2.1 HISTORY OF LEPROSY

2.1.1 Origin and spread of leprosy

The history of leprosy is “interwoven with civilization itself”. Leprosy is a debilitating but treatable disease caused by infection with *Mycobacterium leprae*. The damage to skin and peripheral nerves results in characteristic deformities and disabilities that contribute to an intense social stigma and discrimination of patients and their families. Although leprous nodules were found to contain brown bodies in 1847 (Danielssen et al. 1847), these bodies were mistaken for degenerated fat cells until 1873 when Armauer Hansen re-examined the nodules and recognized that they contained bacilli (Pallamary 1955). However, the proof of an infectious agent was difficult to obtain until the evidence of direct transmission was shown in 1886 (Marmor 2002). First named Bacillus *lepra* (Hansen 1880), the causative agent was assigned to the genus Mycobacterium by Lehmann and Neumann in 1896. The inability to cultivate *M. leprae* in vitro and the lack of a suitable animal model has long hampered leprosy research. Progress was made by Charles Shepard in 1960 with the observation of limited *M. leprae* multiplication after injection into mouse footpads (Shepard 1960) and by Kircheimer and Stoors with the demonstration that the nine-banded armadillo is a natural host for systemic infection (Kirchheimer and Storrs 1971). From a phylogenetic point of view, *M. leprae* is a distinct species (Harmsen et al. 2002). It is not a close relative of *M. leprae murium* (Grange 1996). This unculturable pathogen has undergone extensive reductive evolution, with half of its genome now occupied by pseudogenes. Using comparative genomics, it was demonstrated that all extant cases of leprosy are attributable to a single clone whose dissemination worldwide could be retraced from the analysis of very rare single-nucleotide polymorphisms (Monot et al. 2005). A comparative analysis of the complete genome sequence of Br4923, a Brazilian strain of *M. leprae*, was reported with its use for the discovery of SNPs and other polymorphic markers with phylogeographic potential. This finding was then complemented by genome resequencing of strains from Thailand and the United States. When all the genome sequences were compared, little genomic diversity was uncovered, consistent with the hypothesis that leprosy has arisen from infection with a single clone that has passed through a recent evolutionary bottleneck (Monot et al. 2009). The disease seems to have originated in Eastern Africa or the Near East and spread with successive human migrations. It has been suggested that Europeans or North Africans
introduced leprosy into West Africa and the Americas within the past 500 years (Figure 2.1. modified from (Monot et al. 2009)).

**Figure 2.1: Dissemination of leprosy around the globe,** presenting with the screening of 28 different geographic origins with a total of 400 M. leprae strains, resulting in 16 different M. leprae subtypes, referred to as A-P (shown on left as different colour bars) (Monot et al. 2009). Pillars indicate country of origin of M. leprae sample. Colour of pillars represent colour coded scheme for the 16 SNP subtypes. The thickness of the pillar corresponds to the number of samples (1–5, thin; 6–29, intermediate; >30, broad). The gray arrows indicate the migration routes of humans derived from genetic, archaeological, and anthropological studies with an approximate estimated time of migration in years. The coloured arrows indicate the direction of human migration predicted (modified from (Monot et al. 2009)).

### 2.1.2 Epidemiology

Over the last three decades, significant reduction of global burden of leprosy was achieved with two important events. The first event took place in 1981, when a WHO study group on chemotherapy of leprosy recommended the use of multidrug therapy as the standard treatment for leprosy. The success of multidrug therapy led to the second event in 1991, when the 44th World Health Assembly passed resolution WHA44.9, declaring its commitment to eliminate leprosy as a public health problem by the end of year 2000. This meant achieving a prevalence of less than one case per 10 000 population. The number of countries reporting prevalence rates above one per 10 000 population was reduced from 122 in 1985 to nine at
the beginning of 2004. In addition, more than 14 million leprosy cases were diagnosed and treated with multidrug therapy with very few relapses. Although during 2007 both the Democratic Republic of the Congo and Mozambique reached this important stage, however, pockets of high endemicity still remain in some areas of Angola, Brazil, Central African Republic, Democratic Republic of Congo, India, Madagascar, Mozambique, Nepal, and the United Republic of Tanzania (Figure 2.2). An official report of leprosy, collected from 141 countries and territories during 2010, showed the global registered prevalence of 211,903 cases, while the number of new cases detected during 2009 was 244,796 (excluding the small number of cases in Europe) (WHO 2010). The main principles of leprosy control are based on timely detection of new cases and their treatment with effective chemotherapy in the form of multidrug therapy, which is available free of charge since 1995 in all endemic countries through WHO.

2.1.2.1 Leprosy situation in India

There has been a fall reported in the prevalence rate (PR) from 0.84 in March 2008 to 0.72 at the end of the financial year 2008-09 but no change in prevalence rate 0.71 in 2009-10. (Figure 2.3, Table 2.1). Thirty two out of 35 states and Union Territories have achieved elimination with Bihar at a PR of 1.07. In order to achieve Elimination of Leprosy as a Public Health Problem, the state has to achieve a Prevalence Rate of below one per ten thousand population. Chattisgarh is the only state which has a PR of more than 2. A total of 510 districts (80.57%) out of total 633 districts have reached PR<1/10,000 (Figure 2.4 a).

A total of 1.34 lakh new cases were detected during the year 2009-10, with an Annual New Case Detection Rate (ANCDR) of 10.93 per 100,000 population. This shows ANCDR reduction of 2.32% from 11.70 during 2008-09. (Figure 2.4 b) depicts the trend of leprosy Prevalence (PR) and Annual New Case Detection (ANCDR). The three states /Union territories (UTs) i.e. Bihar, Chhattisgarh and Dadra & Nagar Haveli have remained with PR between 1 and 2.5 per 10,000 population. These 3 states/UTs with 10.5% of country’s population, contribute 19% of country’s recorded caseload and 21.9% of the country’s new cases detected during the year 2009-10 (WHO 2010).
Figure 2.2: Rates of new case detection and prevalence of leprosy in the world (WHO 2010).
Figure 2.3: Declining leprosy prevalence in India (National Leprosy Eradication Programme (NLEP report, 2009)

Table 2.1: Epidemiological Situation in India

<table>
<thead>
<tr>
<th>Prevalence Rate</th>
<th>0.71 per ten thousand population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases on record</td>
<td>0.87 lakh</td>
</tr>
<tr>
<td>MB proportion (As percentage of new cases)</td>
<td>48.50%</td>
</tr>
<tr>
<td>Female proportion (As percentage of new cases)</td>
<td>35.40%</td>
</tr>
<tr>
<td>Child proportion (As percentage of new cases)</td>
<td>9.97%</td>
</tr>
<tr>
<td>Visible deformity (As percentage of new cases)</td>
<td>3.10%</td>
</tr>
<tr>
<td>Number of states which have achieved elimination</td>
<td>32 (out of 35)</td>
</tr>
<tr>
<td>Annual new case detection rate (2009-10)</td>
<td>10.93 per 100,000 population</td>
</tr>
<tr>
<td>Number of cases detected during 2009-10</td>
<td>1.34 lakh</td>
</tr>
</tbody>
</table>
Figure 2.4 (a): Registered leprosy cases in India (March 2010) (National Leprosy Eradication Programme (NLEP) report, 2010).

Figure 2.4 (b): Leprosy Prevalence Rate (PR) and New Case Detection Rate (NCDR) per 10,000 population in India from: 1991-2010 (National Leprosy Eradication Programme (NLEP) report, 2010).
Despite this impressive decline in prevalence, the incidence of new case detection rate per year is still high. Unexpectedly, incidence approximates prevalence, a striking observation in the context of a chronic disease. Although this could partly reflect improved leprosy control and case detection in endemic countries (Lockwood 2002), it is also plausible that the global use of MDT has had limited impact on the transmission cycle of *M. leprae*. It is unclear why the incidence of leprosy is still so high despite effective chemotherapy and the fact that humans are the only relevant reservoir of *M. leprae*.

### 2.1.3 Diagnosis of leprosy

Leprosy is a slow progressing infectious disease, caused by *Mycobacterium leprae* with intermittent hypersensitivity reactions (the so-called lepra reactions) in some patients. It is highly infective with low pathogenicity and virulence and has a long incubation period. The skin, superficial peripheral nerves, anterior chamber of the eyes, and testes are the most frequently affected organs. Its geographic distribution varied in the past. Presently it is endemic mainly in subtropical areas. The clinical diagnosis of leprosy is based on patients having one or more of three cardinal signs (i) hypopigmented or reddish patches with definite loss of sensation (ii) thickened peripheral nerves (iii) acid-fast bacilli on skin smears or biopsy material. Skin smears processed to detect intradermal acid fast bacilli, have high specificity, but low sensitivity, since about 70% of all leprosy patients are smear negative (Lockwood 2002). Nevertheless, skin smears are important because these identify most infectious patients and those at greatest risk of relapse. Histological diagnosis, when available, is considered to be the gold standard for diagnosis. The intradermal injection of 0.1 ml lepromin may provoke a late response (Mitsuda reaction) which is a consequence that follows the phagocytosis of the lepromin's heat-killed *Mycobacterium leprae* by the skin macrophages (histiocytes). This late lepromin reaction may be clinically and histologically evaluated at 4 weeks. During the Sixth International Congress of Leprosy in 1953, it was recommended that five classes of Mitsuda reaction should be clinically read as: an absence of observable or palpable element indicating negative (-) reaction; a perceptible element smaller than 3 mm in diameter – a doubtful (+/- ) reaction; a conspicuous infiltrated element 3-5 mm in diameter as positive (+) reaction; a conspicuous infiltrated element larger than 5 mm - positive (+++) reaction; an ulcerated large nodule – a highly positive (++++) reaction. When histologically evaluated, a positive Mitsuda reaction is defined by the presence of epithelioid cells, usually assuming a tuberculoid or tuberculoid-like structure, where acid-fast
bacilli (AFB) are absent or scarcely found. This reaction indicates, therefore, that the macrophages are able to digest phagocytosed heat-killed *M. leprae*, since this response is a consequence of the destruction of bacilli contained in lepromin by the macrophages which transform themselves into epithelioid elements. In the negative Mitsuda reaction the phagocytosed AFB are not destroyed nor is a tendency to a tuberculoid structure seen (Azulay et al. 1960; Petri et al. 1985). Since the bacilli contained in lepromin are heat-killed, the Mitsuda reaction cannot be considered a replica of the leprosy infection. Nevertheless, this reaction has a high prognostic value, since Mitsuda-positive contacts of leprosy patients are free from the risk of manifesting lepromatous leprosy, which is completely associated with a negative Mitsuda reaction (Dharmendra and Chatterjee 1991). Otherwise stated, a positive Mitsuda reaction indicates that the macrophages are able to destroy either dead or living leprosy bacilli. In contrast, leprosy contacts who persistently exhibit a negative Mitsuda reaction are considered to be at risk of contracting leprosy. The proposal that leprosy might be diagnosed by the presence of an anaesthetic skin lesion alone does not pass critical assessment (report of ILATF, 2002). Although 70% of leprosy skin lesions have reduced sensation, the 30% non-anaesthetic lesions occur in patients with multibacillary disease, (Saunderson and Groenen 2000) who are infectious and have a higher risk of developing disability than those with paucibacillary disease. Therefore other criteria are also used. Neither serology nor PCR has a role for diagnosis at present (report of ILATF, 2002). Antibodies against *M. leprae* specific PGL-I are present in 90% of patients with untreated lepromatous disease, but only 40–50% of patients with paucibacillary disease, and 1–5% of healthy controls (Cho et al. 2001; Roche et al. 1990). PCR for detection of *M. leprae* DNA encoding specific genes or repeat sequences is potentially highly sensitive and specific, since it detects *M. leprae* DNA in 95% of multibacillary and 55% of paucibacillary patients (Kampirapap et al. 1998; Patrocinio et al. 2005). Currently, no specific and sensitive test is available for the diagnosis of asymptomatic *Mycobacterium leprae* infection or for predicting progression to leprosy among exposed individuals.

2.1.4 Classification of the leprosy disease spectrum

The leprosy bacillus has a tropism for macrophages and peripheral nerve Schwann cells, infection of the latter resulting in sensory and motor function loss. Individual differences in the host immune response directed against *M. leprae* strongly correlate with the spectrum of clinical and histological phenotypes delimited by the tuberculoid (TT) and lepromatous (LL)
subtypes (Danielssen et al. 1847) (Figure 2.5). Tuberculoid cases present a limited number of hypopigmented, anesthetic skin lesions with no microscopically discernable bacteria. The correlated Th1-cell-mediated immune (CMI) response (IL2, IFNγ) promotes the formation of delineated granulomas—central areas of infected macrophages, often fused into multinucleate giant cells, surrounded by T cells—that successfully limit bacterial replication. Conversely, LL cases present numerous sensitive or anesthetic skin lesions with high bacillary loads. The correlated Th2-antibody response (IL4, IL10) impedes granuloma formation, allowing for uncontrolled bacterial replication and continuous infiltration of the skin and nerves. Borderline forms, i.e., borderline-tuberculoid (BT), borderline (BB), and borderline-lepromatous (BL), comprise the majority of cases. These individuals present intermediate clinical and histological phenotypes resulting from immunologically unstable responses. With limited accuracy, the Ridley-Jopling designations are approximated in the alternative WHO classification system by reclassifying TT and BT subtypes as paucibacillary (PB), and BB, BL, and LL forms as multibacillary (MB).
Figure 2.5. Clinical and immunological classification of the leprosy disease spectrum. with a wide range of clinical presentations from: tuberculoid (TT) through borderline (BT, BL and BB) forms to lepromatous (LL). The TT and BT patients who have a bacillary index (BI) = 0 are classified as paucibacillary (PB) disease, and BB, BL, and LL patients have a BI > 0 and are classified as multibacillary (MB) disease. Tuberculoid cases present a limited number of hypopigmented, anesthetic skin lesions with no microscopically discernable bacteria. The correlated Th1-cell-mediated immune (CMI) response (with IL2, IFNγ cytokine expression) promotes the formation of delineated granulomas. Conversely, LL cases present numerous sensitive or anesthetic skin lesions with high bacillary loads. The correlated Th2-antibody response (with IL4, IL10 cytokine expression) impedes granuloma formation, allowing for uncontrolled bacterial replication and continuous infiltration of the skin and nerves. There are two main categories of reactions in leprosy: Type I reaction which is called a reversal lepra reaction and Type II lepra reaction, which is reported as erythema nodosum leprosum (modified from (Misch et al. 2010)).
2.1.4.1 Leprosy reactions

The induced Mitsuda reaction is a delayed-type hypersensitivity response (measured 21 to 28 days after inoculation) to intradermally administered leprosy antigens (of which lepromin is one formulation). Patients with tuberculoid leprosy typically have strongly positive Mitsuda reactions, a measure of the presence of functional cell-mediated immunity; in contrast, LL patients commonly have little to no reaction (Danielsson et al. 1847; Ilangumaran et al. 1996; Modlin 2010; Narayan et al. 2001; Sansonetti and Lagrange 1981; Singh et al. 2010). Two types of spontaneous immune reactions, or “reactive states,” can also occur in leprosy. Reversal reactions (RRs) also known as type 1 reactions, represent the sudden activation of a Th1 inflammatory response to \textit{M. leprae} antigens. They occur most frequently, although not exclusively, in borderline categories (BL, BT, or BB categories), often after the initiation of treatment, and reflect a switch from a Th2- toward a Th1-predominant response (Britton and Lockwood 2004; Lockwood et al. 1993; Scollard et al. 2006; Walker et al. 2008). Erythema nodosum leprosum (ENL), also known as a type 2 reaction, is an acute inflammatory condition involving high levels of tumor necrosis factor (TNF) (Sarno et al. 1991), tissue infiltration by CD4 cells and neutrophils (Kahawita and Lockwood 2008), and deposition of immune complexes and complement, resulting in immune-complex-associated vasculopathy, panniculitis, and uveitis (Britton and Lockwood 2004). ENL occurs in LL or BL patients and is more commonly seen in patients with a high bacterial index (Sansonetti and Lagrange 1981). Numerous investigators have measured intralesional and systemic cytokine production during leprosy reactions (Garcia et al. 1999; Jullien et al. 1997; Little et al. 2001; Sieling and Modlin 1994; Yamamura 1992), but those studies did not show a consistent Th1 versus Th2 cytokine pattern for reversal reaction versus ENL (Scollard 2008; Scollard et al. 2006). For example, increased amounts of Th1 cytokines, such as IFN\textgamma, IL-12, and IL-2, have been demonstrated for both reversal reactions and ENL. A major drawback of these studies is the inability to determine whether the measured cytokine response is the cause or the consequence of inflammation. For these reasons, the immune mechanism of these reactions is still poorly understood.
2.1.4.2 Lucio Phenomenon (Erythema Necroticans)

It is unique to diffuse lepromatosis leprosy and usually occurs in Latin American patients. The development of necrotizing vasculitis results in crops of large polygonal lesions characterized by ulcerations and sloughing of large areas of skin. The pathophysiology of this complication is not fully delineated, but it may represent a variant of ENL.

2.1.5 Transmission of leprosy

Leprosy is a curable disease with well-defined etiology. However, it lacks better diagnostic tools and therapeutic strategies, which together with the socio-cultural prejudice becomes an important obstacle to overcome these limitations in early detection and protection of the susceptible population, especially for the household contacts of leprosy patients. It has been suggested that the latter group should be given priority in disease control programs in order to interrupt transmission and reduce physical and social disabilities (Goulart et al. 2008). Household contacts of leprosy patients are the highest risk group for the development of the disease. Although many risk or prevention factors have been identified, these have not been employed in leprosy-monitoring programs.

Transmission of M. leprae infection is not significantly affected by current leprosy control measures. In addition to delayed or missed diagnosis of infectious leprosy patients, the lack of tests to measure asymptomatic M. leprae infection in contacts prevents assessment of transmission of M. leprae. Therefore, a key priority is the development of specific and sensitive diagnostic tools that detect M. leprae infection before clinical manifestations arise (report of ILATF, 2002).

Untreated MB patients are probably the most important source of transmission of M. leprae. Household contacts of MB patients have been estimated to have a 5–10 times greater risk of developing leprosy than that of the general population (Douglas et al. 2004; Douglas et al. 1987; Fine et al. 1997; van Beers et al. 1999). However, in many areas, the number of MB patients is very small and they may not represent the most important source of infection (report of ILATF, 2002). There is increasing evidence that subclinical transmission may occur (Moet et al. 2004), because even in highly endemic countries, no history of close contact with a leprosy patient can be established for many patients (Fine et al. 1997).
Nasal excretion of *M. leprae* by healthy carrier individuals could be responsible for transmission. *M. leprae*-specific DNA sequences have been detected by PCR in nasal swabs from many apparently healthy individuals residing in endemic areas (Goulart et al. 1996; Hatta et al. 1995; Izumi et al. 1999; Klatser et al. 1993; Pattyn et al. 1993; Ramaprasad et al. 1997; van Beers et al. 1994); and large proportions of those who live in endemic areas show seropositivity against *M. leprae* specific antigens (Izumi et al. 1999; Ramaprasad et al. 1997; van Beers et al. 1994).

There is increasing evidence from nasal swab PCR studies of temporary carriage or even subclinical infection (Cree and Smith 1998; Hatta et al. 1995; Job et al. 2008; Klatser et al. 1993; van Beers et al. 1996), indicating that infected persons may go through a transient period of nasal excretion when the mycobacterium is highly infective (Hatta et al. 1995). Patients’ household contacts, neighbours, and social contacts have an increased risk of contracting the disease (van Beers et al. 1999). Nasal carriage of *M. leprae* in healthy people may have important implications for leprosy control, once it is difficult to visualize the widespread exposure without the existence of sources of transmission other than MB patients alone (Klatser et al. 1993). Other strong evidence on the involvement of contacts in the transmission chain is the presence of *M. leprae* DNA in the nasal mucosa biopsies (inferior turbinate) in 10% of household contacts (Patrocinio et al. 2005) and in 4% in the nasal swab (Job et al. 2008), which also confirms that the nose is major port of entry and exit of *M. leprae*. These findings also support the results (Sethna et al. 1997) that have demonstrated the affinity of *M. leprae* for the nasal mucosa and head sinuses, which depend on the bacilli viability and mucosa integrity.

Although nasal carriage may not necessarily result in infection or excretion of bacilli, the finding of nasal carriage evidences the disseminated occurrence of *M. leprae* in contacts (Job et al. 2008; Patrocinio et al. 2005) and leprosy-endemic populations and its probable role as a reservoir for maintenance of bacteria (Sethna et al. 1997).

In a preliminary investigation for the presence of *M. leprae* DNA in blood samples of 110 patients and 434 contacts, the general positivity was 18.2 and 8.9%, respectively. The presence of *M. leprae* DNA in the blood of healthy carriers provides additional epidemiological evidence that the route of *M. leprae* transmission is not only the upper airways, indicating a possible transmission through blood, which may affect blood bank
routine tests in future. This hypothesis is corroborated by case reports in nonendemic areas (Launius et al. 2004; Modi et al. 2003), in which leprosy was acquired after organ transplantation. However, it has been suggested that these two case reports should be carefully investigated to demonstrate that recipient patients did not receive contaminated blood during transfusion, since there is no scientific proof that indirect transmission through the chain of armadillo–dog–man is possible.

Based on earlier findings it is possible to state that there is subclinical infection in leprosy and many times it corresponds to the incubation period of the multibacillary disease (Douglas et al. 2004). It has been found that a maximum duration of seropositivity prior to diagnosis is of 9 years, indicating the long incubation period prior to clinical diagnosis. This group of leprosy patients are likely to pose a serious threat to the control of the transmission of leprosy and have been suggested to be given chemoprophylaxis to prevent the occurrence of new cases and opening the way for a rational program for eradication.

The following approaches for reduction or control of leprosy transmission have been suggested (Goulart et al. 2008): (1) household contacts of leprosy patients must be monitored during the first year after diagnosis of the index case; (2) an additional intradermal BCG booster dose must be given in Leprosy Control Programs for household contacts, aiming for protection against leprosy, mainly against MB forms; and (3) the use of the combination of the three assays may discriminate individuals at a higher risk for developing leprosy from contacts with significant protection factors, which could lead to a closer monitoring program for those at risk, as well as a subsidized new and effective control strategy for leprosy.

2.1.6 Mycobacterium leprae, the etiologic agent of leprosy

2.1.6.1 Basic Characteristics

Cellular morphology: M. leprae is a nonmotile, non-sporeforming, microaerophilic, acid-fast-staining bacterium that usually forms slightly curved or straight rods.

The cell wall core contains peptidoglycan, composed of chains of alternating N-acetylglucosamine and N-glycolylmuramate linked by peptide cross-bridges, which is linked
to the galactan layer by arabinogalactan (Figure 2.6). Three branched chains of arabinan are in turn linked to the galactan. Mycolic acids are linked to the termini of arabinan chains to form the inner leaflet of a pseudolipid bilayer. The outer leaflet is composed of a rich array of intercalating mycolic acids of trehalose monomycolates and mycoserosoic acids of phthiocerol dimycocerosates as well as phenolic glycolipids (PGLs). The dominant lipid in the cell wall which gives *M. leprae* immunological specificity is PGL-1. Recent studies suggest that PGL-1 is involved in the interaction of *M. leprae* with the laminin of Schwann cells, suggesting a role for PGL-1 in peripheral nerve-bacillus interactions (Ng et al. 2000; Scollard et al. 2006).

Most of the genes necessary to build the peptidoglycan-arabinogalactan-mycolate polymer appear to be present in the *M. leprae* genome. A few exceptions are two genes involved in polyprenyl-phosphate synthesis (*dxs-II* and *idi*), a gene (*fabH*) involved in meromycolate synthesis, and a glycosyltransferase gene (*pimB*) involved in the biosynthesis of phosphatidylinositol, phosphatidylinositol mannosides, lipomannan, and lipoarabinomannan.

**Growth:** *M. leprae* has never been grown on artificial media but can be maintained in axenic cultures (Truman and Krahenbuhl 2001). As a result, propagation of *M. leprae* has been restricted to animal models, including the armadillo (Truman 2005) and normal athymic, and gene knockout mice (Krahenbuhl and Adams 2000). These systems have provided the basic resources for genetic, metabolic, and antigenic studies of the bacillus. Growth of *M. leprae* in mouse footpads also provides a tool for assessing the viability of a preparation of bacteria and testing the drug susceptibility of clinical isolates (Shepard and Chang 1962; Truman and Krahenbuhl 2001). The viability of *M. leprae* harvested from several different sources is now known to vary greatly, and many standard laboratory practices, such as incubation at 37°C, rapidly reduce the viability of this organism (Truman and Krahenbuhl 2001). However, *M. leprae* stored at 33°C in 7H12 medium has been shown to remain viable for weeks.
Figure 2.6. Schematic model of the cell envelope of *M. leprae*, where the plasma membrane is covered by a cell wall core made of peptidoglycan covalently linked to the galactan by a linker unit of arabinogalactan. Three branched chains of arabinan are in turn linked to the galactan. Mycolic acids are linked to the termini of the arabinan chains to form the inner leaflet of a pseudolipid bilayer. An outer leaflet is formed by the mycolic acids of trehalose monomycocolates (TMM) and mycocerosoic acids of phthiocerol dimycocerosates (PDIMs) and PGLs as shown. A capsule presumably composed largely of PGLs and other molecules such as PDIMs, phosphatidylinositol mannosides, and phospholipids surrounds the bacterium. Lipoglycans such as phosphatidylinositol mannosides, lipomannan (LM), and lipoarabinomannan (LAM), known to be anchored in the plasma membrane, are also found in the capsular layer as shown (Scollard et al. 2006).
Metabolism: *M. leprae* has the capacity to generate energy by oxidizing glucose to pyruvate through the Embden-Meyerhof-Parnas pathway. Acetyl-coenzyme A from glycolysis enters the Krebs cycle, producing energy in the form of ATP. In addition to glycolysis for energy production, *M. leprae* and *M. tuberculosis* rely heavily upon lipid degradation and the glyoxylate shunt for energy production. In this regard, *M. leprae* contains a full complement of genes for β-oxidation but, compared to *M. tuberculosis*, very few genes are capable of lipolysis. Acetate utilization capability has been lost in *M. leprae* as a carbon source since only pseudogenes are present for acetate kinase, phosphate acetyltransferase, and acetyl-coenzyme A synthase.

Overall, *M. leprae* has many fewer enzymes involved in degradative pathways for carbon and nitrogenous compounds than *M. tuberculosis*. This is reflected in the paucity of oxidoreductases, oxygenases, and short-chain alcohol dehydrogenases and their probable regulatory genes. In addition, other major problems associated with metabolism for *M. leprae* are that the bacilli have lost anaerobic and microaerophilic electron transfer systems and that the aerobic respiratory chain is severely curtailed, making it impossible for *M. leprae* to generate ATP from the oxidation of NADH. In contrast to the reduction in catabolic pathways, the anabolic capabilities of *M. leprae* appear relatively unharmed. For example, complete pathways are predicted for synthesis of purines, pyrimidines, most amino acids, nucleosides, nucleotides, and most vitamins and cofactors (Brosch et al. 2000; Eiglmeier et al. 2001; Scollard et al. 2006; Wheeler 2001).

2.1.6.2 Genome and Proteome

The completion of the genomic sequence of *M. leprae* is a major advance (Cole et al. 2001), which has considerably assisted in elucidation of the unique biology of the organism. The *M. leprae* genome includes 1605 genes encoding proteins and 50 genes for stable RNA molecules (Cole et al. 2001). More than half of the functional genes of the *M. tuberculosis* genome are absent and replaced by many inactivated genes or pseudogenes in *M. leprae* (Figure 2.7). One of the most striking features of *M. leprae’s* genome is that it possesses 1,133 inactivated genes (genes lost through mutation, or pseudogenes), compared to six pseudogenes in *M. tuberculosis* (Cole 1998). When pairwise comparisons of the gene and protein sets of the leprosy and tubercle bacilli (Cole 1998, 1999; Cole et al. 2001; Tekaia et al. 1999) were performed, 1,433 proteins were found to be common to both pathogens. After
removal of proteins that are shared with all other prokaryotes (except Actinomycetes) and eukaryotes the sample contained only 333 proteins. Since these pathogenic mycobacteria occupy similar niches in the human body, where they encounter the same physiological stresses and immune responses, it was conceivable that the products of some of these genes may affect highly specialized functions that could be essential for intracellular growth of mycobacteria. The corresponding proteins or enzymes are suggested to represent novel drug targets. The 333 candidates identified by comparative mycobacterial genomics are subdivided into those proteins that are confined to the genus Mycobacterium (there are 219 of these) and a second group of 114 polypeptides that also occur in Streptomyces or Corynebacteria spp., related members of the Actinomycetales kingdom. The latter proteins confer specific properties on actinomycetes, whereas those that are restricted to mycobacteria are surmised to play an even more specialized role. *M. leprae* seems to have jettisoned genes normally required for replication *ex vivo*, assuming a unique ecological niche with a very limited host range and the need for growth within cells. The largest functional groups of genes in *M. leprae* are those involved in gene regulation, metabolism and modification of fatty acids and polyketides, cell envelope synthesis, and transport of metabolites (Cole et al. 2001; Eiglmeier et al. 2001). Defense against toxic radicals is severely degenerative, as neither *katG* nor the *narGHJI* cluster is functional.

There appears to be limited genetic diversity in *M. leprae*, less than in *M. tuberculosis*, and there is no evidence that the observed genetic variations influence the virulence of *M. leprae*, nerve damage, and eventually fibrosis with axonal death.

### 2.1.7 Treatment

In 1981, the WHO Study Group recommended multiple drug therapy (MDT) for the following reasons (report of a WHO study group. (Geneva 1982)):

i) To address dapsone resistance and to discourage resistance to other drugs to be used.

ii) To promote compliance and to get away from long-term monotherapy such as dapsone. iii) To keep rifampicin in all therapeutic regimens because of its powerful bactericidal action and its effectiveness even when taken once a month. iv) To promote compliance and cost effectiveness. In 1997, the WHO Expert Committee suggested that it might be possible to
reduce duration of MDT for multibacillary disease from 2 years to one year and also recommended the treatment of a single PB lesion with one dose of ROM (rifampicin, ofloxacin, and minocycline) (Bhattacharya and Sehgal 2002).

Figure 2.7. The extent of gene reduction and decay in the genome of M. leprae, depicting (a) the percentage of the total potential open reading frames assigned to major cellular functions; (b) sub-classification of each category and the number of putative functional genes in M. leprae (after eliminating the pseudogenes) for each subclass, followed by the corresponding number in M. tuberculosis. The data were obtained from the databases of the M. leprae and M. Tuberculosis (Vissa and Brennan 2001).

The on-going WHO recommendation of MDT for adults is as follows: i) For paucibacillary disease (PB), 600 mg rifampicin monthly and 100 mg dapsone daily in 6 months. ii) For multibacillary disease (MB), 600 mg rifampicin and 300 mg clofazimine monthly and 100 mg dapsone and 50 mg clofazimine daily in 12 months. iii) For single PB lesion (SLPB),
single dose of rifampicin 600 mg, ofloxacin 400 mg, minocycline 100 mg. However, it has been suggested that because of lack of long-term follow-up, this recommendation has to be considered experimental. Single-lesion leprosy, which is often indeterminate leprosy, heals spontaneously in 80% of patients. It is not easy to determine if those of the 20% who develop classifiable leprosy will benefit from single ROM treatment. It may cure some patients but will only delay the onset of multibacillary disease in others. In the United States, a biopsy of a suspicious lesion is recommended to establish the diagnosis and deliver the appropriate treatment. Other drugs with anti-mycobacterial properties, which may be used if needed as substitutes in the above current therapeutic programs are rifabutin, ofloxacin, sporfloxacin, levofloxacin, minocycline, and clarithromycin (Moschella 2004).

2.1.7.1 Dapsone

The first successful chemotherapy using promin for leprosy was reported in 1943 (Faget et al. 1966) at a leprosarium in Carville, Louisiana, USA. Promin (diamonio-azobenzene 4´-sulfonamide) was introduced in Japan in 1947. Patients were intravenously administrated 5 ml of 30% promin solution. A more effective sulfone, dapsone (4, 4´-diaminodiphenyl sulphone: DDS), replaced promin 6 years later. Dapsone is still a fundamental anti-leprosy compound even in the MDT era. Dapsone inhibits folic acid synthesis by competitive inhibition and is bacteriostatic.

Dapsone, an analogue of p-aminobenzoic acid (PABA), targets dihydropteroate synthase (DHPS), which is encoded by \textit{folP1} and is involved in folic acid synthesis. A relationship between DHPS mutations and dapsone resistance has been demonstrated. Missense mutations at codon 53 (ACC) or 55 (CCC) coding threonine or proline in \textit{folP1} confer dapsone resistance (Kai et al. 1999; Williams et al. 2000).

2.1.7.2 Rifampicin

Rifampicin 3-[(4-methyl-1-piperazinyl)-imino]-methyl] is currently a key bactericidal antibiotic for leprosy treatment. A high bactericidal effect was shown experimentally and clinically in 1970s (Levy et al. 1976; Rees et al. 1970; Shepard et al. 1972, 1974), leading to the introduction of rifampicin for leprosy treatment. A single dose of 1,200 mg or a daily dose of 600 mg for 3 days kills bacilli in patients, and no bacillary growth is shown in mice
inoculated with patient bacilli (Levy et al. 1976).

Rifampicin targets the beta (β) subunit of RNA polymerase, which is encoded by \textit{rpoB}. Rifampicin binding to the β subunit inhibits DNA-dependent mRNA transcription. A correlation between rifampicin resistance and mutations at highly conserved regions in the \textit{rpoB} gene has been shown. Isolates confirmed to be rifampicin-resistant harbor missense mutations at codon 407, 410, 420, 425, and 427 (Cambau et al. 2002; Cambau et al. 1997; Honore and Cole 1993; Matsuoka et al. 2003; Matsuoka et al. 2000; Williams et al. 1994; Zhang et al. 2004). In addition, one isolate had a 6-bp insertion in codon 409 (Honore and Cole 1993). Various mutations detected from isolates confirmed to be rifampicin resistant include codon 407 CAG (Gln) to GTG (Val) (Cambau et al. 2002), codon 410 GAT (Asp) to TAT (Tyr) (Zhang et al. 2004), codon 420 CAC (His) to GAC (Asp) (Cambau et al. 2002; Honore and Cole 1993), codon 425 TCG (Ser) to TTG (Leu) (Cambau et al. 2002; Cambau et al. 1997; Honore and Cole 1993; Maeda et al. 2001; Matsuoka et al. 2003; Matsuoka et al. 2000; Williams et al. 1994) ATG (Phe) (Honore and Cole 1993), and codon 427 CTG (Leu) to GAG (Val) (Cambau et al. 2002). Of 29 mutants, 22 isolates had the mutation TTG at codon 425. The high frequency of this mutation is similar to the frequency of this mutation in \textit{Mycobacterium tuberculosis} (Honore and Cole 1993; Williams et al. 1994). In addition, a codon 416 amino acid substitution of Ser to Cys (TCG-TGT) was found, but results of the mouse footpad susceptibility test were unavailable for this isolate. \textit{M. tuberculosis} with this mutation is resistant to rifampicin (Musser 1995; Williams et al. 1994). Therefore, it has been concluded that this mutation confers rifampicin resistance to \textit{M. leprae}. Incidentally, the mutation Gly408Asp was detected together with a mutation at codon 420 that is known to confer rifampicin resistance. Thus, it has been unclear whether the Gly408Asp mutation is related to rifampicin resistance. A total of 108 rifampicin-susceptible strains determined by the mouse footpad test have not been found to contain any mutations between codons 407 to 427 (Cambau et al. 2002; Honore and Cole 1993; Matsuoka et al. 2007; Williams et al. 1994).

### 2.1.7.3 Clofazimine

Clofazimine [(3-\textit{p}-chloroanilino)-10-(\textit{p}-chlorophenyl)-2,10-dihydro-2-(isopropylimino) phenazine], is bactericidal for \textit{M. leprae}. The mechanism of action is not fully elucidated; however, a possible bactericidal mechanism through the binding of GC-rich domains is
suggested (Morrison and Marley 1976). No molecular background for drug resistance to clofazimine is known. Clofazimine was first used for leprosy treatment in 1962 (Browne and Hogenzeil 1962). It is anti-inflammatory and is also used to control the type 2 reaction (erythema nodosum leprosum: ENL) in MDT. Although clofazimine has been used for leprosy treatment for over four decades, reported drug resistance is rare (Browne and Hogenzeil 1962; Ebenezer et al. 2002; Shetty et al. 1996; Warndorff-van Diepen 1982).

2.1.7.4 Ofloxacin

Ofloxacin (4-fluoroquinolone) is a moderate bactericidal antibiotic for \textit{M. leprae}. Its bactericidal activity for \textit{M. leprae} was first demonstrated in 1986 by the mouse footpad method and subsequently by a clinical trial (Gelber et al. 1992; Grosset et al. 1990; Saito et al. 1986). Ofloxacin binds to the A subunit of DNA gyrase (gyrA) and inhibits DNA replication. Association between mutations within the highly conserved region of gyrA, coded by the \textit{gyrA} gene, and quinolone resistance was revealed in most resistant strains of mycobacterium (Cambau et al. 1994; Takiff et al. 1994). The first ofloxacin-resistant \textit{M. leprae} was found in 1994 (Cambau et al. 1997). Two other cases of ofloxacin resistant \textit{M. leprae} from Japanese relapsed cases were reported in 2000 and 2003 (Matsuoka et al. 2003; Matsuoka et al. 2000). Four other possible ofloxacin-resistant cases have been found (Maeda et al. 2001). Three isolates confirmed to be ofloxacin resistant harbored the mutation Ala-Val (GCA-GTA) at codon 91 (Cambau et al. 1997; Matsuoka et al. 2003; Matsuoka et al. 2000) in the \textit{gyrA} gene. One isolate in which the susceptibility could not be examined by the mouse footpad method had the mutation Gly-Cys (GGA-TGC) at codon 89 (Maeda et al. 2001). Two other amino acid changes, Ser at 91, and Asp at 94 (same codon numbers as \textit{M. tuberculosis}), in \textit{gyrA} of \textit{M. tuberculosis} have been associated with quinolone resistance (Cambau and Jarlier 1996). It seems that mutations at codons 89, 92, and 95 in \textit{gyrA} of \textit{M. leprae} also confer quinolone resistance.

2.1.7.5 Minocycline

Minocycline (7-dimethylamino-6-demethyl-6-deoxy-tetracycline) is the only tetracycline group active against \textit{M. leprae}. Efficacy of minocycline against \textit{M. leprae} was confirmed in 1987 (Gelber 1987). It is bactericidal and its activity is additive when combined with other anti-leprosy drugs (Gelber and Rea 1991; Ji et al. 1991).
Minocycline is used with rifampicin and ofloxacin in MDT only for the single lesion paucibacillary (SLPB) cases. Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit, blocking the binding of aminoacyl transfer RNA to the messenger RNA ribosomal complex (Taylor and Chau 1996). To date, no minocycline-resistant cases are known (Matsuoka 2010).

2.1.8 Vaccination

Anti-leprosy vaccination can be immunoprophylactic or immunotherapeutic (Azulay 2002; Talwar 1999; Zaheer et al. 1993). The aim in immunoprophylactic approach is to restore the host recognition of shared mycobacterial antigens to promote Th1 responses, to induce CD8+ cytotoxic cells, and to downregulate the proportion of T cells producing Th2 interleukins (Naafs 2000). The aim of immunotherapy is to switch off the mechanisms leading to immunopathology and to increase intracellular mechanisms by which bacilli are killed. The first vaccine used was BCG, but its failure to protect certain populations clearly indicated that an improved vaccine against leprosy was needed. Among the vaccines being used or explored are Mycobacterium w., Mycobacterium ICRC (M avium intracellulare), bacillus Calmette-Gue´rin plus heat-killed M. leprae, Mycobacterium tufu and Mycobacterium Habana (Azulay 2002). The enthusiasm for use of vaccines has lessened because of the significantly favorable impact of MDT on leprosy. However, one cannot deny the potential usefulness of a proven effective vaccine in highly endemic countries such as India and Brazil. Some of the best information about vaccination is from India where the Mycobacterium w and Mycobacterium ICRC vaccines have been used in the field (Talwar 1999). The comparative genomics of different mycobacterial species have spurred the identification of new vaccine targets and the successful preliminary trials of rationally constructed knock outs in BCG strain (Brodin et al. 2004) in providing improved resistance against M. tuberculosis infection is a step forward in this direction.

2.1.8.1 Lacunae in leprosy vaccine studies

It is worthwhile to define the lacunae in vaccines currently proposed for prevention of leprosy (Sehgal and Sardana 2007):-
i) Very few well-performed double blind randomized controlled trials with proper follow up are established (Sehgal and Sardana 2007; Setia et al. 2006).

ii) The largest vaccine trial which has been conducted in recent times, has found a maximum efficacy of 67% (BCG+ heat killed *M. leprae* [HKML]) which still does not meet the criteria of usage (Gupte 2001).

iii) Scientific analysis of data has shown that observational studies overestimate the efficacy of vaccines (Setia et al. 2006).

### 2.2 IMMUNOLOGY

*Mycobacterium leprae* probably enters the body by way of the nose and spreads to the skin and nerves via circulation. The host’s immunological response determines the clinical forms that develop. Figure 2.8 depicts genes and gene products involved in the immune response to *M. leprae*.

#### 2.2.1 Innate immunity

An effective innate immune response in combination with the low virulence of the leprosy bacillus may underlie resistance to the development of clinical disease. Dendritic cells (DCs) uptake *M. leprae* and subsequent local production of cytokines and chemokines regulates inflammation and influences the course of the adaptive cell-mediated immunity into a Th1 or Th2 response. Although DCs are known to be effective presenters of *M. leprae* antigens, major histocompatibility complex (MHC) class I and II expression is downregulated in monocyte-derived DCs infected with *M. leprae* bacilli. On the other hand, DCs stimulated with *M. leprae* membrane antigens upregulate both MHC class II and CD40 ligand-associated interleukin-12 (IL-12) production, suggesting that whole live bacilli may suppress the interaction of DCs and T cells (Gulia et al. 2010; Maeda et al. 2003).

The cytokine profile present in the lesion also appears to be correlated with Toll-like receptor (TLR) function: Th1-type cytokines are associated with TLR1 and TLR2 activation, and Th2-type cytokines are associated with inhibition of activation. The expression of TLR1 and TLR2 has been found to be stronger on monocytes and DCs in TT lesions than in the LL counterparts. In addition, *in vitro* studies have shown that the *M. leprae* 19-kDa and 33-kDa
lipoproteins could activate monocytes and monocyte derived DCs through TLR2 (Gulia et al. 2010; Krutzik et al. 2003)

**Figure 2.8.** Genes and gene products involved in the immune response to M. leprae, depicting molecular and cellular interactions known or postulated to play a role in the immune response to M. leprae. This comprises of TLRs activation by Mycobacterium leprae antigens, IL10, IL12, IL4 and TNFa regulatory pathways, generation of Th1 or Th2 cell responses. T-cell-dependent acquired immune responses typically require antigen-presenting cells (APCs) to present antigen-derived peptides (after phagocytosis) within major histocompatibility complex (MHC) molecules to naive T (T lymphocyte) cells, resulting in Helper T 1 (Th1) and Helper T 2 (Th2) responses.

Molecule symbols are: C3, complement factor 3; CR1, complement receptor 1; DEFB1, beta defensin 1; IFNγ, gamma interferon; IL10, interleukin-10; IL12, interleukin-12; LTA4H, leukotriene A4 hydrolase; MHC II, major histocompatibility complex class II; MBL2, mannose binding lectin 2; MRC1, mannose receptor; NOD2, nucleotide oligomerization domain 2; RIP2, receptor-interacting kinase; SLC11A1, solute carrier family 11, member 1 (also known as NRAMP); TCR, T-cell receptor; Th1, T-cell helper type 1; Th2, T-cell helper type 2; TLR, Toll-like receptor; TNF, tumor necrosis factor (modified from (Misch et al. 2010)).
Recent investigations have shown that plasmacytoid DCs are not involved in the immune response against *M. leprae* whereas FoxP3-positive cells (markers of regulatory T cells, or Treg cells) are present in 95% of the cases in a retrospective immunohistochemical study, with an average density of 2.9% of the infiltrate. Their distribution was not related to granulomatous structures or special locations (Gulia et al. 2010; Massone et al. 2010).

### 2.2.1.1 Receptors of innate immunity

Cells of the innate immune system are equipped with germ line encoded pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs), which are shared among groups of pathogens.

*Toll-like receptors*

Mammalian Toll-like receptors (TLRs) are crucial for the recognition of microbial pathogens by macrophages and dendritic cells during innate immunity. TLRs are phylogenetically conserved transmembrane proteins that contain repeated leucine-rich motifs in their extracellular domains. The cytoplasmic signaling domain is linked to the IL-1 receptor-associated kinase, which activates transcription factors such as NF-κβ to induce cytokine production. Ten TLRs have been identified in humans, of which TLR2-TLR1 heterodimers, TLR2 homodimers, and TLR4 appear to be significant in the recognition of mycobacteria. TLRs have been found to be necessary for the optimal production of IL12 (Brightbill et al. 1999), a proinflammatory cytokine responsible for the induction of Th-type immunity, as well as TNFα (Underhill et al. 1999) a cytokine important in cellular activation and granuloma formation and also implicated in the tissue destruction associated with leprosy reactions.

A number of mechanisms have been identified, which regulate TLR function in leprosy. In addition to the ability of IL4 to downregulate TLR2/1 expression, it also inhibits TLR2/1-induced cytokine responses (Krutzik et al. 2003). IL10 had no effect on TLR2/1 expression but strongly inhibit TLR2/1-induced cytokine release (Krutzik et al. 2003). Activation of LILRA2, which is differentially expressed in lepromatous leprosy (L-lep) versus tuberculoid leprosy (T-lep) lesions, inhibited TLR2/1-induced IL12 release but preserved IL10 release (Bleharski et al. 2003). Similarly, oxidized phospholipids inhibited TLR2/1-induced IL-12
release but preserved IL10 release (Cruz et al. 2008). The ability of *M. leprae* to upregulate tryptophan aspartate containing coat protein (TACO), known to be expressed in macrophages containing *M. leprae in vitro* and in disease lesions (Suzuki et al. 2006), was shown to downregulate TLR2-mediated signalling (Tanigawa et al. 2009).

Nucleotide-binding oligomerization domain 2 (NOD2) is a cytoplasmic receptor belonging to the NOD-like receptor family. It not only shares homology to TLRs in containing leucine rich repeats but also encodes a CARD domain. It mediates the response to peptidoglycan, including that derived from mycobacteria, by sensing muramyl dipeptide (MDP) (Girardin et al. 2003; Yang et al. 2007). Triggering of NOD2 by MDP activates NF-κB through the adaptor molecule, RIP2. In addition, MDP can also activate the inflammasome, by recruitment of caspase-1, leading to the proteolytic cleavage and activation of IL-1β (Delbridge and O'Riordan 2007). NOD2 deficiency in a mouse model of tuberculosis (TB) resulted in increased susceptibility to infection at late time points (Divangahi et al. 2008; Gandotra et al. 2007). NOD2 polymorphisms, not those associated with Crohn’s disease, were shown to be associated with susceptibility to TB (Austin et al. 2008) and those associated with Crohn’s disease, were shown to be associated with leprosy (Zhang et al. 2009). NOD2 was not only found to mediate the response to mTB in human monocytes and synergize with lipoprotein in activating monocyte cytokine responses (Ferwerda et al. 2005), but also synergized with transfected DNA in activating the IFN-β pathway (Leber et al. 2008).

**C-type lectin receptors**

These receptors bind specifically to carbohydrate moieties on pathogens (Allavena et al, 2004) and facilitate internalization for antigen processing and presentation. The mannose receptor (also called CD206), is expressed primarily on cells of the myeloid lineage, especially mature macrophages, although not on monocytes, and on some subsets of dendritic cells. The macrophage has been shown to play a role in uptake of virulent mycobacteria (Schlesinger, 1993), and a major mycobacterial ligand for this receptor is lipoarabinomannan (Prigozy et al, 1997). It has also been reported that uptake of mycobacteria via the mannose receptor does not elicit a respiratory burst (Astarie-Dequeker et al, 1999).
DC-SIGN another C-type lectin receptor is expressed on dendritic cells and also recognizes pathogens via the binding of mannose-containing structures (van Kooyk and Geijtenbeek, 2003). In studies using *M. tuberculosis*, DC-SIGN has been shown to be the major receptor on DC for the bacilli (Tailleux *et al.*, 2003). Again, the primary mycobacterial ligand for DC-SIGN was mannose-capped lipoarabinomannan (Koppel *et al.*, 2004; Maeda *et al.*, 2003; Tailleux *et al.*, 2003; van Kooyk and Geijtenbeek, 2003). Some investigators have proposed that virulent mycobacteria may subvert DC function via DC-SIGN possibly through the inhibition of IL12 production (Nigou *et al.*, 2001) and the induction of IL10 (Geijtenbeek *et al.*, 2003). Engagement of DC-SIGN has also been suggested to inhibit TLR signaling (van Kooyk and Geijtenbeek, 2003).

2.2.1.2 Alternative mechanisms of infection

Recent work has suggested that the successful infection and survival of *M. leprae* could be associated with the ability of *M. leprae* to regulate cytokine production or to drive Th1 or Th2 responses. Other pathways including insulin-like growth factor have also been implicated (Rodrigues *et al.*, 2010; Gulia *et al.*, 2010) Lipid droplet (LD) formation and prostaglandin 2 (PGE2) productions are directly correlated, indicating that *M. leprae*-induced LDs constitute intracellular sites for eicosanoid synthesis and that foamy cells may be critical regulators in subverting the immune response in leprosy (Mattos *et al.*, 2010; Gulia *et al.*, 2010).

2.2.2 Adaptive immunity: development of cell-mediated immunity

Second branch of immune response, the adaptive immunity, plays an important role in clearance of pathogens. Cells of the T-cell lineage play an essential role in resistance to *M. leprae*.

2.2.2.1 Role of T lymphocyte subsets in Leprosy

Initial studies to identify and characterize T lymphocytes in lesions have shown striking differences in the CD4:CD8 (T-helper/inducer: T suppressor/cytotoxic) ratio at the poles of the leprosy spectrum. In tuberculoid leprosy lesions, the CD4 population predominates with a CD4:CD8 ratio of 1.9:1 and in lepromatous lesions the CD8 population predominates with a CD4:CD8 ratio of 0.6:1. However, the CD4:CD8 ratios are independent of those in the blood of the patients suggesting selective migration of cells into, proliferation within, or retention in, lesions (Modlin *et al.*, 1986). The proportions and distributions of CD4, CD8
subpopulations and their localization in the granulomas also shows striking differences (Modlin et al., 1988). In tuberculoid lesions, the T-helper/memory: T naïve ratio has been reported to be 1:1 in blood but 14:1 in lesions. In contrast, in the lepromatous lesions one half of the CD4+ cells belong to the naïve T cell subset, with majority of the CD8+ cells belonging to the T-suppressor subset (i.e CD8+ CD28-). CD8+ cells of the T-cytotoxic phenotype (i.e CD8+ CD28+) are predominant in tuberculoid lesions.

The microanatomic localization and association of T cell subpopulations has shown T-helper/memory (CD4+, CD45RA) cells in close association with macrophages in the core of the granuloma with relative restriction of T-cytotoxic (CD8+ CD28+) cells to the mantle surrounding the tuberculoid granuloma. The T naïve (CD4+ CD45RO) or suppressor inducer subset (CD8+ CD28+) are localized to the mantle surrounding the granuloma near CD8+ CD28+ T cytotoxic cells where they perhaps restrict the cell mediated immune response from causing extensive tissue damage (Modlin et al., 1982; Narayanan et al., 1983; Wallach et al., 1984; Longley et al., 1985). In lepromatous granulomas, the T suppressor subset (CD8+ CD28-) cells were reported to be admixed with macrophages and CD4+ cells, which could facilitate suppression of the cell-mediated immune response.

The most puzzling and fundamental immunological issue in lepromatous leprosy that remains unresolved is that of selective and specific unresponsiveness. CD8+ lines and clones derived from lepromatous lesions but not from tuberculoid lesions have been reported to suppress mitogen responses in vitro in an MHC class II restricted manner (Modlin et al., 1986, Salgame et al., 1991). It has also been observed that these CD8+ cells secrete more IL4 which might be responsible for downregulating macrophage activation by IFNγ (Salgame et al., 1991).

MHC class II restricted CD4+, MHC class I restricted CD8+, non MHC CD1 restricted CD4+ T cells and NKT cells are known to play an important role in host defense against leprosy (Yamamura et al., 1991; Salgame et al., 1991; Sieling et al., 1999; Sieling et al., 2000; Gansert et al., 2003; Sieling et al., 2005). Studies with these lymphocyte subsets (cell lines and clones) have also corroborated the fact that there is skewness of particular lymphocyte subset associated with protective immune responses in tuberculoid patients; whereas there is absence or inadequate populations of these protective lymphocytes in lepromatous patients. Simultaneous measurements of memory T cell (MT1 CD45 RA-, CD62L-, CD11a bright;
IFNγ biased) and memory T cell 2 (MT2, CD45RA−, CD62L+, CD11a dim IL4 biased) of peripheral blood of leprosy patients has shown that the ratios of MT1/MT2 differed significantly in patients with tuberculoid and lepromatous leprosy, indicating that cells become polarized for one type or the other in leprosy (Mitra et al., 1999). T cells have been shown to respond to around 30 different antigens, but it is still unknown if these antigens induce protective immunity or contribute to immune dysfunction and disease progression. A systematic comparison between T-cell epitopes recognized by healthy contacts and different groups of leprosy patients in the context of HLA molecules has been proposed to facilitate the understanding of the distinction between protective and harmful T-cell responses in mycobacterial diseases. The availability of the M. lepare genome sequence and comparative genomics of M. leprae with other mycobacterial species has precipitated research in this direction. The functional status of T cells in leprosy has clearly delineated a striking dichotomy (Th1/Th2) in the nature of immune responses elicited by the two polar forms. The genomic profiling of clinically categorized leprosy patients reinforces the fact that the skewness or imbalance between responses of Th1 and Th2 cells is involved in disease progression (Bleharski et al., 2003). The role of cytokines, IL18 (Garcia et al., 1999), IL12R (Kim et al., 2001), IL15 (Jullien et al., 1997), IL10 and IL4 (Seiling et al., 1993), TNFα (Kaplan et al., 1994; Khanolkar-Youmg et al., 1995; Lima et al., 2000), TGFβ1(Goulart et al., 1996) IFNγ (Libraty et al., 1997), costimulatory molecules B7-CD28 (Schlienger et al., 1998), CD40-CD40L interactions (Yamauchi et al., 2000) and signaling lymphocytic activation molecule (SLAM, CD150) (Bleharski et al., 2001; Garcia et al., 2001) in influencing the level and pattern of cell mediated immune response to leprosy has also been investigated. All together these observations suggest that the outcome of leprosy is believed to be determined at an early point following infection which could be partly determined by host genetic factors.

2.2.2.2 Neutrophil recruitment

One of the histological differences between ENL and lepromatous leprosy is the characteristic infiltration of neutrophils in ENL lesions. Lee and colleagues (Lee et al., 2010) investigated the mechanisms of neutrophil recruitment at the site of disease. The gene expression profile of ENL lesions has been shown to integrate pathway of TLR2 and Fc receptor activation, neutrophil migration, and inflammation. Major aspects of this pathway include the following: (a) FcR or TLR2 induction of IL-1b release; (b) endothelial activation,
including the upregulation of E-selectin and subsequent neutrophil binding; and (c) upregulation of inflammatory mediators associated with both neutrophils and monocytes/macrophages. Thalidomide, which is a highly effective agent used in the treatment of ENL and is known to reduce neutrophil infiltration in lesions was shown to target individual events in this inflammatory pathway (Lee et al, 2010).

2.2.3 Cytokines in leprosy

The Th1/Th2 paradigm, based on functional discrimination of T-helper cells according to their pattern of cytokine production, asserts that Th1 and Th2 cells promote a cellular and humoral immune response, respectively (Mosmann et al, 1986). This functional differentiation has offered an attractive hypothesis to explain the differences between tuberculoid and lepromatous responses to M. leprae. Many studies have revealed a predominance of IL2, TNFα, and IFNγ transcripts in tuberculoid lesions and IL4 and IFNγ in lepromatous ones, gene expression profiles consistent with Th1 and Th2 patterns, respectively (Arnoldi et al, 1990; Flad et al, 1990; Mutis et al, 1993; Sieling et al, 1994; Yamamura et al, 1991). CD4\(^+\) clones isolated from TT lesions have been reported to secrete primarily IFNγ, whereas a CD4\(^+\) clone from an LL lesion produces predominantly IL4 (Sieling et al, 1994), and CD8\(^+\) clones isolated from LL patients likewise generate large amounts of IL4 (Salgame et al, 1991).

Circulating leukocytes and T-cell lines from tuberculoid patients stimulated by M. leprae in vitro have also generally been found to produce a Th1 cytokine pattern, while leukocytes and T-cell lines from lepromatous patients have generally been shown to produce a Th2 cytokine pattern (Misra et al, 1995; Nath et al, 2000).

In summary, studies of cytokine gene expression in leprosy lesions thus far have given us a more detailed description of the immunological parameters of the polar types of leprosy, confirming and supporting the original concept that tuberculoid lesions are manifestations of delayed hypersensitivity and cellular immunity and that lepromatous ones result when immune recognition occurs (as indicated by antibody production) but the host is unable to develop cellular immunity to M. leprae (Scollard et al, 2006). However, these studies have not yet revealed the mechanisms by which the cellular immune response is so extraordinarily titrated to produce the entire leprosy spectrum.
2.2.3.1 Mechanisms of nerve injury

*M. leprae* is an obligate intracellular pathogen with a distinct tropism for Schwann cells of the peripheral nervous system and for macrophages (Boddingius, 1974; Schlesinger and Horwitz, 1991; Spierings *et al.*, 2000; Stoner, 1979). The pronounced specificity of *M. leprae* for Schwann cells is related to the tissue-specific expression of laminin-2 on Schwann cells. *M. leprae* contains a phenolic glycolipid (PGL-1) that has been shown to bind to the G domain of the α2 chain of laminin-2 on the membrane of Schwann cells (Ng *et al.*, 2000). The uptake of *M. leprae* into the Schwann cell is thought to occur when the PGL–laminin-2 complex interacts with α-dystroglycan, the laminin-2 receptor located on the Schwann cell membrane (Ng *et al.*, 2000; Rambukkana *et al.*, 1997; Rambukkana *et al.*, 1998). Laminin binding protein 21 (LBP21) also mediates the intracellular entry of *M. leprae* into the Schwann cell (Figure 2.9) (Rambukkana, 2001; Shimoji *et al.*, 1991). A variety of other receptors on monocytes and macrophages have also been proposed to facilitate intracellular entry by *M. leprae*. On monocytes, PGL-1 mediates *M. leprae* phagocytosis via the complement receptor CR3 and serum complement 3 (Schlesinger and Horwitz, 1991). On macrophages, complement receptors 1 and 4 help phagocytose *M. leprae* (Schlesinger and Horwitz, 1991). Another candidate phagocytic receptor on the macrophage is the mannose receptor, which binds mannose and other carbohydrate moieties on mycobacteria (Kery *et al.*, 1992; Schlesinger *et al.*, 1996).

Nerve injury is the hallmark of progressive leprosy infection and involves both myelinated and unmyelinated nerves (Hagge *et al.*, 2002; Job, 1989; Job, 1971). Biopsy specimens taken from affected nerves of leprosy patients has revealed perineural and intraneural inflammation and, in myelinated fibers, eventual demyelination (Scollard, 2008). At the tissue level, the influx of immune cells and interstitial fluid (edema) inside inflexible nerve sheaths has been shown to cause nerve injury through mechanical compression and ischemia (Scollard, 2008). At the cellular level, immunological injury is thought to be a major mechanism of nerve damage. The immune-mediated injury hypothesis has indirect support from *in vitro* studies in which the stimulation of monocytes or macrophages with *M. leprae* induces proinflammatory cytokines such as TNFα, IL12, IL6, IL1β, IL18, and IL15 (Garcia *et al.*, 1999; Jullien *et al.*, 1997; Krutzik *et al.*, 2003; Misch *et al.*, 2008). For example, the 19-kDa protein of *M. leprae*, which is recognized by the TLR2/1 heterodimer, elicits a robust proinflammatory cytokine response (Krutzik *et al.*, 2003) and induces apoptosis in Schwann cells (Oliveira *et al.*, 2003).
In addition, Schwann cells exposed in vitro to necrotic neurons produce TNFα and nitric oxide (Lee et al., 2006), potent inflammatory mediators. In ex vivo studies, human Schwann cells loaded with M. leprae antigen have been shown to be targeted by cytolytic CD4$^+$ T cells (Spierings et al., 2001; Misch et al., 2010). Despite these in vitro and ex vivo observations, the mechanism of nerve injury remains poorly understood, partly due to the lack of good animal models for leprosy and leprosy-induced nerve damage.

![Figure 2.9. Entry of M.leprae into Schwann cells showing, laminin binding protein 21 (LBP21) and phenolic glycolipid 1 (PGL-1) in M. leprae cell wall binding to the α2 chain of laminin-2 (LAMA2) and α-dystroglycan on the Schwann cell membrane. This permits entry and subsequent damage to the peripheral nerves (adapted from Misch et al., 2010).](image)

### 2.3 GENETIC SUSCEPTIBILITY AND INFECTIOUS DISEASES

In majority of infectious diseases only proportions of individuals exposed to the pathogen become infected and develop clinically evident disease. This interindividual variability is determined, at least in part, by the combined effect of host proteins encoded by a series of genes that control the quantity and quality of host parasite interaction and host immune responses. Immunity to infection is difficult to study due to high complexity of host-environment relationship. Host defense not only involves the immunological haematopoietic-derived cells, but also many endothelial and epithelial cell types. Moreover, a host is continually challenged by large numbers of highly diverse and coevolving microorganisms. Despite these difficulties, the study of immunity to infection as in leprosy is of paramount
importance, since it provides an insight into physiological, ecological and evolutionary aspects of immunity (Figure 2.10).

**Figure 2.10. A schematic presentation showing host–environment interaction stages in the course of infection such as leprosy**, depicting how the exposure to an infectious agent (M. leprae) does not always result in infection (leprosy). In case of Mycobacterium leprae infection, >95% of individual resist disease by establishing protective immunity and only the minority develops leprosy. The overall susceptibility/resistance to infection is under host and environmental control. Host factors might be genetic (for example, mutation in a gene involved in immunity to infection) or non-genetic (for example, skin lesion), and might act at the level of exposure to (cutaneous and mucosal barriers) or infection with (innate and adaptive immunity) the microorganism. In leprosy, several genes modulate cell-mediated immunity and some have a role in either susceptibility to leprosy per se or to leprosy type. Environmental factors might be microbial (for example, virulence factors) or related to the mode of exposure (for example, air temperature), and might have an impact at each stage of the interaction (modified from (Casanova and Abel 2004)).
2.3.1 Assessing the genetic contribution to leprosy risk

Leprosy has long been observed to be a disease that aggregates in families (Brown and Stone, 1958; Hansen, 1875; Job, 1980; Propping and Vogel, 1976). In the 19th century, the hereditary versus environmental origins of this illness were vigorously debated (Browne, 1985; Hansen, 1875) driven in part by the social stigma attached to leprosy. The discovery of the *M. leprae* bacillus by Gerhard Henrik Armauer Hansen in 1873 (Hansen, 1875; Harboe, 1973) settled the argument for the time being in favor of an environmental etiology. In the modern era it has become clear that while encounter with the *M. leprae* pathogen is necessary for infection, it is not sufficient, since the majority of exposed individuals do not become infected. Host genetic factors may therefore largely determine which exposed individuals develop disease. Evidence that host genes influence susceptibility to leprosy or its various clinical forms is supported by data from a wide variety of sources. These sources include twin studies, segregation analyses, family-based linkage and association studies, candidate gene association studies, and most recently, genome-wide association studies (GWASs). The most definitive twin study of leprosy by Chakravartti and Vogel, (1973) enrolled 62 monozygous and 40 dizygous twin pairs from three different regions in India and found a 3-fold-greater concordance rate for the type of leprosy disease in monozygotic twins than in dizygotic twins (Chakravartti and Vogel, 1973). Segregation analyses determine whether or not there is a segregation of disease among more closely related individuals (evidence of a “major gene effect”) and what mode of inheritance is at work (dominant, recessive, or additive) (Stein, 2010). A number of segregation studies have been carried out for leprosy (Abel and Demenais, 1988; Abel *et al*, 1995; Feitosa *et al*, 1995; Haile *et al*, 1985; Lazaro *et al*, 2010; Ranque *et al*, 2005; Serjeantson *et al*, 1979; Smith, 1979; Wagener *et al*, 1988) several of which have detected the presence of a recessive or codominant mode of inheritance for leprosy per se or for non-lepromatous-leprosy (Abel and Demenais, 1988; Feitosa *et al*, 1995; Haile *et al*, 1985; Lazaro *et al*, 2010; Smith, 1979). Based on the results of complex segregation analyses, linkage and association studies, a two-stage model of genetic susceptibility to leprosy was proposed (Figure 2.11). The first stage representing an overall susceptibility/resistance to the infection, which is a manifestation of innate resistance mediated by cells of the monocyte lineage. If innate resistance is insufficient and infection becomes established, genetic influence is expressed at the second level, i.e., influencing the degree of specific cellular immunity and delayed hypersensitivity generated by the infected individual (Scollard *et al*, 2006). In addition, the limited genomic diversity between *M.
lepra

isolates implies that microbial factors do not play a major role in determining the clinical outcome. As there is no relevant animal model for human leprosy, forward genetics is the main method used to identify the genes, and consequently the immunological pathways involved in the human response to M. lepra (Figure 2.12).

Figure 2.11. M.lepra infection and the two stage genetic control of susceptibility to infection (Malhotra Ph.D. thesis 2005).
Figure 2.12. Strategies for identifying susceptibility genes that contribute to leprosy phenotypes. There are mainly two methods: Candidate gene study and genome-wide association studies. The role of the variant (common/rare) in candidate gene identified by either approach is further investigated by testing its associations with the leprosy phenotype under study, using population-based (case-control) or family-based designs such as the transmission disequilibrium test (TDT). Then role of associated polymorphism is functionally validated (modified from (Moller et al. 2010)).

2.3.2 Study Design for Complex Diseases

It has been widely presumed for many infectious diseases, including leprosy, that susceptibility is governed by polygenic inheritance, or the additive effect of multiple genes, each with a modest effect on the infectious phenotype. Two study designs are typically used to examine diseases with complex inheritance patterns: linkage studies of families and
association studies (candidate gene or genome wide). Linkage studies look for evidence of the segregation of a genetic marker and a disease trait within families. Genetic association studies assess whether the frequency of a particular genetic variant differs between individuals with a disease compared to unrelated controls.

### 2.3.2.1 Linkage studies

Linkage studies often follow up on the results of segregation analyses in the same study population. Several genome-wide linkage studies of leprosy susceptibility have been performed by using a family based design (Mira et al., 2003; Siddiqui et al., 2001). A major strength of genome-wide linkage studies is the absence of bias: no hypothesis as to which chromosomal loci or genes might be linked to disease status is required. Linkage studies genotype microsatellites or SNPs (single-nucleotide polymorphisms) spaced evenly throughout the genome, typically every 10 centimorgans (cM). Susceptibility loci identified in these studies are then investigated further by higher-resolution mapping of markers or gene alleles and linked to disease traits (Alcais et al., 2007; Mira et al., 2004; Tosh et al., 2002). Linkage studies have also been used to evaluate candidate regions (Jamieson et al., 2004; Wallace et al., 2004) and candidate genes (Abel et al., 1998; Santos et al., 2002) in leprosy. Significance in these studies is reported via Z scores, LOD scores (logarithm of odds), or P values (Lander and Kruglyak, 1995). The proposed criteria for significance in genome-wide linkage studies are somewhat stringent, given the risk of false positives due to the large number of markers studied. For example, one common genome-wide linkage study design relies on sibling pairs. Suggested threshold levels of significance for “suggestive linkage,” “significant linkage,” and “highly significant linkage” for individual markers in these sibling pair genome-wide linkage studies are P values of 7x10^-4, 2 x10^-5 and 3x10^-7, respectively (corresponding to LOD scores of 2.2, 3.6, and 5.4, respectively) (Lander and Kruglyak, 1995). The suggested P value for validating linkage in replication studies (which typically focus on a candidate region of ~20 cM in size) is a P value of 0.01 (Lander and Kruglyak, 1995). Linkage studies are also the most powerful study design for identifying rare variants of genes that confer a large risk to disease (Ardlie et al., 2002; Stein, 2010). Conversely, they have reduced statistical power to detect genes with modest or weak effects on disease risk, even when hundreds of families are included (Altmuller et al., 2001; Ralston and Crombrugghe, 2006; Risch and Merikangas, 1996; Stein, 2010).
2.3.2.2 Genetic association and gene-gene interaction studies

In contrast to linkage studies, association studies evaluate whether common polymorphisms in candidate genes are associated with susceptibility to disease, usually in unrelated individuals. These studies are hypothesis driven and often focus on genetic variants that are predicted to alter protein structure or function. The most common study design is a case-control format with comparisons of one or more polymorphism (single nucleotide, insertions, deletions, or microsatellite [MS] markers) frequencies between cases and controls. A major strength of this study design is the power to find relatively modest effects, generally with smaller sample sizes than family-based studies (Risch and Merikangas, 1996; Stein, 2010). One disadvantage is the problem of population stratification or admixture, where differences in ethnic compositions of the cases and controls can lead to spurious disease associations. Methods to control for population stratification include matching cases and controls for ethnicity and adjusting for ethnicity as a possible confounder in a multivariate logistic regression model. An alternative study design that takes care of the problem of population stratification is the transmission disequilibrium test (TDT). This approach looks for evidence of nonrandom transmission of the candidate allele from a heterozygous parent to an affected child and can be used to corroborate findings of either linkage studies or association studies.

It is important to remember that association, even down to the SNP level, does not necessarily explain an etiology of a disease. Genetic associations at specific loci may derive from neighboring alleles in linkage disequilibrium (LD) with the candidate gene that is being studied. In these situations, the candidate gene SNP serves as a proxy for the association, and if the haplotype structure of the region surrounding the candidate gene is not explored, the alleles most responsible for the disease association remains unascertained. The haplotype structures at specific genetic loci often differ between populations (population-specific linkage disequilibrium). This variability can make it difficult to replicate disease associations when the underlying LD structure has not been evaluated for both populations. Both linkage and genetic association study designs are also vulnerable to the generation of false-positive results from multiple comparisons. This problem is especially relevant in the current era of genome-wide linkage scans and high-throughput genotyping strategies. As a result, replication and validation of findings in independent populations coupled with investigation of the underlying haplotype structure of each population are an essential part of a careful
study design. The candidate gene approach can also be linked to functional studies of the polymorphisms to determine the biological mechanism relevant to disease pathogenesis.

Finally, neither association nor linkage studies are designed to detect rare alleles with weak effects (Stein, 2010). Adequate power has been a limitation for candidate gene association studies and probably accounts in part for a poor track record for replicating SNP associations. The need to include adequate number of cases and controls is particularly important when the frequency of the allele(s) being studied is low (< 5%). Numerous candidate gene association studies have relied on sample sizes of 50 to 100 cases. There is an absence of adequate power to detect a disease association of a variant allele with a population frequency of 5% in these small studies, unless the odds ratio (OR) rises to the level of 3.0 to 4.0 (for example, for an α of 0.05 with 100 cases and 100 controls with a minor allele frequency [MAF] of 0.05, power equals 0.17 for an OR of 1.5, 0.44 for an OR of 2, 0.87 for an OR of 3, and 0.98 for an OR of 4). However, many candidate genes have disease associations with ORs in the range of 1.5 to 2.5 or lower. To have power to detect associations of low-frequency SNPs with a more modest influence on disease susceptibility, study investigators would need to recruit 300 to 600 cases and an equal number of controls (for example, for an α of 0.05 with 500 cases, 500 controls and an MAF of 0.05, power equals 0.58 for an OR of 1.5, 0.97 for an OR of 2, and 1.00 for an OR of 3 to 4).

Because of the complex nature of the immune system and the polygenic nature of complex diseases, it has become increasingly evident that gene-gene interactions play a far more important part in an individual’s susceptibility to a complex disease than single polymorphisms would on their own (Williams et al, 2000; Ritchie et al, 2001; Tsai et al, 2003). Methods for studying gene-gene interactions are based on a multilocus and multigene approach, consistent with the nature of complex-trait diseases, which would provide a new paradigm for future genetic studies of leprosy (Moller et al, 2010).

### 2.4 ROLE OF PARK2-PACRG GENE REGION

PARK2 and PACRG genes both share a common regulatory region and encode the proteins that are involved in the cellular ubiquitination metabolism. Little is known about the specific function of the PACRG gene, but PARK2 protein product, parkin, has been identified as an
ubiquitination E3 ligase involved in delivery of polyubiquinated proteins to the proteasomal complex (Shimura et al. 2000) The only experimental evidence for the involvement of the PARK2 and its co-regulated gene PACRG with the host responses to M. leprae has been provided by the positional cloning in Vietnamese and Brazilian populations (Mira et al. 2004). Where a genome-wide scan of multi-case families from Vietnam with almost equal distribution of PB (44%) and MB (56%) cases detected significant evidence of linkage for chromosome region 6q25–q26 (multipoint LOD=4.31) and also provided a suggestive evidence for regions 6p21 (multipoint LOD=2.62), 20p12 (LOD=1.13), and 13q22.1 (LOD=1.68) (Mira et al. 2003). A separate study of 208 families confirmed that two markers in the 6q25-27 region were strongly linked to leprosy susceptibility. Further scanning of 6.4-megabase region of 6q25-27 using 81 SNPs found 17 SNPs that were associated with leprosy susceptibility. These identified SNPs were in or near the core promoter region of PARK2 and PACRG and were in strong linkage disequilibrium with each other. Two SNPs, PARK2_e01(2599) and rs1040079, accounted for the entire association at this locus. A separate case-control study from Brazil identified 9 SNPs, significantly associated with leprosy risk and validated the PARK2_e01(2599) and rs1040079 alleles and a third SNP, PARK2_e01(2697) (Miller et al. 2004). However, Malhotra et al. did not find a significant association (after conservative Bonferroni correction) between leprosy and SNPs in the PARK2 or PACRG coregulatory region, including PARK2_e01(2599) and rs1040079 in case-control study from India (Malhotra et al. 2006). The identification of PARK2 and PACRG as major leprosy risk genes in Brazilian and Vietnamese populations and their apparent absence in Indian population highlights the heterogeneity of risk alleles for infectious diseases across different ethnic groups. PARK2 codes for parkin, an E3 ubiquitin–protein ligase that is involved in the delivery of poly-ubiquitinated proteins to the proteasome complex (Shimura et al. 2000). Mutations in the PARK2 gene cause autosomal recessive early-onset Parkinson’s disease (Kitada et al. 1998). The function of PACRG is unknown, but is linked to the ubiquitin–proteasome system. Both genes are expressed in varying degrees in different tissues, including Schwann cells and monocyte-derived macrophages, the primary host cells for M.leprae, suggesting the involvement of the both PARK2 and PACRG gene in the ubiquitin-mediated proteolysis of the important immune-regulatory molecules.

Different pathway analyses also show the importance for these genes in the pathogenesis of the disease (Schurr et al. 2006; Zhang et al. 2009) However, attempts to replicate the results in other populations have failed in the past (Li et al. 2012; Malhotra et al. 2006) suggesting
the possible involvement of different variants in diverse populations responsible to provide susceptibility towards leprosy. This possibility could arise due to the change in LD structures across the populations for the SNPs distributed in the specific genomic regions.

The present study with the rationale, as mentioned already, selected the group of SNPs to saturate the regulatory region of the PARK2 and PACRG genes to find out the variant LD structure, if any, in Indian population as compared to Brazilian and Vietnamese; and study the unexplored variants that may be responsible for an association with leprosy or its sub-types in the studied population.

2.5 INFERENCES FROM THE STUDIES CONDUCTED SO FAR AND THE RATIONALE FOR OUR STUDY

While it is encouraging that regions of linkage have been identified reaching genome-wide statistical significance, however, many of these results have not been replicated in other populations, reflecting existence of genetic heterogeneity between populations. Frequencies of the disease related polymorphisms have differed between populations and exist only in some populations and not in others. Such heterogeneity also is evident in the apparent differences in relative frequency of multibacillary (MB) and paucibacillary (PB) disease between populations, with proportions of MB disease being lowest in Africans, higher in Asians and highest among Caucasians. Besides, other explanations, such as exposure to other factors (e.g. environmental/mycobacteria) which modify the risk of the disease and associated immune response, cannot be discounted.

India carries the majority of the global burden of leprosy and ironically the role of anti-inflamatory cytokines and their receptor genes in susceptibility to leprosy have not been studied extensively. The present case control association study of functional polymorphisms in the PARK2 and PACRG gene regulatory regions was carried out to genetically dissect the basic elements involved in the interplay between *M. leprae* and its human host. Since, leprosy is a complex disease caused by multiple genes, it was considered pertinent to explore gene–gene interactions between the studied variations of the candidate genes in view of the polygenic nature of the disease.
With this background, the present work entitled “Genetic Predisposition to Leprosy: A study of PARK2-PACRG gene regulatory region in Indian Population” was undertaken laying down the following objectives.

### 2.5.1 OBJECTIVES

1. Screen some known SNPs in the chromosome region 6q25-26, spanning the coding and the regulatory region of PARK2 and PACRG genes to find an association with Leprosy and its sub-types.

2. Study the level of significance and the strength of association of individual single nucleotide polymorphisms (haplotypes or interacting alleles among the selected polymorphisms) with susceptibility / resistance to leprosy, through a case-control study in the individuals representing Indian population, belonging to the States of Uttar Pradesh, Delhi and Bihar.

3. Replicate the results in the ethnically distinct population of eastern Indian, Orissa, if possible.

4. Examine the extent of genetic heterogeneity associated with leprosy susceptibility among the selected polymorphisms for the populations under study and the populations already studied.

5. Carry out the bio-informatics analysis of the associated SNPs and to evaluate their functional role by *in-vitro* luciferase reporter expression assay.