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Interferon gamma (IFNγ) & interleukin-4 (IL-4) gene variants & cytokine levels in pulmonary tuberculosis

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***Background & objectives*: Cytokine gene polymorphisms may alter Th1/Th2 balance with major implications in tuberculosis. The aim of our study was to find out whether Interferon γ +874A and IL-4 -590T polymorphisms were associated with susceptibility to pulmonary tuberculosis as well as the level of IFNγ and IL-4 in south Indian population.**

***Methods*: Interferon γ +874A and IL-4 -590T promoter polymorphisms were studied in 129 pulmonary tuberculosis (PTB) patients and 127 normal healthy subjects (NHS) and were associated with culture filtrate and live *Mycobacterium tuberculosis* induced IFNγ and IL-4 production in peripheral blood mononuclear cells (PBMCs). IL-4 gene variants were also associated with IgG antibody levels against *M. tuberculosis* culture filtrate antigen.**

***Results*: The variant IFNγ genotypes and IFNγ levels between genotypes did not differ significantly in patients and controls. Significantly increased frequency of variant IL-4 'CT' genotype in PTB patients ($P<0.05$) and 'CC' genotype in control group ($P<0.01$) was observed. IL-4 levels were detectable in very few subjects and the IgG levels did not differ between the three IL-4 genotypes.**

***Interpretation & conclusion*: The study suggests a lack of functional association of Interferon γ +874A polymorphism in tuberculosis in south Indian population. The higher frequency of IL-4 'CT' genotype in PTB suggests a possible association of IL-4 -590T promoter polymorphism with susceptibility to tuberculosis, and the 'CC' genotype may be associated with protection.**

Key words Cytokine - IFNγ - IL-4 - polymorphism - tuberculosis

Mycobacterium tuberculosis is a successful intracellular pathogen that can persist in the human host in the face of a robust immune response. Attempts have been made to identify the host genes associated with tuberculosis susceptibility to understand the host pathogen interaction. Various studies relating cytokine production with tuberculosis disease progression have shown that a preferential production of Interferon γ (IFN-γ) occurs in mild disease whereas in advanced stages, the level of Interleukin -4 (IL-4) increases with a concomitant decrease in IFNγ production[1]. Such a shift in balance between Th1 and Th2 cytokines may be

disrupted by polymorphisms in the cytokine genes and this may decide the disease outcome and progression.

Interferon γ, a Th1 type of cytokine is important in the initial protective immune response against *Mycobacterium tuberculosis* infection. Individuals defective in the genes for IFNγ or IFNγ receptor have been shown to be prone for mycobacterial infections including *M. tuberculosis*[2]. Inter-individualistic differences have been seen in the production of IFNγ and some studies have shown a depressed production of IFNγ in active tuberculosis[3,4]. An earlier study has reported a significant functional association of IFNγ + 874 A/T polymorphism with development of tuberculosis in a Spanish population[5].

Interleukin-4, a Th2 type of cytokine is generally elevated in advanced stages of tuberculosis and downregulates protective Th1 responses[6]. In progressive disease it causes increased rather than diminished immuno-pathology[7]. A single nucleotide polymorphism in the IL-4 promoter region represents a C to T transition 589 base pairs upstream of the transcription start site. This polymorphism has been shown to be associated with enhanced promoter strength and increased binding of nuclear transcription factors to the promoter and also with different levels of IL-4 activity[8] and increased IgG levels against specific antigens[9].

In the present study, we have investigated the association of single nucleotide polymorphisms in the intronic region of Interferon γ (+874 A) gene and promoter region of IL-4 gene (-590T) to study the influence of these polymorphisms in tuberculosis susceptibility in a south Indian population. The genotypes were further associated with the levels of IFNγ and IL-4 produced spontaneously and upon antigen stimulation *in vitro* to assess the influence of these polymorphisms on cytokine production.

Material & Methods

Study subjects: The study group included 129 pulmonary tuberculosis (PTB) patients (mean age ± SD: 36 ± 12.3 yr) and 127 normal healthy subjects (mean age ± SD: 30 ± 9.2 yr). Patients attending Tuberculosis Research Centre, (TRC) Chennai from January 2004 to December 2005, with respiratory symptoms and radiographic abnormalities suggestive of PTB and sputum positive for *M. tuberculosis* by both smear and culture were included. All the patients were in the initial stages of the disease. Among the 129 patients, 74 were males (mean age ± SD: 39 ± 12 yr) and 55 females (mean age ± SD: 32 ± 11.3 yr). Blood samples were collected after informed consent was obtained and before starting chemotherapy. The study had been approved by the ethical committee of the institute.

Normal healthy subjects (NHS) were volunteers who were clinically normal at the time of blood collection. The NHS group comprised 74 males (mean age ± SD: 32 ± 8.5 yr) and 53 females (mean age ± SD: 27 ± 9.4 yr). All the patients and normals were south Indians and belonged to the same ethnic origin.

Peripheral blood mononuclear cell (PBMC) separation: Twenty milliliters of venous blood was collected, defibrinated and the PBMC were separated by Ficoll - Hypaque density gradient centrifugation[10]. Cell viability was checked using trypan blue dye exclusion and the PBMCs were used for cytokine culture and DNA extraction.

In vitro cytokine production: PBMCs were cultured in sterile 48 well tissue culture plates (Costar, Cambridge, MA, USA) at a concentration of 2 million cells/ml in Rosewell Park Memorial Institute (RPMI) medium supplemented with 2 per cent autologous serum for 72 h at 37°C and 5 per cent CO2. The cultures were stimulated with live *M. tuberculosis* H37Rv, culture filtrate antigen (CFA) of *M. tuberculosis* (10 µg/ml) and phytohaemagglutinin (PHA) (Sigma, St. Louis, USA) (1 µg/ml). The culture supernatants were harvested at the end of 72 h and stored at -80°C until use. IL-4 and IFNγ levels were estimated in the supernatants using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA).

DNA extraction: DNA was extracted from a portion of lymphocytes by a salting out procedure as described earlier[11]. The concentration and purity of DNA was estimated spectrophotometrically.

Genotyping: IFNγ genotyping was done by using amplification refractory mutation system-polymerase chain reaction (PCR) method as described before [5,12]. DNA was amplified in two different PCR reactions with a generic antisense primer (5'-TCAACAAAGCTGATACTCCA-3') and one of the two allele specific sense primers [Primer A (sense) 5' TTCTTACAACACAAAATCAAATCA-3' and Primer T (sense) 5'-TTCTTACAACACAAAATCAAATCT-3']. The PCR reaction conditions were 10 cycles (95°C for 1 minute, 95°C for 15 seconds, 62°C for 50 seconds, and 72°C for 40 seconds), followed by 20 cycles (95°C for 20 seconds, 56°C for 50 seconds, and 72°C for 50 seconds). To check for the success of PCR amplification an internal control of 426 bp was amplified using a pair of primers designed from the nucleotide sequence of human growth hormone. The primer sequences for internal control were Primer 1 (sense) 5'-CCTTCCAACCATTCCCTTA-3' and Primer 2 (antisense) 5'-TCACGGATTTCTGTTGTGTTTC-3'.

IL-4 genotyping was performed as described earlier[13]. Briefly, PCR reaction was performed with specific primers and the reaction conditions were 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min. The primers amplified nucleotide sequences of -680 to -491. The forward primer sequence was 5,-GTAAGGACCTTATGGACCTGC-3' and the reverse primer sequence was 5'-CATCTTGGAAACTGTCCTGTC-3'. The PCR products were denatured and blotted onto nytran membranes. Genotyping was performed using sequence-specific oligonucleotide probing (SSOP) with eighteen-mer oligonucleotide probes with the sequence IL-4-590T, 5'-GAACATTGTTCCCCAGTG-3',; IL-4-590C, 5'-GAACATTGTCCCCCAGTG-3'.

IgG antibody estimation: The optical density (O.D.) value at 490 nm of IgG antibody (1:100, 1:500 and 1:1000 dilutions) against *M. tuberculosis* H37Rv culture filtrate antigen (5 µg/ml) was estimated in the serum samples by enzyme linked immunosorbent assay (ELISA).

Statistical analysis: Genotype frequencies of IL-4 and IFNγ genes were estimated by direct genotypic counts and expressed as percentage. Comparison between patients and controls was done using 2x2 contingency tables and χ^2 test with Yates correction (Stat calc programme, Epi Info Version 6.04, CDC, Atlanta, GA, USA). The frequencies were also tested

Table. Variant allele and genotype frequencies of IFN γ (+874A) and IL-4 (-590T) gene in normal healthy subjects (NHS) and pulmonary tuberculosis (PTB) patients

IFNγ and IL-4 polymorphisms	Allele frequencies		Genotypes	Genotype frequency (%)	
	NHS (n=127)	PTB(n=129)		NHS (n=127)	PTB (n=129)
IFNγ (+874 T/A)	A - 0.64	0.68	AA	43.3 (55)	47.2 (61)
	T - 0.36	0.36	AT	40.9 (52)	41.9 (54)
			TT	15.8 (20)	10.9 (14)
IL-4 (-590T)	C - 0.89	0.79	CC	80.3 (102)*	64.3 (83)
	T- 0.11	0.21	CT	16.5 (21)	31 (40)**
			TT	3.2 (4)	4.7 (6)

*P=0.006, **P=0.01
IFNγ - AA, homozygotes of allele 'A'; TT, homozygotes allele 'T'; AT, heterozygotes of alleles 'A' & 'T'
IL-4 - CC, homozygotes of allele 'C'; TT, homozygotes of allele 'T'; CT, heterozygotes of allele 'C' & 'T'

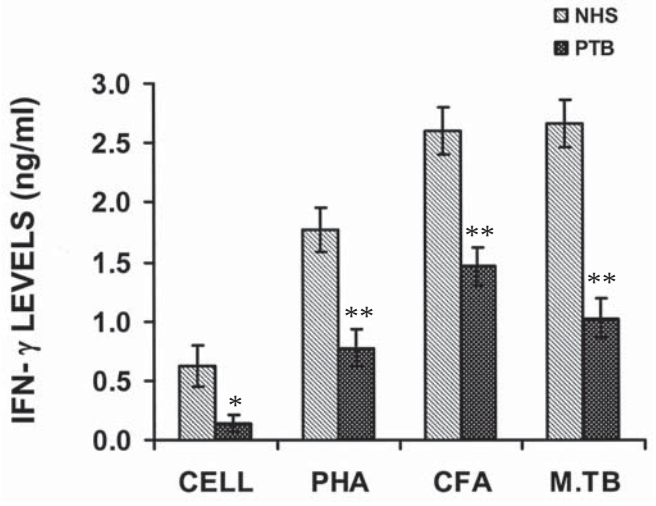

Fig. 1. Spontaneous and induced production of Interferonγ (IFNγ) by peripheral blood mononuclear cells (PBMC) *in vitro*, in normal healthy subjects (NHS) and pulmonary tuberculosis (PTB) patients. Peripheral blood mononuclear cells were cultured for 72 h to determine spontaneous IFNγ release or stimulated with phytohaemagglutinin (PHA), culture filtrate antigen (CFA) or live *M. tuberculosis* to estimate the antigen specific IFNγ release. Results are expressed as mean ± standard error (SE). Number of subjects studied in each group: NHS=59 and PTB=60. *P**<0.05, ***<0.001 compared to NHS.

for Hardy-Weinberg equilibrium by calculating allele frequencies using Pearson's χ^2 test with one degree of freedom. Results of cytokine and antibody assays were expressed as arithmetic mean ± SE and analyzed by student's t test. $P<0.05$ was considered statistically significant.

Results

The frequencies of the three IFNγ genotypes (AA, AT and TT) and the allele frequencies did not differ significantly between the PTB and NHS group. All the three genotypes conformed to Hardy Weinberg equilibrium (Table).

The spontaneous and induced IFNγ levels were significantly higher in NHS when compared to PTB patients (Cells, $P<0.05$; CFA, PHA, *M. tuberculosis* $P<0.001$) (Fig. 1). No significant difference was observed in IFNγ levels between the three IFNγ genotypes of PTB and NHS groups (Fig. 2).

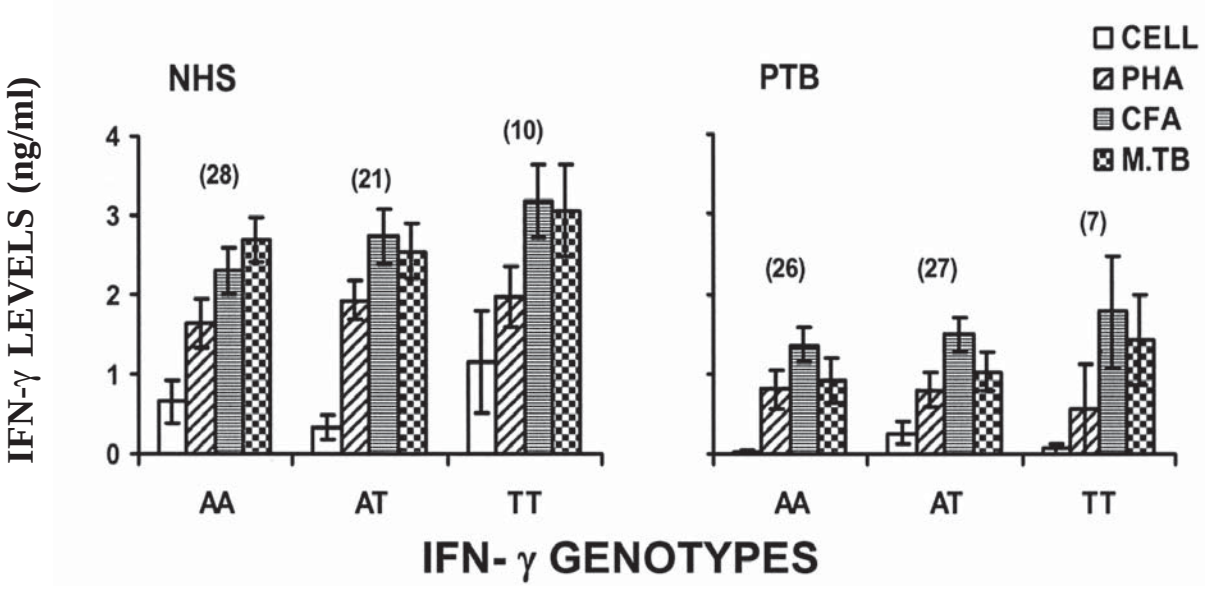

Fig. 2. Variant IFNγ genotypes and IFNγ production in NHS and PTB. PBMCs were cultured for 72 h to determine spontaneous IFNγ release or stimulated with phytohaemagglutinin (PHA), culture filtrate antigen (CFA) or live *M. tuberculosis* to estimate the antigen induced IFNγ release. Results are expressed as mean ± SE. Numbers in parentheses represent the number of subjects studied in each group.

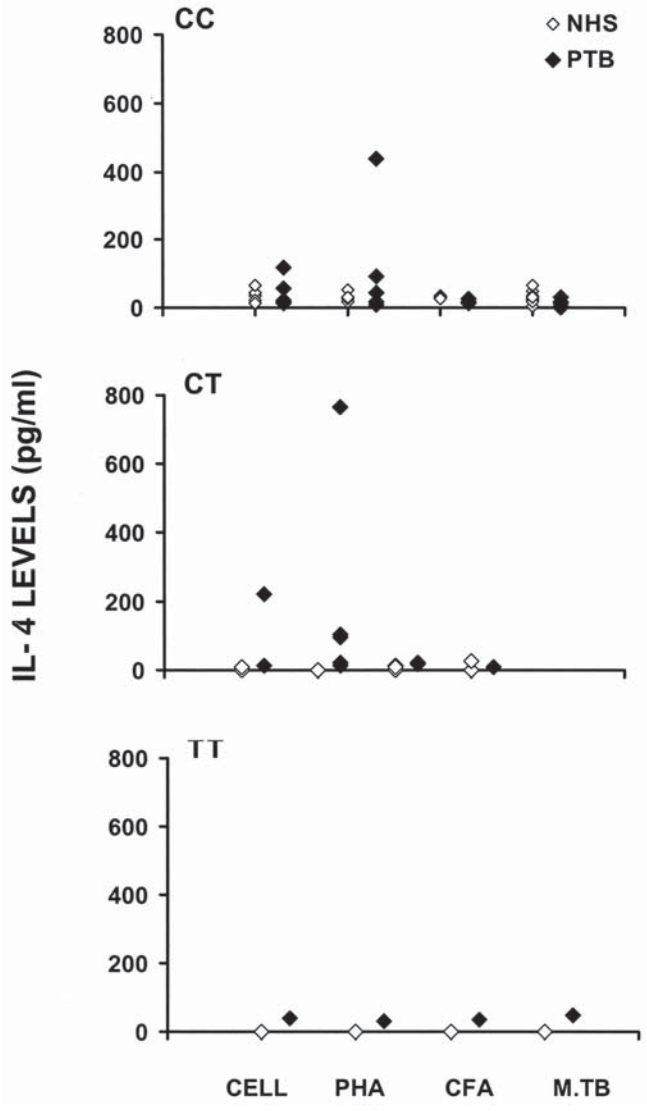

Fig. 3. IL-4 levels in NHS and PTB patients with variant IL-4 genotypes. PBMCs were cultured either with or without the mitogen and the antigens for 72 h and IL-4 level was estimated in the supernatants by ELISA. This graph represents the results only in few subjects where the levels were detectable. Number of subjects responded/total number of subjects studied for cytokine levels in each group: NHS: CC-11/68, CT-4/17 and TT-1/4; PTB: CC -8/44, CT-5/20 and TT-1/3.

Among the three variant IL-4 genotypes, the frequency of heterozygous genotype 'CT' was significantly higher in PTB patients than controls (P<0.05, odds ratio (OR) 2.3; 95% confidence interval, 1.2-4.32). Significantly higher frequencies of the genotype 'CC' were observed in controls (P<0.01, OR-0.44; 95% confidence interval, 0.24-0.81) when compared to patients. The homozygous genotype 'TT' for the rarer allele 'T' was low in numbers and did not differ much in the frequencies between the two groups. All the 3 genotypes conformed to Hardy-Weinberg equilibrium. The frequency of the 'C' allele of IL-4 was 0.79 and 0.89 and that of 'T' allele was 0.21 and 0.11 in patients and controls respectively. When the allele frequencies were compared, the frequency of allele 'T' was significantly higher in patients and that of allele 'C' was significantly higher in the control group (P<0.01) (Table).

The IL-4 levels were low or undetectable in the culture supernatants in most of the patients and control subjects. Nearly, 15-20 per cent of NHS and PTB patients were IL-4 producers to various stimulations. Individuals with variant IL-4 genotypes did not vary significantly in their IL-4 production (Fig. 3).

The IgG antibody levels against *M. tuberculosis* H37Rv CFA was significantly higher in patients (P<0.001) when compared to normals. Among the three variant IL-4 genotypes, no difference in the IgG antibody levels was observed in both patients and controls (Fig. 4).

Discussion

In the present study, single nucleotide polymorphisms in the IFNγ and IL-4 genes were associated with *in vitro* cytokine production in pulmonary tuberculosis patients and normal healthy subjects. There was no significant difference in the frequency of IFNγ genotypes between NHS and PTB, showing a lack of association of IFNγ (+874 T/A) polymorphism with tuberculosis susceptibility.

The spontaneous, mitogen and antigen induced IFNγ production was significantly higher in NHS when compared to patients. A decreased IFNγ production has been reported earlier in tuberculosis patients[3,4] and it may be due to the initial T cell anergy seen in the disease. Such an inadequate IFNγ

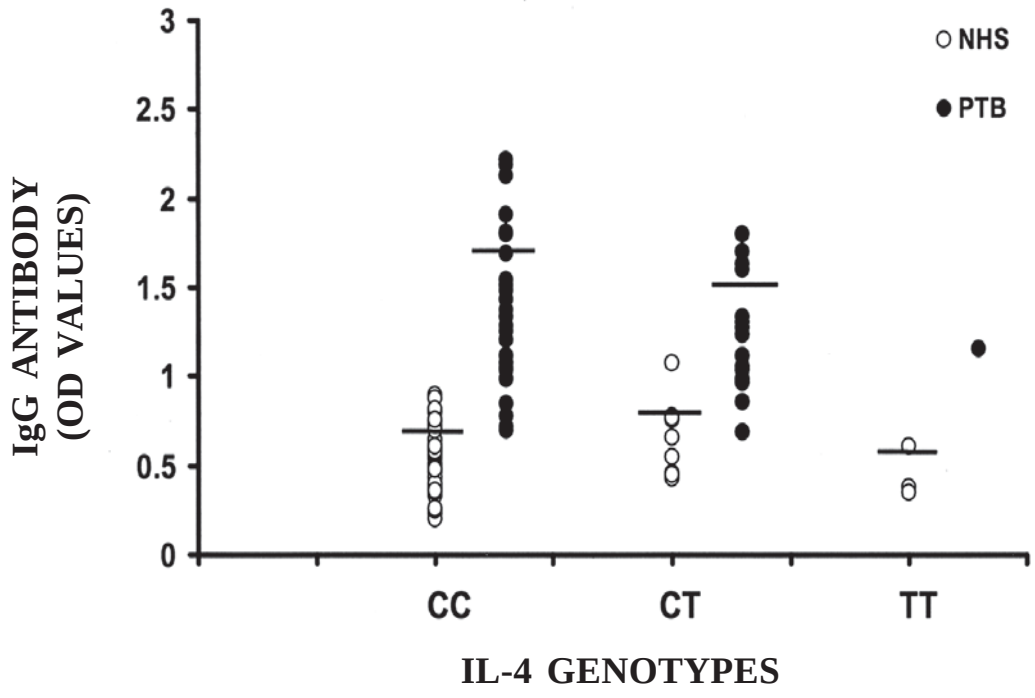

Fig. 4. The optical density (OD) values at 490 nm of IgG antibody at 1 : 1000 serum dilution against *M. tuberculosis* H37Rv culture filtrate antigen in NHS and PTB patients with variant IL-4 gene variants. The levels were also measured at 1:100 and 1:500 dilutions, but no significant difference was observed between the three genotypes in any of the given dilution. Number of subjects studied in each group: NHS: CC-53, CT-10 and TT-3; PTB: CC -27, CT-16 and TT-1.

production may result in failure of macrophage activation, which could lead to active disease progression. When the IFNγ levels were compared between the IFNγ genotypes, no significant difference could be found. On the other hand, a study by Lo´pez-Maderuelo *et al*[5] has revealed a functional association between the IFN-γ (+874 A/T) polymorphism and development of pulmonary tuberculosis in Spanish whites. In their study, patients with tuberculosis and tuberculin-reactive contacts that were homozygous for the IFNγ 874A allele were shown to produce significantly less IFN-γ than those with other allele combinations. Our results did not match with these findings and this may be due to ethnic differences in genotypic frequencies among various populations. Functional variants in the closely linked alleles of IFNγ gene may also influence the cytokine production and need to be studied.

There is substantial evidence that an IL-4 response in tuberculosis downregulates protective Th1 responses. A study by Wangoo *et al* demonstrated that a pre-existing Th2 response was sufficient to undermine the efficacy of the dominant Th1 response[14]. IL-4 can downregulate inducible nitric oxide synthase (iNOS), Toll-like receptor 2 (TLR2) and macrophage activation and this may determine whether the infection becomes latent or progressive[15-17].

A single nucleotide polymorphism at position -590 in the IL-4 promoter region has been shown to be associated with enhanced IL-4 promoter strength and altered IL-4 activity and production[9]. Such a functional polymorphism in the IL-4 gene may alter IL-4 levels and thereby influence the IL-4 dependent events which determine disease progression. Based on this hypothesis, we investigated the association and functional significance of the IL-4 -590 polymorphism in pulmonary tuberculosis.

In the present study, the genotype frequency of the heterozygous 'CT' genotype of IL-4 gene was

significantly higher in patients with a 2.3 fold risk, whereas the homozygous 'CC' genotype showed significantly higher frequency in the NHS group. Similarly, higher frequencies were found for the 'T' allele in PTB and the 'C' allele in NHS suggesting that 'T' allele may be associated with susceptibility and the 'C' allele with resistance to tuberculosis. It has been shown in an earlier study that the 'T' allele is associated with delayed acquisition of X4 variants but not disease progression in HIV patients[18].

An increased binding of transcription factors has been shown with IL-4 -590 T polymorphism[8]. This may alter the gene expression and have functional effects on the rate of cytokine production which in turn could influence disease susceptibility.

In view of this concept, we tried to associate the IL-4 genotypes with IL-4 production *in vitro*, but very few subjects responded by producing detectable levels of IL-4. The overall low IL-4 levels may be due to the low concentrations and low mRNA copy numbers at which IL-4 is active[19] or may be due to a downregulation of Th2 cell proliferation because of the Interferon γ secreted on stimulation with mycobacterial antigens.

Since IL-4 is involved in B cell proliferation and antibody production, IgG antibody levels to CFA was measured in the serum samples. The levels were higher in patients as expected, but there was not much difference between the three IL-4 genotypes studied.

In conclusion, the present study suggests a possible association of the IL-4 -590T promoter polymorphism with susceptibility to pulmonary tuberculosis. However, no association of IL-4 genotypes with IL-4 levels was seen. Interferon γ gene (+874 T/A) polymorphism neither associated with tuberculosis susceptibility nor with IFNγ secretion. A similar approach with other cytokine genes may elucidate the regulatory role played by these genes in immunity to tuberculosis.

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