to be heat shock or stress related proteins. The presence of stress proteins among the major immune targets is suggestive of the fact that stress response may be a natural response during infection (Young, 1990).

**Small Heat Shock Protein, sHsp18**

The 18 kDa antigen (sHsp18) is one of the five most immunogenic protein antigens of *M. leprae* identified by Young *et al.* (1985). This antigen is of particular interest because it appears to be a good stimulator of CD4+ T-cell responses (Booth *et al.*, 1988). Crude lysate from the recombinants expressing an epitope of the 18 kDa protein was found to stimulate the proliferation of *M. leprae* specific human T-cell clones derived from *M. leprae* vaccinated volunteers (Mustafa *et al.*, 1986). sHsp18 has been suggested to be involved in protective immunity against *M. leprae* infection and is considered as an important antigen in the immune response to *M. leprae* (Dockrell *et al.*, 1989). Moreover, when most of the antigenic proteins of *M. leprae* exhibited immunologic cross reactivity at the level of antibodies with proteins from other mycobacteria (Engers, 1985), the epitope of 18 kDa protein recognized by L5 (formerly L7.15) monoclonal antibody appeared to be specific for *M. leprae* (Britton *et al.*, 1985;Engers, 1985). Hence, a considerable amount of studies has been carried out on this antigen.

**shsp18 gene**

Initially, a portion of the gene encoding the sHsp18 protein was isolated by probing a λ.gt11 recombinant DNA library with the monoclonal antibody (mAb) directed against the *M. leprae* proteins (Young *et al.*, 1985). Subsequently, the entire gene was isolated and sequenced by Nerland *et al.* (1988). Recombinant *E. coli* harboring a pUC18 plasmid construct carrying the 611 bp fragment of the *shsp18* coding sequence along with the upstream putative regulatory sequence was shown to express high
levels of a 16 to 18 kDa protein and this protein was recognized by L5 mAb (Booth et al., 1988). Later, Dellagostin et al., (1995) examined the upstream region of *shsp18* gene and identified a single transcriptional start point to be located 65 bp upstream of the start codon. The -10 and -35 sequences upstream of the transcriptional start site were found to be similar to the *E. coli* σ^70^ consensus promoter sequences. Additionally, this start site was shown to be recognized and utilized in *M. smegmatis*. The upstream region of *shsp18* gene was also shown to harbor a transcription repression responsive element(s) acting as an operator. This region can further be divided into two independent functional regions, suggesting a bipartite structure of these elements (Kim and You, 2004).

The gene encoding sHsp18 protein has been shown to be polymorphic at a single site in a population of leprosy patients from the same endemic geographical location. The 154^th^ base of *shsp18* exists either as T or C and consequently the protein exists as two class of proteins, one with serine at the 52^nd^ amino acid (class I, S-type) and the other with proline in this position (class II, P-type). Analysis of the SNP of *shsp18* of *M. leprae* isolated from different patients showed that a particular patient has either TCA or CCA containing gene and not a mix of both. Thus, the clonal expansion of bacteria carrying only one type of sHsp18 antigen gene indicates that the secondary infection from a different strain of *M. leprae* is absent. Alternatively, it could also be due to the preferential survival of only one type of *M. leprae* strain in leprosy patients (Shabaana et al., 2003).

**sHsp18 protein**

The *shsp18* gene encodes a protein with predicted molecular mass of 16,607 Da (Booth et al., 1988) which exhibited striking sequence similarity to a family of 17 kDa soybean heat shock proteins (Nerland et al., 1988). sHsp18 protein belongs to the α-crystallin family of low molecular mass small heat shock proteins (de Jong et al., 1993). The members of this family are characterized by the presence of a conserved sequence of about 80 amino acids, which is generally referred to as the α-crystallin domain. A variable N-terminal region precedes the α-crystallin domain and a short C-terminal tail extends downstream this domain (Narberhaus, 2002). In the 148 amino acid sHsp18, the α-crystallin domain spans from residues 32 to 117 residues (de Jong et al., 1993; Narberhaus, 2002) as shown in figure R11.
Figure R11: Sequence of sHsp18. Different regions in the sequence are marked. Arrow indicates the 52\textsuperscript{nd} amino acid which corresponds to the SNP site.
The polymorphism at the 52nd amino acid lies in this α-crystallin domain. A homology modeled structure has been proposed for sHsp18 using sHsp16.9 of wheat and Acr1 of *M. tuberculosi*s as the substrate for modeling. The structure for both the ‘S’ and ‘P’ type sHsp18 protein were modeled and superimposition of these two structures showed distinct structural changes at the polymorphic site (Rehna *et al.*, 2008).

The molecular mass of α-crystallin type small heat shock proteins ranges from 12 to 43 kDa. However, their active entity usually is a large oligomer consisting of multiple subunits (Narberhaus, 2002). Recombinant sHsp18 with a monomeric mass of 16.7 kDa (and 19.3 kDa with His-tag) was shown to refold to form a nonamer, *in vitro* (Lini *et al.*, 2008).

Subcellular localization of sHsp18 has been reported in *M. leprae*. Marques *et al.* (1998) showed the presence of sHsp18 in more than one subcellular fraction of the armadillo-derived *M. leprae*, namely the membrane and cell wall fraction. Subsequently, a proteomic approach revealed the presence of as many as seven spots for sHsp18 in the membrane fraction and four in the cytosolic fraction (Marques *et al.*, 2004). The *shsp18* gene was cloned in an *E. coli* overexpression vector and the recombinant sHsp18 on overexpression was also shown to localize predominantly to the outer membrane fraction and to a lesser extent to the periplasm (Lini *et al.*, 2008). In the absence of signal sequences and transmembrane regions, the mechanism of transport of sHsp18 is unclear and intriguing.

sHsp18 has been shown to be an extremely resistant protein. It was found to resist a wide range of temperatures and chemical modifications without the loss of its main characteristic, that is, to act as a source of T-cell epitopes (Costa *et al.*, 2002).

**Immunological properties of sHsp18 antigen**

Antibody production as well as lymphocyte proliferation has been detected in response to sHsp18 in leprosy patients, long-term leprosy contacts and normal controls (Dockrell *et al.*, 1989; Hussain *et al.*, 1992; Ilangumaran *et al.*, 1994).

sHsp18 protein was first of the five immunogenic antigens (Young *et al.*, 1985) demonstrated to stimulate a high proportion of *M. leprae* specific T-cell clones and these clones were isolated from healthy vaccinated volunteers immunized with
M. leprae (Mustafa et al., 1986). Peripheral blood leukocytes from both PB and MB patients have been shown to proliferate in response to sHsp18 (Launois et al., 1993; de la Barrera et al., 1995). Further, sHsp18 was found to be capable of inducing cytotoxic T-cells from PBMCs of leprosy patients and normal individuals (de la Barrera et al., 1995). Backstrom et al. (1992) studied the in vivo and in vitro responses to sHsp18 in different strains of mice and found that distinct Th-1 subpopulations (DTH effector cells and 18 kDa-specific proliferative T-cells) were stimulated in response to this protein.

Antibodies against sHsp18 have been demonstrated in leprosy sera (Vikerfors et al., 1993). Using an ELISA-based assay with specific mAb, antibodies to M. leprae sHsp18 were found to be restricted to IgG1 and IgG3 antibodies with higher seropositivity in lepromatous patients compared to the patients with tuberculoid disease (Hussain et al., 1994). Roche et al., (1992b) and Vikerfors et al., (1993) have shown that sHsp18 antigen ELISA displayed cross reactivity to sera from patients with tuberculosis.

M. leprae sHsp18 is represented among the antigenic targets of human T-cell responses induced by M. leprae immunization (Baumgart et al., 1996). Immunization of mice with sHsp18 was found to be ineffective in conferring protection (Gelber et al., 1994) although it induced humoral and cellular immune response (Baumgart et al., 1996). The biological properties of the recombinant sHsp18 irradiated with γ-rays were also assayed by immunization of mice and it was shown that γ irradiation can significantly potentiate the T-cell response. This data suggest that the irradiated recombinant antigen could be used in a more sensitive standardized skin test to monitor M. leprae infection (Pinho et al., 1995).

**T-cell and B-cell Epitopes of sHsp18**

Study of the epitope specificity and interlinked function of responding T-cells may help to improve the understanding of immunopathogenesis of nerve and skin lesions and may advance the search for immunodiagnostic and vaccine subunits. The epitopes of sHsp18 has been well characterized. T-cells from leprosy contacts were found to respond variably to a diverse range of antigens but most frequently to the 18 kDa fraction (Booth et al., 1993). This highly immunogenic protein was predicted
to contain multiple epitopes capable of stimulating T-cells based on their propensity to form amphipathic helices. Three of the predicted epitopes were present in the 111 amino acid carboxy terminal region (Booth et al., 1988). An epitope analysis of sHsp18 by Hussain et al. (1992) suggested that the major human B- and T-cell epitopes are located within the segment 38-148. The epitope of sHsp18 protein recognized by the L5 mAb appears to be specific for *M. leprae* (Britton et al., 1985) and this L5 binding epitope in the sHsp18 protein has been mapped to position 110-115 within the sequence. Peptides 111-125, 121-148 and 91-126 were all found to elicit T-cell response. However, residues 116-120 appeared to be the central part of the T-cell stimulating epitope of the sHsp18 protein. Thus, the B-cell (110-115) and T-cell (116-120) epitopes are present adjacent to each other on the sHsp18 protein (Harris et al., 1989). Of the predicted epitopes, the highly amphipathic peptide was found in the N-terminal region (Booth et al., 1988). The peptide 38-50 serves as an immunodominant epitope recognized by CD4+ T-cell clones and this peptide was recognized by memory T-cells 8 years after immunization with *M. leprae*. Other T-cell epitopes of this protein include the peptides spanning 1-38 and 41-55 residues. While the epitope 38-50 exhibited limited cross reactivity, epitopes 1-38 and 41-55 were broadly cross reactive. Amongst these epitopes, 1-38 and 39-50 were presented by one of the two HLA-DR molecules expressed from self HLA-DRB1 genes. Epitope 41-55 was recognized in the presence of autologous as well as HLA-DR and HLA-DQ mismatched allogenic APC (Mustafa et al., 2000). Studies indicate that sHsp18 contains an *M. leprae*-specific B-cell determinant and a T-cell epitope that is shared with *M. tuberculosis* (Harris et al., 1989) and BCG (Dockrell et al., 1989).

**Homologues of sHsp18**

A monoclonal antibody previously thought to be specific for *M. leprae* was found to cross react with a cultivable *Mycobacterium, M. habana* TMC5135 (now identified as *M. simiae* serovar1) and the epitope was present on a protein of identical molecular mass of 18 kDa (Lamb et al., 1990). Subsequent DNA hybridization analysis of mycobacterial DNA with *M. leprae shsp18* gene derived probe revealed that *M. simiae, M. intracellulare, M. kansaisti, M. terrae, M. avium, M. scrofulaceum, M. gordonae* and *M. chelonae* appeared to posses homologous sequences. Further, the 18 kDa protein sequence of both *M. avium* and *M. intracellulare* shared 86% similarity with *M. leprae* sHsp18 sequence (Booth et al., 1993). The sHsp18 from
Streptomyces albus also exhibited a striking similarity to the sHsp18 of *M. leprae* with 52% amino acid similarity (Servant and Mazodier, 1995). While the other strongly immunogenic antigens such as the Hsp60, Hsp70, SOD are all highly conserved in *M. leprae* and *M. tuberculosis* (Young et al., 1988; Thangaraj et al., 1990), the sHsp18 antigen homolog could not be detected in *M. tuberculosis* DNA (Booth et al., 1993).

**Analysis of the expression of shsp18 and its utility**

*shsp18* gene has been used as a target for several studies for the detection of *M. leprae* from leprosy samples by PCR (Fiallo et al., 1992; Sharma et al., 1996; Scollard et al., 1998; Donoghue et al., 2001) and RT-PCR (Chae et al., 2002; Shabaana et al., 2003; Lini et al., 2009). PCR based assay using primers for sHsp18 gene of *M. leprae* has been used to assess the application of gene amplification in biopsy and skin-scraping specimens from leprosy cases across the disease spectrum. The overall sensitivity of the assay ranged from 71% to 93%. The test was found to be absolutely specific by the absence of any false positivity in control specimens as well as with purified DNAs from mycobacterial as well as non-mycobacterial organisms, grown from the specimens (Sharma et al., 1996). A similar PCR-based assay for the *shsp18* gene was employed to differentiate leprosy from other cutaneous granulomatous disease. The specificity of this assay was 100% with a sensitivity in the range of 50-83% (Scollard et al., 1998). Detection of the sHsp18 antigen gene using nested primers that yielded an amplicon of smaller length was found to be very sensitive (100-fold) in detecting *M. leprae* DNA which is damaged or present at a very low level. The possible applications of the use of such primers were to detect DNA in samples from treated lepromatous leprosy patients and from archaeological samples of human remains showing typical leprosy palaeopathology (Donoghue et al., 2001). DNA-PCR and reverse transcription (RT)-PCR for the sHsp18 gene have been used to examine the efficacy of multi-drug therapy in leprosy. One dimensional densitometric analysis of agarose gels of PCR products from the longitudinal study showed a gradual reduction of the *shsp18* amplicon after 12-24 months of MDT. RT-PCR for mRNA of the sHsp18 protein was able to successfully track the bacterial RNA changes in biopsies and confirmed a decrease in *shsp18* transcripts in *M. leprae* from patients after MDT for 12 months (Chae et al., 2002). Semi-quantitative RT-PCR analysis of *shsp18* mRNA expression showed that this gene was expressed
even in the tuberculoid end of the leprosy spectrum with the highest expression seen in LL cases. The expression of shsp18 mRNA was demonstrated in majority of the reactional cases which implies that the pathogen-specific genes might play a role in reactional conditions. Transcription of the shps18 gene was thus used to indicate that metabolically viable bacteria are present in the biopsy material (Shabaana et al., 2003). A real time PCR based assay was used to quantify the copy number of bacterial DNA and RNA from leprosy patients across the spectrum (Lini et al., 2009). The authors have analyzed the changes in the expression of shsp18 DNA and mRNA in different states of leprosy and reversal reactions and have also validated the usefulness of this method in monitoring the chemotherapy of leprosy.

**Functional studies**

sHsp18 is a stress induced protein and stress proteins have been identified as targets of the immune response for a variety of pathogens, including mycobacteria, coxiella, plasmodia, shistosomes. This fact suggests that the stress response may be a natural response of the infectious pathogen to the hostile environment in the host cells (Nerland et al., 1988).

*M. leprae* shsp18 gene with its upstream region has been examined by Dellagostin et al. (1995). The promoter fragments of this gene were relatively strong in E. coli but showed very low level of expression in *M. smegmatis*. The recombinant BCG strains carrying the shsp18 promoter fragments were used to infect murine macrophages and the promoter activity during the intracellular growth was assessed. The shsp18 promoter fragments showed the highest relative intracellular activation, *i.e.* ten-fold higher activation than the activation of BCG hsp60 or *M. leprae* 28 kDa antigen gene. The *M. leprae* shsp18 promoter fragment gave very low levels of expression in *M. smegmatis* and BCG grown in liquid culture, but in BCG growing within macrophages it was induced to high levels. These results indicated that the shsp18 gene is specifically activated during intracellular growth and may therefore be involved in the survival of *M. leprae* within macrophages. The intracellular upregulation found for the shsp18 gene is consistent with the known characteristic of this protein as a major antigen in natural infection (Dellagostin et al., 1995).

The purified recombinant sHsp18 has been demonstrated to be a molecular chaperone, *in vitro* by its ability to prevent enzymes from heat inactivation. The physical
interaction of sHsp18 with its substrates were demonstrated which was required for stabilizing and restoring their biological activity over a wide range of physiological temperatures. Further, this molecular chaperone activity was not lost even after the pre treatment of the protein at 100 °C for 5 min (Lini et al., 2008).

Thus, many studies on sHsp18 have clearly indicated a role for this protein antigen in the immune response to *M. leprae*. In addition to the immunological properties, an understanding of the cellular function of this protein in *M. leprae* is equally important. During the course of reductive evolution *M. leprae* has lost enormous number of genes but has still preserved the *shsp18* gene. Moreover, *shsp18* is the only one in the family of small heat shock proteins that has been retained which in turn implies the significance of this gene in the *M. leprae* life cycle.

Genes that are selectively expressed during intracellular growth may be involved in intracellular survival and virulence of *M. leprae*. The identification and characterization of such genes may provide new targets for chemotherapy, immunotherapy and/or prophylaxis. And, *shsp18* is one such gene that has been shown to be specifically activated intracellularly (Dellagostin et al., 1995). Since no other functional aspects of this gene/protein has been deciphered so far, characterization of this protein antigen will throw light on the importance of this single copy gene in the virulence of *M. leprae* which is the objective of this study.