Summary

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Conclusions
Microbes and host defence systems have developed intricate genetic pathways and the study of complex interactions between them allows dissecting their role in the pathogenesis. The immune system has evolved primarily to combat infection and in leprosy this response correlates with the broad clinical spectrum of the disease (Ridley and Jopling, 1966), triggering further complications, such as nerve damage (Pfaltzgraff, 1973; Myers, 1987). It is, therefore, pertinent to understand the molecular mechanisms involved in leprosy onset and progress, an important challenge to control the disease and reduce the rate and severity of disabilities.

Overall only a minority (<5%) of individuals exposed to M. leprae develop clinically relevant disease, while the majority develop protective immunity (Schurr et al, 1991). Factors reported to influence the risk of infection include age, sex, crowding, socioeconomic conditions, urbanization and race/ethnic group (Naafs, 2001). The risk of developing disease after infection is strongly dependent on any condition that modifies the balance established in the body between the leprae bacilli and host immune defences. Thus, factors that have been shown to influence this balance include age, sex, immunosuppressive treatment, malnutrition, and bacille Calmette-Gue'rin vaccination. In addition, there is increasing evidence that genetic factors, in part, determine differences in host susceptibility to infection with M. leprae and that they might contribute to the pattern of clinical disease (Wagener et al, 1988; Abel et al, 1988; Feitosa et al, 1995). A recent genome-wide study observed a significant association between SNPs in the genes, CCDC122, C13orf31, NOD2, TNFSF15, HLA-DR and RIPK.2 and a trend towards an association with a SNP in LRRK2 (Zhang et al, 2009). Another genome-wide association study found consistent associations between genetic variants in both TLR1 and the HLA-DRB1/DQA1 regions with susceptibility to leprosy (Wong et al, 2010). Besides, other candidate gene studies have found several immunogenetic polymorphisms with a moderate effect on risk of susceptibility to leprosy and its polar clinical forms (Fitness et al, 2002). Differential susceptibility to leprosy is most likely determined by several genes. The associations of candidate genes in different populations necessitate examining the role of polymorphisms in candidate genes of innate and adaptive immune system, such as cytokines which are continually selected by distinct environmental and natural selection forces. Here 51 SNPs, located within five anti-inflammatory cytokine (IL10, IL6, IL4, IL13, TGFBI) and nine anti-inflammatory cytokine receptor (IL10RA, IL10RB, IL6R, IL4R, IL13RA1, IL5RA, IL5RB, TGFBR1, TGFBR2) genes were analyzed to genetically dissect the basic elements of interplay between M. leprae and its human host.
Summary and Conclusions

IL10 is a potent anti-inflammatory and immunosuppressive cytokine, regulating protective immunity in leprosy (Misra et al, 1995; Lima et al, 2000). The analysis of eight SNPs in *IL10* suggested that promoter SNPs, rs1800872 (-592C>A) (P=0.006, OR=0.76, 95% CI=0.61-0.92), rs1800871 (-819C>T) (P=0.005, OR=0.75, 95% CI=0.62-0.92) and intron 3 boundary SNP rs1554286 (P=0.0001) OR=1.66, 95% CI=1.30-2.14), were strongly associated with leprosy in North Indian samples. In a replication study involving Orissa samples, observation on rs1554286 supported the finding of a significant association with leprosy (P=0.016, OR=1.98, 95% CI=1.13-3.50). Interestingly, the bioinformatics analysis predicted the creation of a splicing factor binding site by the change from C to T (U) in rs1554286 SNP site, possibly playing a role in alternate splicing; which would require confirmation through wet lab experiments in future.

A highly frequent haplotype TTTATAT of *IL10* SNPs provided risk (P= 0.00004, OR=1.23, 95% CI=1.04-1.45) towards leprosy. Haplotype analysis of the SNP allele rs1554286 T at the intron 3 boundary in complete LD with functional promoter SNPs (rs1800872, rs1800871 and rs1800896) represented by ATA haplotype, provided risk towards leprosy (OR=1.21). The ATA haplotype has been shown to downregulate the IL10 expression (Turner et al, 1997) which could downregulate Th1 response and consequently protective immune response. Interestingly, the low producer haplotype of *IL10* in strong association with the PB form of leprosy, in present study, could be explained on the basis of a possible overactive Th1 cytokine response.

Apart from cytokines, their receptor chains, *IL10RA* and *IL10RB* were also explored in the study. *IL10RB*, located in a class II cytokine receptor gene cluster together with IFNa receptor 1 (IFNAR1), 2 (IFNAR2) and IFNγ receptor 2 (IFNGR2) on chromosome 21q22 (Frodsham et al, 2006), but not *IL10RA*, played a possible role in susceptibility to leprosy. The two SNPs, rs3171425 and rs7281762 within and downstream to 3'UTR of *IL10RB* gene played this significant role in susceptibility towards leprosy. Remarkably, 3'UTR downstream polymorphism rs7281762 was observed to be associated with tuberculosis as well, an observation made for the first time and of relevance especially when both the mycobacterial species occupy similar niches in human body where they encounter same physiological stresses and immune responses. The functional implication of rs7281762 (replicated in Orissa population and associated with tuberculosis too) was validated by luciferase assay, revealing ‘A’ allele upregulate IL10RB expression, hence STAT3-dependent pathway of IL10 production.
Furthermore, the SNP–SNP interactions reflected highly significant risk (additive effect) (P=2×10^{-6}, OR=2.12, 95% CI=1.56-2.85) towards leprosy in presence of rs1554286 TT and rs7281762 GA+AA genotypes. We observed rs1554286 TT was associated with low IL10 expression and rs7281762 GA+AA with increased IL10Rβ expression, providing risk towards leprosy. IL10Rα has a dominant role in ligand binding and signal transduction; whereas IL10Rβ participates in the initiation and transduction of the signal (Lutfalla et al, 1993). IL10Rα can sustain the STAT activation even in the absence of the ligand (Finsterbusch et al, 2011). Since, TT of rs1554286 was associated with low IL10 expression and its interaction with GA+AA of rs7281762 provided risk towards leprosy, we conclude that in spite of increased expression of IL10Rβ and probably an enhanced STAT3 activation, IL10 production remains low because of its genotype status. Alternatively, we propose because of an influence of a neighbouring SNP in a gene like IFNGR2 present in strong LD with IL10RB, an over-expression of interferon receptor combined with increased downstream signalling effect of the pro-inflammatory cytokine, IFNγ, due to low producing genotype of IL10, would illicit aggravated pro-inflammatory immune response. This would be favourable for the PB form of leprosy. Both these explanations, though hypothetical and to be proven, support the risk observed in IL10-IL10RB interaction study.

The investigation of TGFBR2 polymorphisms showed for the first time a possible role of synonymous SNP rs2228048 and 3'UTR downstream SNP 744751 providing susceptibility to leprosy. Interestingly, rs2228048 was found to be associated with another mycobacterium afflicted disease, tuberculosis. The functional implication of rs744751 was validated by luciferase assay in HeLa (P = 0.005), HEK (P = 0.046), HepG2 (P = 0.03), revealing that 'C' allele overrepresented in patients could down regulate TGFBR2 gene expression. It is quite likely that change in expression of TGFBR2 could alter TGFβ driven downstream signalling in the studied leprosy patients. Haplotype combination TAG of three TGFBR2 SNPs: rs2228048, rs6550008, rs744751, providing higher risk (P=0.0003, OR=1.51, 95% CI=1.15-1.93) towards MB compared to PB form of leprosy, suggested it to be one of the genetic markers in the development of disseminated disease.

The present study reported IL6 promoter SNP −597 G/A (rs1800797) providing a significant risk towards leprosy per se (P =0.008, OR=1.33, 95% CI=1.1-1.64) which was also replicated in Orissa samples (=0.01, OR=4.9, 95% CI=1.50-16.05). This is for the first time that IL6 has been shown to be involved in susceptibility towards leprosy. In vitro analysis showed ‘A’ allele of rs1800797 in IL6 promoter which was overrepresented in controls, upregulating IL6 expression while ‘G’ downregulated the expression. Remarkably, low producer allele of IL6
promoter was associated with PB form of leprosy. This result apparently indicated that low producer status of IL6 enhanced Th1 cytokine production, since IL6 inhibits the production of TNFα and IL1β, which may enhance intracellular killing of microorganisms and development of granulomas (Denis and Gregg, 1991; Shiratsuchi et al, 1991). IL6 also has been reported to promote the growth of mycobacteria in peripheral blood monocytes (Schindler et al, 1990; Aderka et al, 1989). Thus, low IL6 production could result in enhanced cell mediated immune response, as observed in the PB form of leprosy, the interpretation which corresponds with our observation.

Further, an interaction between rs1554286 TT of IL10, associated with low IL10 production (Turner et al, 1997) and the low IL6 producing -597G allele of rs1800797 provided a significant risk towards leprosy. These results corroborate with the fact that the outcome of mycobacterial infection involves complex interactions of the proposed and many other host genes. On the basis of our observations we conclude that differential susceptibility to leprosy is critically influenced by IL10, IL10RB, TGFBR2 and IL6 polymorphisms. It will be interesting, in future, to investigate the effect of these polymorphisms on cytokine production in patients and control subjects with defined genotype backgrounds.

Copy number variations (CNVs) have been clearly shown to have the potential to directly or indirectly influence a healthy individual’s susceptibility to disease. Since no CNV studies have been performed in leprosy so far, we attempted to find CNV in a cytokine gene IL10, the low producer genotype of which was implicated in our study for its association with leprosy. An absence of alteration in gene copy number was found in patients and controls. Nevertheless, there is a need to explore copy number changes in other immune-regulatory genes, since an immune response to M. leprae infection is mainly mediated by the balanced activity of cell mediated immune response and the altered profile is always likely to shift the protective immunity to detrimental response by shifting Th1-Th2 response. This shift mainly depends on the altered levels of cytokines, such as IFNγ, IL4, TNFα, TGFβ and IL10 secreted by T lymphocytes in response to antigenic stimuli.

An improved understanding of the pathogenesis of leprosy and an effective treatment for it shall significantly be influenced by our ability to untie the effects of host genetic factors in response to M. leprae infection. Our study highlights that susceptibility to leprosy is critically influenced by polymorphisms in anti-inflammatory cytokine and receptor genes. All the candidates for a given pathway/network work in unison and influence the final outcome of disease pathogenesis. In conclusion, the present findings provide an insight into leprosy pathogenesis and add to the information regarding the complex puzzle of genetic factors.
involved in infectious diseases, especially leprosy. There is also a need to investigate genetic predisposition to leprosy at the genome wide level to dissect common and population specific predisposing gene(s) associated with leprosy susceptibility.