Discussion
The widespread implementation of multidrug therapy, shorter treatment courses, and improved access to leprosy diagnosis has meant that over the past two decades the global prevalence has fallen by almost 90%, and more than 14 million patients have been cured. Paradoxically, although 113 of 122 countries endemic for leprosy in 1985 have reached the elimination target yet, leprosy remains a major public-health problem in nine countries in Africa, Asia, and Latin America with India carrying almost 60% of the global leprosy burden. Worryingly these countries show no decline in case-detection rate, and now countries such as Tanzania having reached their elimination target in 1997, have crept back onto the endemic list (editorial, 2005). There are compelling reasons to study leprosy because the disease poses a significant health and economic burden in third world countries and since leprosy is a spectral disease in which the pathology and immune response are inextricably linked thus, providing a unique critical model to investigate immunoregulatory mechanisms in humans.

Susceptibility to leprosy involves both genetic and environmental components (Chakravati et al, 1973; Shields et al, 1989). Evidence that host genetic factors contribute to susceptibility to leprosy comes from epidemiological data, segregation and twin studies. Numerous case control studies have identified variants in VDR (Roy et al, 1999), HLADR2 specificities (Rani et al, 1993; Mehra et al, 1995; Roy et al, 1997; Joko et al, 2000; Shaw et al, 2001; Mira et al, 2003), TAPI and TAP2 (Rajalingam et al, 1997), CTLA4 (Kaur et al, 1997), COL3A (Kaur et al, 1997), SLC11A1 (Shaw et al, 1993; Roger et al, 1997; Roy et al, 1999; Alcais et al, 2000; Meisner et al, 2001; Meisner et al, 2001; Ferreira et al, 2004) and TNF-α (Roy et al, 1997; Santos et al, 2000; Moraes et al, 2001; Shaw et al, 2001) to be associated with leprosy or its subtypes. Genome wide linkage scans so far have led to the identification of a chromosomal region 10p13, for loci controlling susceptibility to the PB form of leprosy (Siddiqui et al. 2001), chromosome 20p12 (Tosh et al, 2002), chromosome 21q22 (Wallace et al 2004), chromosome 17q11-21 (Jamieson et al, 2004) and a chromosomal region 6q25-26 (Mira et al, 2004) harboring variants in the common regulatory region of PARK2 and PACRG genes, as risk factors for susceptibility to leprosy per se. However, many of these associations have not been replicated reflecting genetic heterogeneity between populations. The different histories of discrete populations and geographical and ecological diversity also suggest that genes controlling human immunity have evolved differently in different regions of the world. Although India carries the majority of the global burden of leprosy and is one of the most genetically diverse
populations of the world, the role of host genetic components in controlling susceptibility to leprosy, beside human leukocyte antigen complex (HLA), has not been intensively investigated. The present study was designed to investigate the role of functional polymorphisms in candidate genes known to play an important role in innate and adaptive immunity to genetically dissect the basic elements of interplay between pathogen and its human host in leprosy. The results of the study are discussed under the following sections.

5.1 Polymorphisms in the candidate genes involved in innate immune response (TLR2, SLC11A1, PARK2 and PACRG, IL-10 and IL-12p40) and their association with leprosy

5.1.1 TLR2 association

Our investigation for TLR2 gene by an in-silico analysis of the upstream region homologous to the third exon of TLR2, followed by experimental validation by a direct PCR sequencing and genotyping method, using primer pair PP1 (Kang et al, 2001), depicted that PP1 amplified both exon 3 of TLR 2 and the homologous region upstream (psi) of this gene with nucleotide substitutions. This explained the unusual observation of the presence of heterozygosity in all samples studied with primer pair PP1 in our study. This was further substantiated by the genotyping results obtained with another primer pair PP2, designed specifically for the exon 3 of TLR2 intracelluar signaling region, which did not amplify the upstream homologous region and showed the absence of any mutation including Arg677Trp, in the conserved region of TLR2 in all the patients and the controls studied. These results suggested the nonspecificity of primer pair PP1, used previously (Kang et al, 2001). It is apparent through our study that the variant that was associated with lepromatous leprosy (Kang et al, 2001), in all likelihood, could have resulted from the variation present in the duplicated region of exon 3 present approximately 23 kb upstream to TLR2 gene with 93% homology with the authentic exon 3 of TLR2 gene. Interestingly, TLR2 Arg677Trp polymorphism has not been associated with any other disease except lepromatous leprosy in Korean population.

Since the level of TLR2 expression and its inducible regulation may influence response to microbial infection, we therefore investigated variants in the promoter region that could possibly regulate TLR2 expression. An absence of any variant in the promoter region probably suggested that other regions of TLR2 such as, the microsatellite polymorphism in
the intron2 (Yim et al, 2004) might confer modifying effects or susceptibility to this infectious disease.

Our observation suggests that TLR2 polymorphism needs to be studied with caution in future because of the presence of variations in the duplicated (pseudogene) region representing exon3 of TLR2 gene. The absence of any variant in the conserved promoter and intracellular signaling regions of the TLR2 gene in our study indicates a need to investigate other regulatory mechanisms, which could control TLR2 function.

5.1.2 SLC11A1 (NRAMP1)

On the basis of the strong influence of variation in the Nramp1 gene on susceptibility to BCG infection in inbred strains of mice, there has been considerable interest in the relevance of the human homolog NRAMP1 in susceptibility to human mycobacterial diseases. A number of polymorphisms have been described in SLC11A1 (Liu J et al, 1995), and genetic variants in intron 4 (INT4) and the 3'untranslated region (3'UTR) of the SLC11A1 gene were linked to significantly increased risk of pulmonary tuberculosis in West Africans (Bellamy et al, 1998). However, ethnicity needs to be considered in assessing this problem. Allelic frequency in the 3'UTR of SLC11A1 reportedly differs between Korean patients with tuberculosis and healthy individuals (Ryu et al, 2000). In the Japanese population, 5' promoter with (GT)ₙ repeats was found to be significantly associated with tuberculosis (Gao et al, 2000). In contrast, a large study of Brazilian families did not find the SLC11A1 locus to represent a major susceptibility gene (Shaw et al, 1997). Information on the role of SLC11A1 in susceptibility to leprosy has been limited and heterogeneous. The study conducted in Indian population did not find any evidence of association of SLC11A1 promoter polymorphism with leprosy susceptibility (Roy et al, 1999).

In our study of SLC11A1, no significant association was obtained between the leprosy per se and its clinical types and any of the three polymorphisms typed. The SLC11A1 variants studied were relatively poorly informative in this population. The lack of association with the SLC11A1 polymorphisms in this population does not rule out an association with the gene in other ethnically different populations and a weak association that would not be detected in a study of this size. Racial differences have been noted in associations between D543N and 3'UTR alleles. A 3'UTR del/D543N G haplotype has been reported in West Africans but is absent in Europeans and Asians. The D543N A allele is always associated
with the 3'UTR del allele in West Africans, Europeans, and Asians (Bellamy et al., 1998; Ryu et al.; Gao et al., 2000; Shaw et al., 1997). Our study agreed with these observations. We did not investigate the effect of functional promoter (CA)$_n$ repeat polymorphism and a non conservative SNP at codon 543 in exon 15 (D543N) in our study. However, the polymorphisms at the INT4 and 5'(CA)$_n$ loci are known to be in linkage disequilibrium as are the polymorphisms at D543N and 3'UTR loci. In conclusion, our results do not show any involvement of SLC11A1 variants in conferring risk to leprosy in Indian population and the results of this study are consistent with a small family studies of leprosy from Polynesia (Roger et al., 1997) and the Indian study (Roy et al., 1999) which found no evidence of association to the SLC11A1 region.

5.1.3 PARK2 and PACRG

Single nucleotide polymorphisms (SNPs) in the regulatory region shared by PARK2 and PACRG have been identified as major risk factors for leprosy susceptibility in two ethnically distinct populations. These five single nucleotide polymorphisms in the common regulatory region of PARK2 and PACRG genes have been identified as major risk factors for leprosy in two ethnically distinct populations (Mira et al., 2004). Among them, PARK2_e01 (-2599) and rs1040079 were the two most significantly associated SNPs. Independently these SNPs conferred relatively lower risk in a dominant manner whereas these SNPs in cis as a haplotype showed significant dominant effect in susceptibility to leprosy per se (Mira et al., 2004). The risk allele T of SNP 28kb target_2_1 showed differential associations with susceptibility to leprosy between Brazilian and Vietnamese population (Mira et al., 2004). It was, however, interesting to find in our study that the susceptibility to leprosy per se is not spread over the whole 80 kb block region in Indian population, instead it is confined to one major risk SNP PARK2_e01 (-2599) which showed significant recessive effect with susceptibility to leprosy, though with a modest effect (OR = 1.42), when compared to the observed dominant effects of the risk haplotype in the previous study (O.R = 5.0) (Mira et al., 2004). There was a significant difference in the prevalence of the risk allele T of PARK2_e01 (-2599) in Indian population and the two populations (Brazilian and Vietnamese). We did not find a significant association with the other risk SNPs or haplotypes with leprosy per se or with different clinical forms of leprosy in an Indian population, suggesting heterogeneity in association of these SNPs with susceptibility to leprosy in different populations. The association of nonfunctional variants depends upon
the patterns of LD across the relevant chromosomal region, which may differ between populations and contribute to heterogeneity among associations. The strength of LD among the 4 markers (B, C, D, E) studied in our population was comparable to the strength of LD observed for same markers for Brazilian and Vietnamese population. These observations highlight the differences in relative importance of these SNPs as susceptibility loci in disease manifestation in the Indian population and the populations studied previously. A number of association studies in the past have also suggested the prevalence of differential genetic susceptibility between populations (Roy et al 1997; Santos et al 2000; Santos et al 2002; Wallace et al 2004). This is supported by genome wide linkage scans of several complex diseases, such as type 2 diabetes, where both different and overlapping chromosome regions were linked in different populations. It has been shown that such differential susceptibility could extend between different caste groups within a population. The genetic heterogeneity in linkage of chromosomal region 20p12 with the susceptibility to PB form of leprosy between two population groups of South India corroborates the existence of genetic diversity between caste groups in India (Tosh et al, 2002). In our study, the risk of population stratification bias due to differences in the ethnic background between patients and controls and variations of allele frequencies according to ethnic background was minimized by including patients and controls of the same ethnic background. Further, the samples were analyzed independently with 2 genomic control markers (whose mean heterozygosity was 48%) not known to be associated with leprosy, resulting in no association with cases and controls.

The involvement of PARK2 and PACRG locus in Indian population highlights the ubiquitous role of this locus in susceptibility to leprosy per se although the effect of the SNPs in this region in regulating genetic susceptibility to leprosy appears to be differential in Indian population and other populations. It will be interesting to investigate whether the spectrum of variations within other regions of PARK2 and PACRG loci, apart from the presence of a global risk SNP PARK2_e01 (-2599), are also involved in disease susceptibility in Indian population. Also, it will be worthwhile to examine the role of other modifier gene(s) in the background of risk alleles in PARK2 and PACRG locus in providing susceptibility to leprosy.
5.1.4 IL12 p40 (IL12B)

The identification of differential cytokine patterns in patients with leprosy have provided evidence that the imbalance in the expression of Th1 and Th2 cytokines may be a central mechanism in the development and progression of leprosy and Interleukin-12 plays a key role in this process. Polymorphisms in the human cytokine genes have been associated with different levels of protein production. Recent studies have shown that the 1188 3'UTR IL-12 p40 polymorphic site is biologically relevant. Seegers et al (2002) have demonstrated that the presence of the rarer allele was correlated with increased IL-12 p70 secretion by stimulated monocytes. A logical progression from these studies was to evaluate whether SNPs located on the genes coding for IL-12p40 are associated with the development of leprosy.

In the current study, our investigation of a functional polymorphism in the IL-12 p40 subunit gene at position 1188 in the 3' UTR region in patients with leprosy and healthy controls showed no differences between these populations with regard to their allelic distributions. However, interestingly an absence of IL12 promoter homozygotes for the 4bp long allele in our population was observed. The significant deviation from HWE of this locus can indicate inbreeding, population stratification, and even problems in genotyping. The problem of inbreeding and population stratification was ruled out by selection of unrelated random patients and controls and by genotyping these loci for genomic control markers which showed no association with disease. The technical artifact of genotyping was ruled out by sequencing of the clones for the long and short allele. Thus the significant deviation from HWE is indicative of a selection pressure at this locus in our population which needs to be further assessed.

To the best of our knowledge our study is the first report in Indian population which have investigated the role of IL12p40 gene in susceptibility to leprosy. Although we did not observe an association between the polymorphic loci examined and leprosy, we cannot exclude that other polymorphic variations within these genes (Huang et al, 2000) or their receptors (Ohyama et al, 2005; Lee et al, 2003) could provide susceptibility to leprosy. Further studies are therefore required to evaluate whether other polymorphisms in genes regulating IL-12 production are involved in leprosy susceptibility. The present study, although negative, shall hopefully direct subsequent work in this direction.
5.1.5 IL-10

The analysis of the entire region encompassing -4 to 0.5 kb, using genotyping data of the six IL-10 promoter SNPs suggested that the extended haplotype, -3575T, -2849G, -2763C, -1082A, -819C, -592C, was protective and was associated with resistance to leprosy per se and to the development of severe types of leprosy. The haplotype -3575T, -2849G, -2763C, -1082A, -819T, -592A was also significantly associated with severity of leprosy. The results of individual SNPs suggests that the effect of proximal promoter SNPs (-1082 A>G, -819 C>T, -592 C>A) in determining risk/protection to leprosy is more pronounced than distal SNPs (-3575T>A, -2849G>A, -2763C>G). The genotypes defined by these proximal promoter haplotypes also showed significant preponderance of ACC/ACC haplotype in controls and ATA/ATA haplotype in patients. The proximal promoter polymorphisms have been reported to define ‘high’ (-1082G/-819C/-592C), ‘medium’ (-1082A/-819C/-592C) and ‘low’ (-1082A/-819T/-592A) expressing genotypes for IL-10 (Turner et al, 1997). The increased production of IL-10 in controls and relatively decreased production of IL10 in leprosy patients defined by these genotypes do not correlate well with the pathophysiological role of IL10 in leprosy infection in our study. We did not investigate the effect of IL10 polymorphisms on cytokine production in patients and control subjects. Therefore it is still important to analyze whether the genotypes that appear to be associated with increased or decreased levels of IL10 could be used to select patients and controls in our population in whom these increased or decreased levels are observed. The mosaics of distal and proximal promoter elements reflecting high and low responses is, however, still unknown. It will be interesting to find out how the extended haplotype influences the expression of IL-10 in in-vivo conditions.

Although the frequency of -819T/T was found to be increased in Brazilian leprosy patients (Moraes et al, 2004), it did not show significant differences between patients and controls suggesting differential involvement of these proximal SNPs in susceptibility to leprosy. The frequency differences in the studied Indian population and that of Brazil for IL-10 promoter SNP genotypes and haplotypes probably explains the involvement of different genotypes and haplotypes in the resistance/susceptibility to leprosy or its types. The present study showed that the frequency of the haplotypes, -3575A, -2849G, -2763C, and -3575T, -2849A, -2763C defined as a marker of resistance and susceptibility to leprosy, respectively in Brazilian population (Moraes et al. 2004) were observed in a very low frequency both in controls and leprosy patients in our population. Since the rare haplotypes, when pooled
together showed an overall significant effect, the specific effect of this haplotype in disease resistance and severity cannot be ruled out. However, the overall contribution to disease resistance at the population level for this haplotype seems limited. The haplotype -3575T, -2849G, -2763C, -1082A, -819C, -592C with a relatively high frequency among patients and controls in our study suggested it to be a significant contributor to the disease resistance in our population though with a modest effect (OR = 0.58). These results corroborate with the fact that the outcome of mycobacterial infection involves complex interactions between several other host genes and also highlights the role of IL10 in early and late phases of leprosy infection.

The involvement of IL-10 polymorphisms in the outcome of leprosy in two ethnically distinct populations suggests that IL-10 region needs to be further investigated both in genetic and functional studies.

5.2 Polymorphisms in the candidate genes involved in adaptive immune response (IL4, TNF-alpha and TGF-beta1) and their association with leprosy

5.2.1 IL-4

The T-helper-cell 1 and 2 (Th1 and Th2) pathways, defined by cytokines interferon-gamma (IFN-γ) and interleukin-4 (IL-4), respectively, comprise two alternative CD4+ T-cell fates, with functional consequences for the host immune system. IL-4 is known to elicit a number of different biological responses and has a major contribution in humoral immune response. The 5'UTR region of IL-4, screened for polymorphism, showed a reported -33C>T polymorphism. There was however no significant difference observed in the frequencies of heterozygous -33C/T and homozygous -33T/T genotypes between different categories of patients and controls. Observed Odds ratio did not reveal any risk for heterozygous C/T and homozygous T/T genotypes. The role of -33 C>T polymorphism on IL-4 production are contradictory. Although our findings show a negative association of -33C>T polymorphism with leprosy susceptibility, nevertheless it will be interesting to investigate the involvement of other polymorphic loci within IL-4 with leprosy susceptibility.
5.2.2 TNF-alpha and TGF-beta 1

The only TNF-α polymorphism associated with leprosy susceptibility or resistance is a G to A substitution in the promoter, at position −488 (Roy et al. 1997; Santos et al. 2000; Santos et al., 2002). Our results suggested for the first time a possible role of TNF-α−418 G/A allele in susceptibility/resistance to leprosy. The TNF-α−488−418 G-G haplotype was associated with susceptibility whereas the TNF-α−488−418 A-G and TNF-α−488−418 G-A haplotypes were associated with resistance to leprosy in our study. The effect of -418A allele was more pronounced in providing resistance to leprosy per se whereas the -488 A allele was significantly associated with providing resistance to MB leprosy but not PB leprosy. The major hypothesis as to how TNF-α influences a chronic infection such as leprosy predicts that the requirement for TNF-α production to activate macrophages to kill microorganisms means that polymorphisms leading to low production of TNF-α would be associated with disease. TNF-α−418 and -488 A allele might regulate TNF-α gene transcription and alter the levels of protein that are produced (Bayley et al. 2001) and thereby promote host resistance to local infection by increasing the production of TNF-α at the infection site. Because TNF-α promotes anti-microbial mechanisms, it is possible that the increased frequency of TNF-α−418 and -488G allele, which represses TNF-α transcription, leads to disease susceptibility. The significant preponderance of TNF-α−418 and -488 G/A heterozygotes in controls when compared with leprosy patients also suggests that the optimal level of TNF-α response against M.leprae infection is maintained by TNF-α−418 and -488 heterozygotes, supported by the results of the in vitro studies which have shown that healthy TNF2 -488 heterozygotes release more TNF-α in response to lipopolysaccharide than do non-carriers (Louis et al. 1998). Strong linkage disequilibrium, in some populations is known to exist within MHC class II and TNF regions (gene order: HLA-DQB1, HLA-DQA1, HLA-DRB1,TNF, LTA). The pattern of linkage disequilibrium between TNF and other close by genes has not been investigated in our study and the possibility of this association with leprosy resistance/susceptibility of TNF-α−488 and −418A/G alleles due to linkage disequilibrium cannot be ruled out.

The balance of pro and anti-inflammatory cytokines play a critical role in determining the outcome of leprosy infection. The decreased production of TNF-α might result from an increased production of TGF-β1, an anti-inflammatory cytokine that inhibits the synthesis of proinflammatory cytokines and suppresses T cell activation. The increased expression of TGF-β1 in dermal lesions of lepromatous leprosy patients and reduced expression in
tuberculoid leprosy patients (Kiszewski et al 2003) highlights the role of its differential expression in clinical outcome of leprosy. Our results provide for the first time a suggestive evidence of TGF-β1 promoter polymorphism in susceptibility to leprosy type. The association of TGF-β1 −1349 T allele with susceptibility to multibacillary form of the disease and not with paucibacillary leprosy suggests that this polymorphic allele could be one of the genetic markers in development of disseminated disease. The T allele at position −1349 is associated with increased transcriptional activity and serum levels whereas C allele has reduced transcriptional activity and is associated with decreased serum TGF-β1 levels (Silverman et al 2004). Further studies are needed in a large sample size to confirm our findings regarding TGF-β1 polymorphisms.

Ideally, the risk genotypes in different candidate genes present together in affected individuals should be present in the large proportion of diseased cases. Although the penetrance of −488G, −418G haplotype in leprosy patients is high with 93% patients possessing this haplotype, the proportion of controls with this haplotype (83%) is also high suggesting that the outcome of mycobacterial infection is due to a complex interaction between several host genes. It will be interesting to investigate the effect of other pro and anti-inflammatory cytokines in the background of −488G, −418G haplotype in association with leprosy susceptibility.
To sum up, our findings of the molecular typing of functional polymorphisms in non MHC candidate genes involved in innate and adaptive immunity in leprosy highlight the ubiquitous role IL-10 promoter polymorphisms in critically determining the outcome of *M. leprae* infection. The differential associations of candidate genes (TNF-α, PARK2 and PACRG, SLC11A1) in our study when compared with different populations suggests that distinct environmental and natural selective factors have probably resulted in population-specific immunogenetic adaptations to clinical leprosy. In this regard, it is interesting to note that, although malaria has resulted in ethnic-specific adaptations of common erythrocytic variants and hemoglobinopathies associated with resistance to plasmodial parasite infection in populations from Africa, Asia, and the Mediterranean (Kwiatkowski, 2000), other associations of candidate genes of less clear functional impact on susceptibility or resistance to malaria, including polymorphisms of the MHC (Hill et al., 1991), TNF-α (McGuire et al., 1994; Knight et al., 1999), intracellular adhesion molecule-1 (Fernandez-Reyes et al., 1997) and nitric oxide synthase 2 genes (Burgner et al., 1998), appear to be geographically heterogeneous or contradictory (Mazier et al., 2000). Here, we have shown that the frequencies of genetic variants of IL-12p40, SLC11A1 (NRAMP1), IL-10, and PARK2 and PACRG genes are also population-specific, thus underscoring the need for understanding the frequency of a particular polymorphism in specific populations before assigning to it specific infectious-disease associations.