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HLA-DR2 subtypes & immune responses in pulmonary tuberculosis

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Background & objectives: HLA-DR2 has been shown to be associated with the susceptibility to pulmonary tuberculosis and altered antibody and lymphocyte response in pulmonary tuberculosis. In the present study, the influence of DR2 subtypes on antibody titre and lymphocyte response ,to *Mycobacterium tuberculosis* culture filtrate antigens (10 µg/ml) was studied in 22 patients with active pulmonary TB (ATB), 50 inactive (cured) TB (ITB) patients and 36 healthy control subjects.

Methods. HLA-DR2 gene was amplified by polymerase chain reaction (PCR) and dot-blotted. Genotyping of DRBl*1501, *1502, *1503, *1601 and *1602 was carried out using sequence specific oligonucleotide probes (SSOPs) and detected by chemiluminescence method. Antibody titre as well as lymphocyte response to *M.tuberculosis* antigens were measured by enzyme linked immunosorbent assay (ELISA) and lymphocyte transformation test (LTT) respectively.

Results: The allele frequency of DRB1*15Ol was significantly increased in pulmonary tuberculosis patients as compared to controls *(P<0.05).* No marked difference in the antibody titre and lymphocyte response to *M. tuberculosis* antigens was observed between the DRBl *1501, *1502 and *1503 positive or negative controls, ATB and ITB patients. DRBl *1501 and *1502 positive as well as negative ATB patients showed a higher antibody titre as compared to controls and ITB patients. ITB patients with *1502 showed a higher lymphocyte response as compared to *1502 positive controls (P<0.001) and ATB patients (P<0.05). Similarly, an increased lymphocyte response was observed in *1501, and *I503 negative ITB patients compared to *1501 and *1503 negative controls and ATB patients.

Interpretation & conclusion: The present study revealed that DRBl *1501 may be associated either alone or with other DR2 alleles, with the susceptibility to pulmonary tuberculosis. None of the DR2 alleles influenced the antibody and lymphocyte response to *M tuberculosis* culture filtrate antigens. This suggested that HLA-DR2 gene/gene products as a whole may influence the immune response in pulmonary tuberculosis.

Key words HLA-DR2 subtypes - immune response - *Mycobacterium tuberculosis* - pulmonary tuberculosis

Genetic susceptibility in infectious diseases, especially in mycobacterial diseases is well documented[1-5]. The innate/genetic immunity is thought to play a key role in determining the direction of the adaptive immune response, especially in chronic infectious diseases in humans[6].

Studies on the host genetics in pulmonary tuberculosis by various groups in India, including ours have revealed the association of HLA-DR2 with the susceptibility to pulmonary tuberculois[2-4]. With the advances in molecular biological techniques, the subtypes of HLA-DR2 have been documented[7-10]. These studies have shown that the molecular subtypes HLA-DRBI *1501 either alone or in combination with *1502 are associated with the susceptibility to pulmonary tuberculosis in India[9,10]

Our earlier study revealed that HLA-DR2 is associated with a trend towards an augmented humoral immune response to antigens of *Mycobacterium* tuberculosis[11]. Moreover, HLA-DR2 has been shown to be associated with a low spontaneous lymphocyte response and a high responder status to *M. tuberculosis* antigens[11]. The influence of the HLA-DR2 subtypes on humoral and cell mediated immune response has not been studied in normal subjects, active tuberculosis patients and cured tuberculosis patients in a comprehensive manner. The present study was carried out to find out the HLA-DR2 subtype/subtypes that are associated with the susceptibility to pulmonary tuberculosis, as well as the influence of these subtypes on the antibody titre (humoral immune response) and lymphocyte response (an *in vitro* correlate of cell-mediated immune response) to culture filtrate antigens of *M. tuberculosis* in patients with active disease and cured patients (inactive stage of the disease).

Material & Methods

Study subjects: Subjects included were 72 pulmonary tuberculosis patients (PTB) (22 active and 50 inactive PTB) and 36 healthy controls. Among the active TB patients, 21 were males and 1 was a female, aged 37.4±2.6 yr and 25 yr respectively. Among the inactive TB patients, 38 were males and 12 were females aged 38.5±1.3 yr and 39.5±2.8 yr respectively. Of the controls, 15 were males and 21 were females, aged 36.4±2.0 yr and 36.7±1.7 yr respectively.

(i) Active pulmonary tuberculosis patients (ATB) — Patients attending the clinic at the Tuberculosis Research Centre (TRC), Chennai, with respiratory symptoms and radiographic abnormalities suggestive of pulmonary TB were studied. These patients (22) were sputum positive for *M. tuberculosis* by smear and culture. Blood was drawn before starting anti-tuberculous treatment.

(ii) Inactive tuberculosis patients (ITB) (cured patients) — These patients (50) were selected from among the subjects of an earlier chemotherapy study. Patients classified as suffering from active pulmonary tuberculosis had received supervised short course chemotherapy of 6 to 8 months duration and had been followed for five years after treatment. At the time of blood sample collection, all these cured patients were in the quiescent stage of the disease.

(iii) Controls — The controls comprised family contacts (16) (spouses who were living together with the patients before, during and after treatment for a period of 10-15 yr) and staff (20) working at TRC for more than 3 years. All the family contacts and other control subjects were clinically normal at the time of blood sample collection. The patients and the spouses were not consanguineous with each other. The other control subjects were not related to any of the patients.

The patients and the controls were randomly selected and belonged to the same ethnic group. They were Tamil speaking south Indians (belonging to different communities/castes) living in and around Chennai.

For genotype analysis, ATB and ITB patients were included as a single group as TB patients, irrespective of the stage of disease that they presented with viz., active or inactive, whereas for the immune functions ATB and ITB (cured TB) were considered separately.

This study was conducted during 1995-1998.

Processing of blood sample: About 20 ml of heparinised venous blood was collected from each individual and subjected to Ficoll-Hypaque density gradient centrifugation. The upper layer of plasma was separated and used in ELISA for measuring the

antibody titre. Peripheral blood mononuclear cells (PBMC) were collected and separated into portions and used for HLA-DR serological typing, lymphocyte transformation test (LTT)[4,11] and DNA extraction.

Extraction of human genomic DNA: Genomic DNA was extracted from PBMC using a saltinig out procedure as described elsewhere[12].

Genotyping of HLA-DR2 subtypes: DNA typing of DRB1 *1501, *1502, *1503, *1601 and *1602 HLA-DR2 subtypes was carried out using polymerase chain reaction (PCR) and sequence specific oligonucleotide Probes (SSOP)[9]. The second exon of the DRB gene was amplified using DR2 group specific primers (DRB1-DR2) in a Programmable thermalcycler (MJ Research Inc., Watertown, MA, USA). The primers used were *(i)* 5'-TTC CTG TGG CAG CCT AAG AGG-3'; and *(ii)* 5'-CCG CTG CAC TGT GAA GCT CT-3'.

Twenty five microlitres of the PCR reaction mixture containing 100ng of genomic DNA, 200 μM of dNTPs (Gibco-BRL, Grand Island, NY, USA) 20 pmol of each primer (Genei, Bangalore, India), 1 unit of Taq DNA polymerase and buffer (Genei, Bangalore, India) was used for PCR amplification using the following cycling conditions: 96°C for 30 sec (denaturation), 61°C for 30 sec (annealing) and 72°C for 60 sec (extension) for 30 cycles. The PCR product (265 base pair) was checked in a 0.8 per cent agarose (Gibco-BRL, Grand Island, NY, USA) gel containing 0.1 per cent ethidium bromide.

Biotinylated SSOPs: All the probes were biotinylated at the 5' end

(i) DRB8603-DRB1*1501:5'-Biot-AAC TAC GGG GTT GTG GAG-3'

(ii) DRB8601-DRB1*1502+*1601+*1602:5'-Biot-AAG TAC GGG GTT GGT GAG-3'

(iii) DRB 7011-DRBl* 1501+*1502:5' Biot-GAC ATC CTG GAG CAG GCG - 3'

(iv) DRB2814-DRB1*1503:5'-Biot-CTG GAC AGA CAC TTC TAT AA-3'

(v) DRB7002-DRBl*1601:5'-Biot-GAC TTC CTG GAA GAC AGG-3'

(vi) DRB 7003 - DRB1*1602:5'- Biot - GAC CTC CTG GAA GAC AGG-3'

All the probes were purchased commercially (Gibco-BRL, Grand Island, NY, USA).

Dot blotting and oligonucleotide hybridisation: The PCR product (10 µl) was denatured by adding 90 µl of freshly prepared denaturing solution [9.9 ml of Tris EDTA (ethylene diamine tetraacetic acid), *(pH* 8.0), 0.66 ml of *0.5M* EDTA *(pH* 8.0), 0.88 ml of *6M* NaOH]. The mixture was placed on ice during denaturation for 10 min, and 100 µl of *2M* ammonium acetate was added to the denaturing mixture to stop the reaction. The DNA was dot-blotted on Nytran membrane (Boehringer Mannheim GmbH, Mannheim, Germany) using a dot-blot apparatus (Gibco-BRL, Grand Island, NY, USA). The membrane was baked for 2 h at 80°C or ultra-violet (UV) cross-linked in a UV cross-linker (Bio-Rad, Hercules, CA, USA) at 250 mJ and stored at -20°C until use.

Hybridisation and stringency wash: The membrane was blocked with 10 ml blocking solution [4X sodium chloride, sodium phosphate, EDTA (SSPE), 1 per cent casein hydrolysate (SRL, Mumbai. India) and 0.1 per cent lauryl sarcosine hydrolysate (SRL, Mumbai, India)] in a hybridisation oven (Hybaid Ltd., UK) at room temperature. Prehybridisation was done with 10 ml of hybridisation solution *[3M* TMAC (tetramethylammonium chloride; SRL, Mumbai, India), 50 mM Tris *(pH8.0),* 0.1 per cent SDS (sodium dodecyl sulphate), 2m *M* EDTA] for 45 min at 55°C in the hybridisation oven.

Biotinylated SSOPs (5 pmol/ml) were added to the hybridisation solution and hybridisation was carried out overnight at 55°C. Membranes were then washed with 25 ml of wash buffer (2X SSPE, 0.1% SDS) at room temperature and stringency wash step was carried out with 10 ml of TMAC for 15 min at various temperatures as described earlier[9] (DRB 8603: 62°C; DRB 8601: 62°C; DRB 7011: 62°C; DRB 2814: 63°C; DRB7002: 62.5°C and DRB 7003: 61.5°C).

After stringency wash, the membrane was washed in wash buffer. The washed membrane was blocked

with 20 ml of blocking solution (NaCl 125 m*M*, NaH_2PO_4 17 m*M*, Na_2HPO_4, 8 m*M*, SDS 173 *mM*) for 15 min at room temperature. The signal was detected using Phototope Detection Kit 6K (New England Biolabs Inc, Beverly, USA). Blocking solution containing streptavidin (1 µg/ml) was added to the membrane (50-60 µl/cm^2) and incubated at room temperature for 20 min. The membrane was washed two times, 10 min each in wash solution-I (1 : 10 diluted blocking solution, 0.5 ml/cm^2) at room temperature.

This was followed by biotinylated alkaline phosphatase (0.5 µg/ml in blocking solution, 50 µl solution per cm^2 membrane) incubation for 20 min at room temperature. The membrane was washed twice in 20 ml of wash solution-I1 [Tris-HC1 10 *mM* (pH 9.5), NaCl 10 *mM*, $MgCl_2$ lm*M]* and drained. The membrane was treated with Lumigen-PPD substrate for 5-7 min. The membrane was kept wet in cling film and incubated at room temperature for 15 min. The chemiluminescent signal was detected on X-ray film after exposing the membrane for 15 and 30 min.

The membrane was stored until further use, at -20°C in a wet condition to ensure that it did not dry, as drying of the membrane leads to irreversible probe and DNA hybridisation.

Stripping: The membranes were washed with 20 ml of stripping buffer-1 [20m M EDTA and 2X sodium chloride, sodium citrate (SSC)] for 30 min at 65°C. The second wash was done with 20 ml of stripping buffer-I1 (2X SSC and 0.1% SDS) for 15 min at room temperature. The membranes were then washed in stripping buffer-I11 (0.2 *M* NaOH, 0.1% SDS) at 37°C for 30 min. The stripped membranes were reprobed with the other probes.

Five microgram per ml of *M. tuberculosis* H_{37}Rv culture filtrate antigen was used in ELISA to measure the IgG antibody titre in plasma samples[11,13] and 10 µg/ml concentration of antigen was used in LTT to stimulate the lymphocyte culture[11,13]. The results of these experiments were compared with the HLA-DR2 subtypes.

Statistical analysis: The frequencies of HLA-DR2 subtypes were determined using direct allelic count. The significance was analysed using X^2 test (Statcalc program, Epi Info, Version-5; USD; Stone Mountain, GA, USA). The results of antibody titre and lymphocyte response are expressed as mean ± standard error (SE) and the significance was assessed using Student's 't' test. Multiple comparison with analysis of variance (ANOVA) was done using Microsoft Excel, version 6.0 (Microsoft Corporation, Seattle, WA, USA).

Results

No marked difference in the antibody titre was observed between DR2 subtypes *1501, *1502 and *1503 positive and negative control subjects, ATB and ITB patients. However, a decreased antibody titre was seen in *1503 positive ATB patients than *1503 negative patients. An increased antibody titre to *M. tuberculosis* culture filtrate antigens was seen in DRB1 *1501 and *1502 positive or negative ATB patients when compared to *1501 and *1502 positive or negative control subjects as well as ITB patients (Fig. 1) (Control vs ATB-*1502 positive-$P<0.01$; *1503 negative-$P<0.02$; ATB vs ITB-*1501 and *1502 positive and *1503 negative - $P<0.05$, other comparisons were not significant). Further analysis using ANOVA between antibody titres of controls, ATB patients and ITB patients was significant ($P<0.001$). However, comparisons of the three subtypes (DRB1 *1501, *1502 & *1503) within the three categories of study subjects were not significant.

A significantly increased allele frequency of DRB1-*1501 was seen in pulmonary TB patients as compared to control subjects ($P<0.05$) (Table). However, the corrected *P* value for the number of DR2 alleles studied was not significant ($P_c>0.05$). A decreased frequency of alleles *1502 and *1503 was observed in patients when compared to control subjects. However, this difference was not significant. The frequencies of *1601 and *1602 were less or almost nil when compared to *1501. *1502 and *1503 in both controls and patients (Table).

No difference in the lymphocyte response to *M. tuberculosis* culture filtrate antigens was observed between *1501, *1502 and *1503 positive and negative control subjects, ATB and ITB patients.

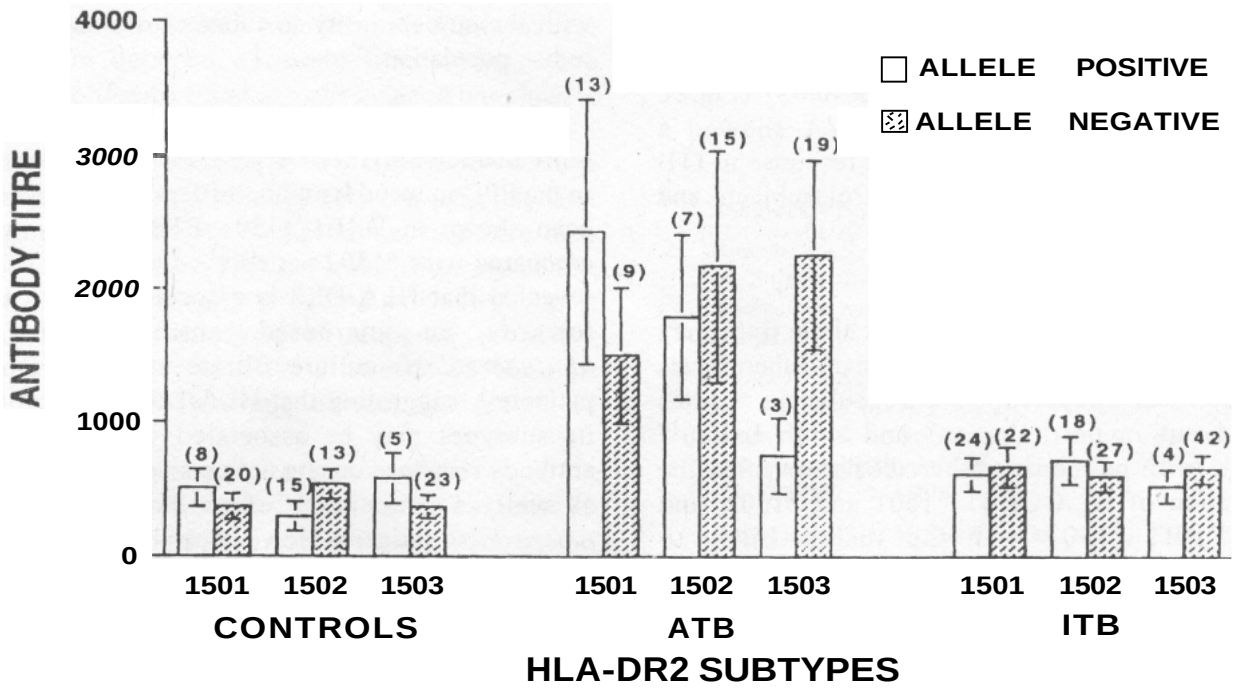

Fig. 1. Comparison of antibody titre to *M. tuberculosis* H_{37} Rv culture filtrate antigens in subjects positive for various HLA-DR2 alleles among controls, ATB and ITB patients. Control vs ATB - *1502 - *P<0.01;* ATB vs ITB - *1501 - *P<0.05*, *1502 - *P<0.05*. Numbers in parentheses represent the number of subjects studied in each group.

Table. Allele frequency of HLA-DR2 subtypes in pulmonary TB patients and control subjects

HLA-DR2 subtype	Allele frequency (%)			
	Control (N=36)		TB (N=72*)	
	n	%	n	%
1501	11	30.5†	41	53.2†
1502	19	52.8	27	35.1
1503	6	16.7	7	9.1
160l	0	0	0	0
1602	0	0	2	2.6

n, no. of subjects positive for each allele; N, total number of subjects studied; * Five patients were homozygous for DR2 and their genotypes were 1502/1503, 1501/1502, 1502/1602, 1502/1502 and 150211503; †Odds ratio – 2.6; *P < 0.05; Pc* >0.05

An increased lymphocyte response was seen in ITB patients with *1501 and *1502 positive and negative subjects (as well as *1503 negative ITB patients) when compared to control subjects and ATB patients. A significantly increased antigen induced lymphocyte response was seen in ITB patients with *1502 as compared to *1502 positive control subjects (P<0.00l) as well as ATB patients with *1502 allele *(P<0.05)*. Similarly, *1501 negative and *1503 negative ITB patients showed a higher lymphocyte response than *1501 and *1503 negative control subjects and ATB patients (control vs ITB- *1501

negative - *P < 0.05;* control vs ITB *1503 negative - *P<0.008;* ATB vs ITB - *1501 negative - *P<0.02;* ATB vs ITB- *1503 negative - *P<0.01)* (Fig.2). Multiple comparisons with ANOVA showed a significantly increased lymphocyte response in ITB patients than *1501 negative control subjects and *1501 negative ATB patients *(P<0.001).*

Discussion

In the present study an increased allele frequency of DRB1 *1501 was seen in pulmonary tuberculosis patients when compared to control subjects. Studies carried out in north Indian[9] and south Indian[10] patients with pulmonary tuberculosis, revealed the association of HLA-DRBl *1501 and *1502, and HLA-DRB1 *1501 with the susceptibility to pulmonary tuberculosis. Our study also reveals the association of DRB1 *1501 with the susceptibility to pulmonary tuberculosis in south India. This suggests that allele DRBl *1501 may either alone or in combination with other DR2 alleles be associated with the susceptibility to tuberculosis in our south Indian population.

High tuberculin response has been shown in individuals with HLA-DR15[14]. Further, an increase in the PPD induced lymphoproliferative response has been shown in DRBl *1501 PTB patients, when compared with *1502 patient[15]. Our earlier studies revealed that HLA-DR2 is associated with a trend towards an increased antibody titre to *M. tuberculosis* culture filtrate antigens in ATB patients[''], suggesting that HLA-DR2 as a whole or its subtypes may be associated with augmented antibody response during active stage of the disease as well as persistence of antibody titre to *M. tuberculosis* antigens. However, in the present study the DR2 alleles (subtypes) did not influence the antibody as well as lymphocyte response to *M. tuberculosis* antigens independently. This suggests that HLA-DR2 as a whole may Influence the immunity to tuberculosis.

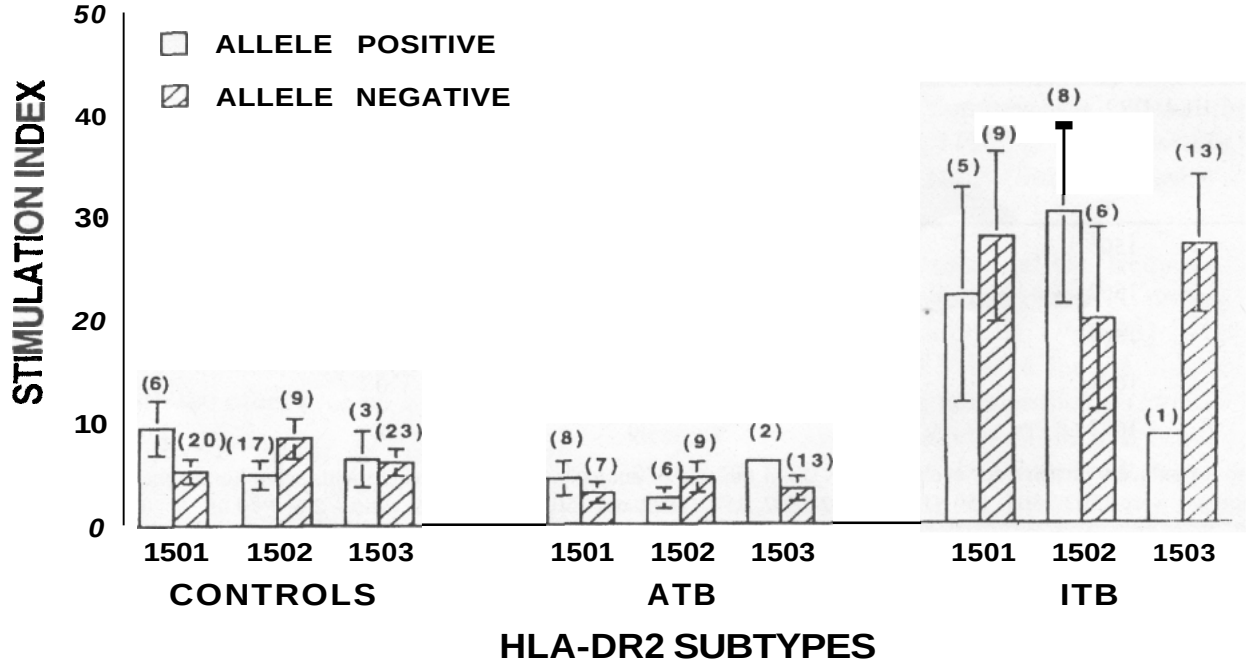

Fig. 2. Comparison of proliferative responses against *M. tuberculosis* $H_{37}Rv$ culture filtrate antigens among the controls, ATB and ITB patients positive for the different HLA-DR2 alleles. Controls vs ITB - *I502 - P<0.01; ATB vs ITB - *1502 - *P<0.05.* Numbers studied in each group are represented in parentheses.

Our earlier studies also revealed an inverse correlation between antibody titre, spontaneous lymphoproliferative and antigen induced lymphocyte response in HLA-DR2 positive active TB patients[11]. In the present study also, irrespective of the disease status an inverse correlation between antibody titre and antigen induced lymphocyte response was observed with various DR2 alleles. This may be due to a shift from an early, predominantly Th1 type of response to a Th2 type of response in active pulmonary tuberculosis and a reversal of Th2 type of response to Th1 type of response in cured patients.

Moreover, an increased lymphocyte response to *M. tuberculosis* antigens was seen in DRBl *1501, *1502 and *1503 positive as well as negative ITB patients as compared to ATB patients and control subjects, suggesting that ITB patients may regain the immune status and retain a strong memory T cell response (irrespective of whether they are positive or negative for the allele) as well as altered Thl and Th2 type of response to *M. tuberculosis*. Irrespective of HLA-DR status, (DR2 positive or DR2 negative) altered level of cytokines released by different subsets of T helper cells and/or other factors that are released by the monocytes and/or lymphocytes during the active stage of the disease may be responsible for the low lymphocyte response in active TB patients. Such suppressive factors[16,17] may be released both by the host as well as the pathogen – *M. tuberculosis* during the active stage of the disease. Such factors may not be produced in cured patients, which in turn may be associated with increased lymphocyte response to *M. tuberculosis.*

The present study suggests that DRBl *1501 allele either alone or with DRBl *1502 or HLA-DR2 as a whole along with other HLA or non-HLA genes may be associated with the susceptibility to tuberculosis and regulate the immune responses in pulmonary tuberculosis irrespective of the disease status.

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