

Mycobacterium bovis BCG Scar Status and HLA Class II Alleles Influence Purified Protein Derivative-Specific T-Cell Receptor V β Expression in Pulmonary Tuberculosis Patients from Southern India

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Received 12 September 2002/Returned for modification 19 February 2003/Accepted 29 April 2003

Purified protein derivative (PPD) RT23-recalled T-cell receptor (TCR) V β expression was studied in the peripheral blood of 42 pulmonary tuberculosis patients and 44 healthy controls from southern India, a region where tuberculosis is endemic. Forty-eight-hour whole-blood cultures in the presence or absence of PPD-RT23 were set up, and at the end of the culture period total RNA was extracted and cDNA was synthesized. Expression of various TCR V β families was assessed by using family-specific primers. PPD-specific expression (usage) of TCR V β families 4, 6, 8 to 12, and 14 was found in more controls than patients. Among the responders (individuals who showed PPD-specific expression), endemic controls had significantly higher responses than the patients had for TCR V β families 2, 3, 7, 13, and 17. The majority of the patients did not show usage of most of the TCR V β families, and this was attributed to T-cell downregulation. A four-way nested classification analysis revealed that TCR V β family 1, 5, 9, 12, and 13 usage in the context of HLA class II high-risk alleles (DRB1*1501, DRB1*08, and DQB1*0601) and *Mycobacterium bovis* BCG scar status were the determining factors in susceptibility and resistance to tuberculosis. The healthier status of controls was attributed to the wider usage of many TCR V β families readily recalled by PPD, while the disease status of the patients was attributed to TCR V β downregulation and the resultant T-cell (memory cell?) unresponsiveness. Host genetics (HLA status) and BCG vaccination (scar status) seem to play important roles in skewing the immune response in adult susceptibility to pulmonary tuberculosis through TCR V β usage.

Pulmonary tuberculosis, a major infectious disease in the world, was declared a global emergency in 1993 (36). The emergence of multi-drug-resistant *Mycobacterium tuberculosis*, overcrowded, poor living conditions, and human immunodeficiency virus coinfection have made this a serious health problem in India and other developing countries. The genetic hypothesis that not all individuals infected with the tuberculosis organism develop the disease requires further research (17, 21a). Tuberculosis is a paradigm of conflicts of several factors, including genetics, immunopathology, epidemiology, and host and pathogen diversity (11). Investigating the relative contributions of these factors should lead to better intervention strategies. A major constraint for such analyses is the lack of availability of necessary readouts to evaluate these parameters in the same cohort.

Studies in southern India have identified the high-risk alleles HLA DR2 (DRB1*1501) and DQB1*0601 as predisposing factors for sputum-positive, far-advanced pulmonary tubercu-

losis (4, 24, 27). Furthermore, expression of the Th2 cytokines interleukin-10 (IL-10) and IL-4 is associated with *Mycobacterium bovis* BCG scar-negative non-DR2 status in patients (7). It has also been demonstrated that there is a spectrum of immune reactivity (delayed-type hypersensitivity versus serum antibodies) in hospital contacts and healthy individuals in southern India, and this finding has been confirmed in other countries where tuberculosis is highly endemic, such as Indonesia and Brazil (3, 12, 22). However, we are unaware of the epitope specificities of the responses.

Immunologists work under the premise that the epitopes responsible for disease may be different from those affording protection. Thus, the *M. tuberculosis* epitopes recognized by patients might be different from those recognized by healthy adult controls (immune individuals living in an endemic region). If this is true, the patients must differ from endemic controls in terms of T-cell receptor (TCR) usage for mycobacterial antigens. We should then be in a position to recall this memory in vitro by using a wider antigen, such as the purified protein derivative (PPD) routinely used in skin tests to evaluate sensitization or infection. By restricting the antigen we may not recall global memory (with all the T cells recognizing various epitopes of *M. tuberculosis*) and may not identify the difference between patients and controls.

An important step in an adaptive immune response is recognition of the peptide major histocompatibility complex by the TCR (8, 25, 37). The TCR repertoire of an individual is the

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outcome of thymic education in the context of the host major histocompatibility complex. Certain TCR Vβ families are more common in individuals with certain HLA class I and class II molecules (repertoire) (28), and HLA identical siblings are more similar in terms of their TCR repertoires than HLA nonidentical siblings (1). The repertoire is also influenced postnatally by environmental exposure to specific and cross-reacting antigens (23). The TCR repertoire of an individual is the memory of experiences of the immune system, including exposure to infectious and innocuous antigens. Thus, it should be possible to identify the immunological memory for a particular antigen by studying TCR Vβ expression in the presence and absence of the antigen *in vitro*.

In this paper we describe the differences between endemic controls and patients (disease status) in terms of PPD-recalled TCR Vβ expression. We found a correlation among HLA class II high-risk alleles, BCG scar status, and PPD-specific TCR Vβ usage.

MATERIALS AND METHODS

Study population. (i) Pulmonary tuberculosis patients. A total of 42 adult pulmonary tuberculosis patients, who were born and brought up in Madurai District in southern India, were enrolled from a state-run public government hospital in Singampunari, Madurai. Ethical clearance from the institutional ethical committee was obtained for the study, and sampling was performed with informed consent. The male/female ratio of the patients was 24:18, and the mean age was 34.7 ± 2.2 years. All the patients showed symptoms of pulmonary tuberculosis with radiological lesions and a compatible clinical picture. The patients were treated with ethambutol, isoniazid, rifampin, and pyrazinamide by following the directly observed therapy protocol implemented in India. The Blood samples were collected during weekly visits to the hospital, and at the time of sampling patients had been treated for various lengths of time (see Table 3).

(ii) Healthy controls. Forty-four healthy endemic controls, all students and staff of Madurai Kamaraj University living in Madurai District, were enrolled for the study. None of these controls had any previous history or symptoms of pulmonary tuberculosis. Their mean age was 26.6 ± 0.76 years, and the male/female ratio was 26:18.

HLA class II typing. HLA DRB1* and DQB1* alleles of the patients and controls were studied by the PCR-sequence-specific oligoprobe method by using primers and probes from the 11th International Histocompatibility Workshop and Conference and the 12th International Histocompatibility Workshop and Conference (2, 18).

Whole-blood cultures. Samples (3 to 5 ml) of peripheral blood were obtained from the patients and controls in heparin vacutainers (455 051; Greiner, Frickenhausen, Germany) and were transferred to the laboratory. Whole-blood cultures were set up on the same day (7). Briefly, 50 µl of whole blood, diluted to a volume of 200 µl with RPMI 1640 medium (31870-025; Life Technologies, Gibco-BRL, Gaithersburg, Md.) and supplemented with 2 mM glutamine (G-1517; Sigma, St. Louis, Mo.), was cultured in quadruplicate for 48 h with or without 20 U of PPD-RT23 in a CO₂ incubator at 37°C in the presence of 5% CO₂ with 95% humidity. After 48 h, the cultures were harvested, and two of the quadruplicate samples were pooled to obtain two aliquots, washed by centrifugation, and frozen at -70°C in lysis buffer.

RNA extraction and cDNA synthesis. Total RNA was extracted from one aliquot of the duplicate aliquots of each sample. RNA was extracted by a single-step acid-phenol-chloroform extraction method (6), and all of the glassware and all of the plastic ware were treated with 0.1% diethyl pyrocarbonate (BDH Laboratory Supplies, Poole, United Kingdom). The RNA was primed with 1 µg of oligo(dT) primer (12- to 18-mer; 27-7858-02; Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom) at 65°C for 10 min and cooled immediately on ice. cDNA was synthesized by using Moloney murine leukemia virus reverse transcriptase (E70456Y; Amersham Pharmacia Biotech) in a block heater at 37°C (9), diluted to a volume of 100 µl with diethyl pyrocarbonate water, and stored frozen.

PCR for TCR Vβ genes. TCR Vβ family-specific primers were synthesized by using the primer sequences described by Hawes et al. (16) and Struyk et al. (32) (Table 1). A total of 24 TCR Vβ family-specific 5' primers, one TCR constant-region (TCRC) 5' primer, and one constant-region 3' primer were synthesized by

TABLE 1. Primers used to amplify various TCR Vβ families and TCRC^a

Primer	Sequence
Forward primers	
TCRBV1.....5'	AAGAGAGAGCAAAGGAAACATTCTTGAAC 3'
TCRBV2.....5'	GCTCCAAGGCCACATACGAGCAAGGCGTCG 3'
TCRBV3.....5'	AAAATGAAAGAAAAGGAGATATTCCTGAG 3'
TCRBV4.....5'	CTGAGGCCACATATGAGAGTGGATTGTCA 3'
TCRBV5A.....5'	ATACTTCAGTGAGACACAGAGAAAC3'
TCRBV5B.....5'	TTCCCTAACTATAGCTCTGAGCTG3'
TCRBV6.....5'	CTCAGGTGTGATCCAATTC3'
TCRBV7.....5'	ATAAATGAAAGTGTGCCAAGTCGCTTCTCS 3'
TCRBV8.....5'	AACGTTCCGATAGATGATTCAGGGATGCC 3'
TCRBV9.....5'	CATTATAAATGAAACAGTTCCAAATCGCTT 3'
TCRBV10.....5'	CTTATTCAGAAAGCAGAAATAATCAATGAG 3'
TCRBV11.....5'	TCCACAGAGAAGGGAGATCTTCTCTGAG 3'
TCRBV12.....5'	CTGAGATGTCACAGACTGAGAACCACCGC 3'
TCRBV13.....5'	CAAGGAGAAGTCCCAAT3'
TCRBV14.....5'	GTGACTGATAAGGGAGATGTTCTCGAAGGG 3'
TCRBV15.....5'	GATATAAACAAAGGAGAGATCTCTGATGGA 3'
TCRBV16.....5'	CATGATAATCTTTATCGACGTGTTATGGGA 3'
TCRBV17.....5'	GATATAAACAAAGGAGAGATCTCTGATGGA3'
TCRBV18.....5'	CATCTGTCTTCTGGGGGCGAGTCTCTCAA 3'
TCRBV19.....5'	GCACAAGAAGCGATTCTCATCTCAATGCC3'
TCRBV20.....5'	TCTAATATTCATCAATGGCCAGCGACCCT 3'
TCRBV21.....5'	GATTCACAGTTGCCCTAAGGAT 3'
TCRBV22.....5'	ATGCAGAGCGATAAAGGAAG 3'
TCRBV23.....5'	ATCTCAGAGAAGTCTGAAAT 3'
TCRBV24.....5'	GATTTTAAACATGAAGCAGA 3'
5'TCRBC.....5'	CCGAGGTCGCTGTGTTGAGCCAT 3'
Reverse primer	
3'TCRBC ^b5'	CTCTTGACCATGGCCATC 3'

^a Data from references 16 and 32.

^b 3'TCRBC was used as the reverse primer in all 25 reactions along with a TCR Vβ family-specific or constant region 5' primer. There were two forward primers for TCR Vβ family 5 (TCRBV5A and TCRBV5B).

Genosys, Parnisford, Cambridgeshire, United Kingdom. The 3' and 5' constant-region primers were used to amplify the TCRC. The 3' TCRC primer was used with one of the TCR Vβ family-specific 5' primers to amplify and identify expression of a given TCR Vβ family. Five-microliter portions of the primer pairs were predotted in 96-well plates, stored frozen at -70°C, and used within 1 week of dotting. The PCR mixture (total volume, 20 µl) was dispensed into each well. Each reaction mixture contained 2 µl of cDNA template, 0.5 U of *Taq* DNA polymerase, each primer at a concentration of 0.2 µM, each deoxynucleoside triphosphate at a concentration of 0.4 mM, 4 mM MgCl₂, and 1× PCR buffer. Twenty-five PCRs were performed for each sample with a Hybaid thermal cycler (OmniGene HBTR3CM; Hybaid Ltd., Middlesex, United Kingdom). The temperature profile was as follows: initial denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 1 min, annealing at 54.5°C for 1 min, and extension at 72°C for 1.5 min; and then a final extension at 72°C for 10 min. The amplified products were electrophoresed at 100 V for 30 min in a 1.5% agarose gel with ethidium bromide. The gels were observed under UV illumination and documented by using a Kodak Digital Science gel documentation and analysis system (Kodak ds EDAS 120; Eastman Kodak Company, Rochester, N.Y.). The TCR Vβ products were around 700 bp long, and the TCRC product was 300 bp long (Fig. 1). The band intensities were measured in pixels by using the Kodak Digital Science one-dimensional image analysis software.

Quality control measures. To avoid inter- and intraexperimental variations, RNA extraction and cDNA synthesis for a batch of 10 samples (in the presence and absence of PPD; 20 cDNAs) were performed simultaneously. The PCR, electrophoresis, and measurement of the TCR Vβ and TCRC band intensities (in pixels) were all performed simultaneously. The TCRC amplicon served as an amplification control. The net intensities of the TCR Vβ bands in no-antigen control and PPD-induced cultures were normalized to the TCRC band intensity of each sample and expressed as percentages of the TCRC band intensity. The antigen (PPD)-specific TCR Vβ expression was obtained by subtracting the percentage of the TCRC band intensity for the no-antigen control from the percentage of the TCRC band intensity for the PPD-stimulated cultures. A sample was considered positive for a TCR Vβ family if the antigen-specific TCR Vβ expression was more than 5% of the TCRC band intensity.

Pilot experiments with whole-blood cultures in two control samples set up in quadruplicate and stimulated with phytohemagglutinin (PHA), PPD, or nothing revealed that PHA induced expression of all TCR Vβ families except families 21,

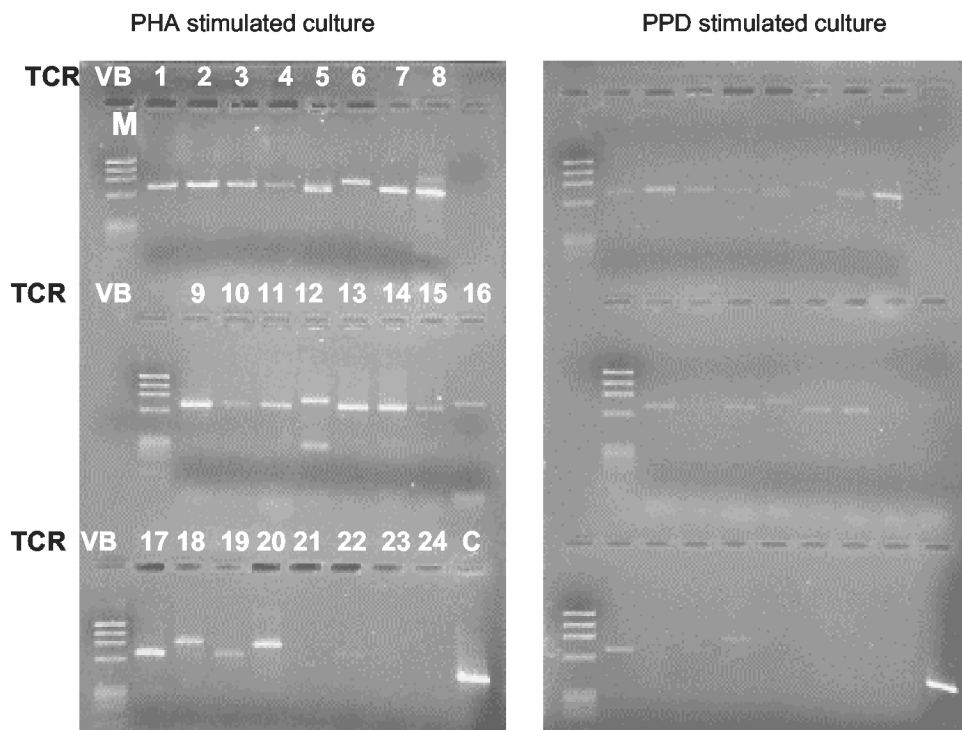


FIG. 1. Agarose gels showing expression of various TCR V β families in PHA- or PPD-stimulated, 48-h whole-blood cultures from a healthy control. M, marker ϕ X-HaeIII.

23, and 24 in both donors. The failure with families 21, 23, and 24 was attributed to PCR failure due to primers under the conditions used, and hence these three TCR V β families were not tested in further experiments. To test the reproducibility of the assay, whole-blood cultures of 10 samples were set up in quadruplicate in the presence of PPD, two cultures were pooled, and the two corresponding cDNAs were synthesized. Expression of TCRC and TCR V β families was studied in both cDNAs, and the concordance in TCR V β expression in the two aliquots of the 10 samples was analyzed. Except for TCR V β families 5, 11, 14, 18, and 19, all of the TCR V β families showed more than 80% concordance. The lower concordance in some of the TCR V β families may have been due to difficult primers under the conditions used. The quantitative expression data for various TCR V β families obtained with the two cDNA aliquots of the 10 samples were concordant. Repeat assays for 11 TCR V β families gave correlation coefficients of >0.8 ($P = 0.003$ to $P = 0.0001$), and repeat assays for six TCR V β families gave correlation coefficients between 0.7 and 0.8 ($P = 0.02$ to $P = 0.006$). In another set of experiments, PCRs with two different samples for TCRC and V β were performed twice on two different days. Although some of the data were outside the 5% confidence interval, there was a good correlation between the repeat assays (Fig. 2) ($R = 0.602$, $P = 0.003$; $R = 0.739$, $P < 0.0001$). The results indicated that the methodological approach employed in this study was sufficient to interpret the results obtained.

Statistical analyses. A paired t test, a Wilcoxon signed rank test, a Mann-Whitney test, and a chi-square test were performed when necessary (29). Log-linear analyses were performed by using BMPD statistical software (13).

RESULTS

Forty-four endemic controls and 42 pulmonary tuberculosis patients were studied for TCR V β expression in 48-h cultures that were stimulated with PPD or without PPD (no-antigen control). TCRC was expressed in all samples, and the expression was significantly higher in PPD-stimulated cultures than in the no-antigen controls ($P < 0.0001$). It is for this reason that the expression data were normalized to the corresponding TCRC data (see Materials and Methods). The TCR V β fam-

ilies expressed in the no-antigen control cultures represented the repertoire, and the difference between the expression in the presence of the antigen and the expression in the absence of the antigen represented the usage.

Comparison of TCR V β expression in controls and patients.

The numbers of controls and patients expressing various TCR V β families in no-antigen controls were compared (Fig. 3A). We found that 5 to 50% of the samples expressed at least one of the TCR V β families and that the number of individuals expressing a particular TCR V β family did not differ significantly between controls and patients except for TCR V β family 17 ($P = 0.048$) (Fig. 3A). In the presence of PPD as many as 65% of the controls expressed selected TCR V β families. Furthermore, more controls than patients expressed many TCR V β families; eight TCR V β families (TCR V β families 4, 6, 8 to 12, and 14) were used by more controls than patients (Fig. 3B).

A quantitative analysis of the PPD-specific TCR V β expression data in responders (the individuals who used a TCR V β family) was also performed (data not shown). The TCR V β expression levels were significantly higher in responding controls than in responding patients for TCR V β families 2, 3, 7, 13, and 17 (Mann-Whitney test: $P = 0.05$, $P = 0.019$, $P = 0.027$, $P = 0.021$, and $P = 0.037$, respectively).

Thus, there could be two different mechanisms of TCR involvement in protection or the disease process. First, PPD-specific expression of TCR V β families in more controls than patients indicated that there was antigen-specific immune recognition of selected epitopes in protection. Second, the higher level of selected TCR V β family expression by controls indi-

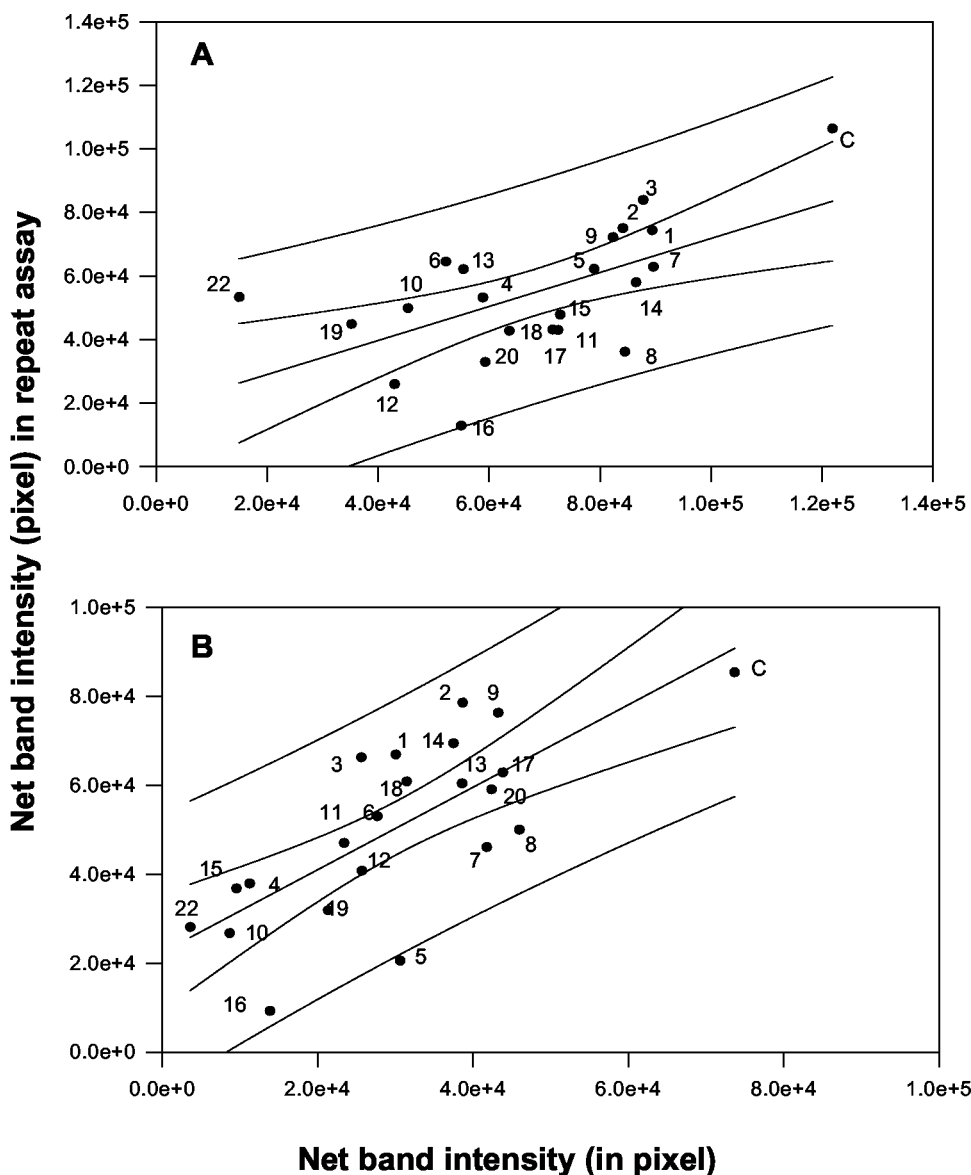


FIG. 2. Concordance of repeat assays of TCR Vβ expression in response to PHA in two different individuals (A and B). Whole-blood cultures were set up in quadruplicate with 0.4 μg of PHA and harvested after 48 h by pooling two wells. RNAs were extracted, cDNAs were synthesized from both aliquots and pooled, and PCRs for TCR Vβ families were performed by using TCR Vβ family-specific primers twice on different days. The net intensities for the repeat experiments (in pixels) are compared. The numbers near the data points indicate TCR Vβ families. The regression line, 95% confidence interval, and prediction interval are indicated. C, TCR constant region.

cated that there was adaptive immunity and immunological memory in the controls. The absence of these phenomena in patients may indicate that there was TCR Vβ downregulation due to antigenic load (25) and, presumably, restricted TCR Vβ usage in diseased patients.

The observation described above was further supported by a comparison of TCR Vβ expression in the absence and in the presence of antigens in controls and patients. Figure 3 shows that more controls (12 to 26 of the 44 controls) expressed TCR Vβ families 1, 5, 6, 9, 10, 11, 13, 14, and/or 18 in the presence of antigen (PPD-specific expression) than in the absence of antigen (1 to 12 of the 44 controls) ($P = 0.039$, $P = 0.009$, $P = 0.021$, $P = 0.01$, $P = 0.019$, $P = 0.027$, $P = 0.016$, $P = 0.005$, and $P = 0.013$, respectively). However, in patients only TCR

Vβ family 2 showed significant usage (12 and 26 of 42 patients; $P = 0.004$).

Correlation among HLA high-risk allele status, BCG scar status, and PPD-specific TCR Vβ expression. Previous studies in our laboratory showed that there is a high-risk association of HLA DRB1*1501, DRB1*08, and DQB1*0601 with pulmonary tuberculosis (24). Furthermore, cytokine IL-4 expression and IL-10 expression were also associated with disease in non-DRB1*02 patients who were not vaccinated with BCG (7). The TCR Vβ expression data presented here were therefore analyzed in the context of the BCG scar status and HLA high-risk allele status.

Tables 2 and 3 show the control and patient samples studied, HLA DRB1 and DQB1 allele data, BCG scar status, PPD-

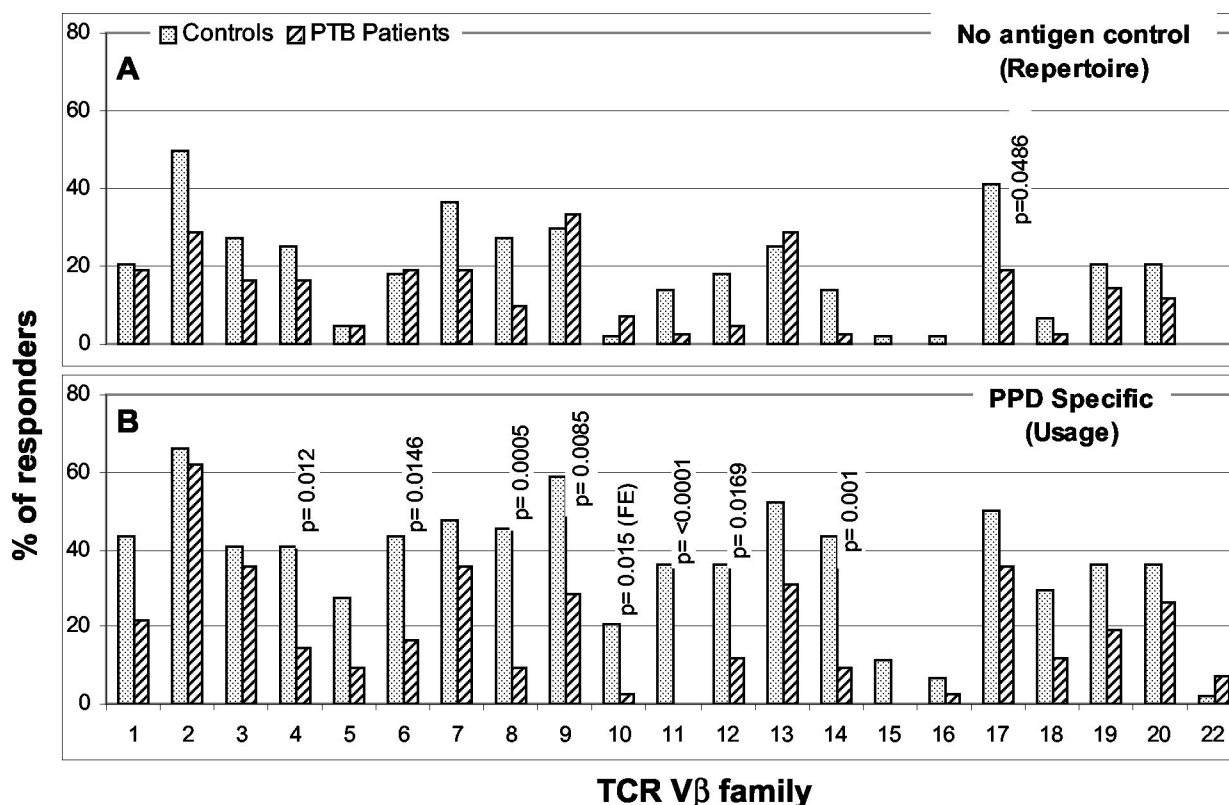


FIG. 3. TCR V β responders in healthy controls and pulmonary tuberculosis patients. The data indicate the percentage of responders in each TCR V β family. (A) No-antigen control; (B) antigen-specific usage. *P* values were obtained based on a chi-square analysis with the Yates correction.

specific expression of various TCR V β families, and cytokine expression data. The control and patient samples were divided into four groups, based on high-risk allele status and BCG scar status. The number of individuals responding (>5% of TCRC expressed) to a particular TCR V β family in a group was determined and defined as the number of responders. The total number of TCR V β families used by each individual was also determined. Among the controls, the total number of responders and the total number of TCR V β families used were more evenly distributed in all four groups (Table 2). Nonetheless, among the patients, the majority of the responders (9 of 11 responders) expressing more than five TCR V β families were in the BCG scar-negative high-risk allele group (Table 3). Most of the patients in the other three groups did not respond; only 5 of 25 patients expressed more than five TCR V β families.

In order to determine whether chemotherapy has any effect on TCR unresponsiveness in patients, we compared the number of TCR V β families expressed in patients treated for ≤ 50 days ($n = 19$) and the number of TCR V β families expressed in patients treated for > 50 days ($n = 14$). This analysis revealed that there was recovery from TCR V β unresponsiveness in the BCG scar-negative HLA high-risk allele carrier patient group following treatment. Of the 15 patients in this group, 7 were in the group that was treated for ≤ 50 days and expressed five or fewer TCR V β families, 3 were in the group that was treated for ≤ 50 days and expressed more than five TCR V β families, none was in the group that was treated for > 50 days

and expressed five or fewer TCR V β families, and 5 were in the group that was treated for > 50 days and expressed more than five TCR V β families ($P = 0.0256$, as determined by the Fischer exact test). The cytokine data did not exhibit any relationship to the TCR V β families expressed.

Nested classification analysis of HLA status, BCG status, TCR V β expression, and disease status. The interactions among the four parameters in question (viz., disease status [D] [healthy versus pulmonary tuberculosis], HLA status [H] [high-risk allele carriers versus non-high-risk allele carriers], BCG scar status [B] [positive versus negative], and TCR V β family usage status [T] [positive versus negative]) were assessed in a nested classification analysis (13). The number of individuals expressing a particular TCR V β family in a group of controls and patients was defined as the number of responders in Tables 2 and 3 and used for the analysis.

All possible log-linear models were considered and tested for significance. Four models fit the observed data well (Table 4). TCR V β families 1, 5, 9, 12, and 13 fit model 1 (TH, TD) well (i.e., interaction of the TCR V β families with HLA status and with disease status). TCR V β families 4, 6, 8, 10, 11, 14, and 18 fit model 2 (TD, H, B) well, implying that there were interactions between the TCR V β families and disease status, which were independent of HLA status and BCG scar status. TCR V β families 7 and 19 fit model 3 (TH, B, D) well (i.e., interaction between the TCR V β families and HLA status, independent of BCG status and disease status). TCR V β families 2, 3, 15, 16, 17, 20, and 22 fit model 4 (T, H, BD) well (i.e.,

TABLE 3. HLA class II alleles, BCG scar status, sputum status, treatment duration, PPD-specific expression of various TCR Vβ families, and cytokine expression data in various groups of patients

Group ^a	Sample no.	Identifi- cation no.	BCG scar status	Alleles ^b		% of TCRC band intensity for the following TCR Vβ families ^c :																				No. of TCR Vβ families expressed	No. of treatment days	% of β-actin ^d																	
				DRB1*	DRB1*	DRB1*	DOB1*	DOB1*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17			18	19	20	22	Gamma interferon	IL-4 IL-10												
a	1	6758	-	15011	09	0601	02	BL	9	16	7	7	3	32	27	38	9	6	0	6	31	8	0	0	17	13	21	5	9	17	20	4	0	-4											
	2	6777	-	08	07	0503	02	BL	49	41	26	27	1	5	14	66	5	0	1	6	15	29	0	4	21	16	8	1	0	14	27	5	0	13											
	3	6761	-	08	06	0503	0612	BL	15	25	27	9	14	-10	21	12	2	-3	2	3	3	4	4	4	8	13	6	8	8	12	130	70	12	1	1										
	4	6792	-	15011	04	0601	0302	BL	7	23	1	1	1	8	2	2	10	0	3	10	8	7	4	3	8	4	5	10	0	10	53	15	0	-7	0	-7									
	5	6784	-	15011	0601	15021	0601	0604	BL	3	18	15	10	0	8	0	13	-3	0	-2	0	0	0	0	12	1	-9	8	0	8	165	135	6	-35	6	-35									
	6	6775	-	07	15021	0601	03032	BL	11	17	16	0	6	4	5	4	2	0	0	8	3	0	0	0	9	0	0	0	0	7	62	65	-5	-4	7	62	65	-5	-4						
	7	6782	-	15011	01	0602	0501	BL	4	15	7	4	0	0	0	0	11	0	0	7	0	0	0	11	0	0	0	0	0	6	NA ^e	57	4	-9	4	-9	4	-9							
	8	6781	-	15011	03	0601	02	BL	6	18	5	0	0	0	0	6	3	13	0	0	0	0	0	0	8	0	0	0	0	6	34	213	10	14	14	10	14	14	10	14					
	9	6772	-	08	03	0503	02	BL	4	15	2	3	0	-1	-3	-3	35	4	-2	8	-2	0	0	0	41	0	8	12	0	6	66	52	10	20	20	6	66	52	10	20					
	10	6790	-	15011	BL	0601	BL	BL	1	3	0	1	0	0	9	0	-1	0	0	6	0	0	0	0	0	0	0	9	16	0	4	30	26	0	0	0	4	30	26	0	0				
	11	6779	-	15011	04	03032	0602	BL	0	20	3	4	0	4	5	0	0	0	0	0	13	0	0	0	0	0	0	0	0	0	3	35	112	1	-27	1	-27	1	-27	1	-27				
	12	6767	-	08	BL	0304	BL	BL	-6	22	1	0	3	0	20	0	2	-7	0	0	0	0	0	0	8	0	4	0	0	3	35	130	21	-7	7	35	130	21	-7	7	35	130	21	-7	7
	13	6755	-	15011	11	0601	BL	BL	0	10	6	4	0	1	1	1	1	-4	0	-3	0	2	0	0	0	-5	0	-11	0	2	NA	22	2	-3	NA	22	2	-3	NA	22	2	-3			
	14	6769	-	11	15021	0601	BL	BL	0	1	-2	2	5	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	2	36	8	0	-29	8	0	-29	8	0	-29					
	15	6786	-	15011	06	0602	BL	BL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	63	103	-57	17	63	103	-57	17	63	103	-57			
	16	6757	-	08	06	0503	BL	BL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	126	21	-109	50	126	21	-109	50	126	21	-109			
	17	6763	-	08	12	0604	BL	BL	-5	-13	-7	-7	-8	-8	-5	-26	-24	0	-6	-13	0	-6	0	0	-16	-5	0	0	0	5	156	0	-40	5	156	0	-40	5	156	0	-40				
b	1	6756	-	BL	01	0604	0501	BL	6	9	6	4	0	0	2	3	11	0	0	10	2	0	0	0	0	0	4	2	0	5	432	0	-4	5	432	0	-4	5	432	0	-4				
	2	6780	-	06	01	0612	0501	BL	0	5	7	4	0	12	12	0	1	0	0	0	0	0	0	2	0	0	0	0	0	5	36	121	4	17	5	36	121	4	17	5	36	121	4	17	
	3	6789	-	12	BL	06	03	BL	0	31	6	0	0	-1	0	0	3	0	0	0	-9	0	0	0	0	0	5	6	0	4	1	103	7	1	4	1	103	7	1	4	1	103	7	1	
	4	6766	-	03	BL	0604	BL	BL	0	9	0	0	0	0	0	0	2	6	0	0	0	-1	0	0	4	0	0	0	2	2	180	24	0	-30	2	180	24	0	-30	2	180	24	0	-30	
	5	6771	-	11	01	0503	03032	BL	0	6	2	0	0	0	0	0	-3	0	0	-6	-7	-3	0	6	-18	0	0	-3	0	2	90	14	6	72	2	90	14	6	72	2	90	14	6	72	
	6	6785	-	06	01	0503	BL	BL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	60	21	0	-44	0	60	21	0	-44	0	60	21	0	-44	
	7	6773	-	07	01	02	0501	BL	-1	-1	-6	-8	0	-5	-3	0	-16	-2	0	0	-16	0	0	0	-3	0	0	-3	0	0	NA	288	0	0	NA	288	0	0	NA	288	0	0			
	8	6760	-	04	12	03	BL	BL	-1	0	-1	-12	0	-8	-6	-8	-11	0	0	0	-4	0	0	0	-1	-3	-6	0	0	210	52	0	-15	0	210	52	0	-15	0	210	52	0	-15		
	9	6770	-	03	11	BL	BL	BL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	281	1	-6	17	281	1	-6	17	281	1	-6				
	10	6752	-	12	07	0301	BL	BL	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	184	0	2	NA	184	0	2	NA	184	0	2				
	11	6764	-	03	07	02	BL	BL	-6	-9	-10	-10	0	-7	-9	-3	-2	0	-2	0	-1	0	0	0	-6	0	0	0	0	0	NA	134	30	-11	0	NA	134	30	-11	0	NA	134	30	-11	
	12	6787	-	06	BL	0612	BL	BL	0	0	0	1	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	19	0	-5	0	NA	19	0	-5	0	NA	19	0	-5	
c	1	6799	+	15011	03	0601	02	BL	4	7	5	6	0	-5	0	0	1	0	1	0	5	0	0	0	9	2	0	0	5	35	82	0	0	5	35	82	0	0	5	35	82	0	0		
	2	6800	+	07	15021	0601	02	BL	0	4	0	0	7	0	0	0	11	0	0	0	0	0	0	0	8	0	5	0	0	4	NA	87	0	0	NA	87	0	0	NA	87	0	0			
	3	6794	+	15011	BL	0601	0610	BL	-11	5	0	-2	1	0	6	1	1	-11	0	0	-4	-3	0	0	-1	1	4	6	0	3	36	137	0	-20	3	36	137	0	-20	3	36	137	0	-20	
	4	6797	+	15011	04	0601	0302	BL	0	8	0	0	0	0	19	0	0	0	0	0	9	0	0	1	0	0	2	0	3	NA	149	13	7	NA	149	13	7	NA	149	13	7				
	5	6791	+	15011	BL	0601	0502	BL	-6	10	0	-3	-8	4	2	0	12	0	0	4	-11	-4	0	0	-1	0	3	0	0	2	34	280	0	0	34	280	0	0	34	280	0	0			
	6	6776	+	15011	08	0601	0604	BL	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	180	77	-9	-27	180	77	-9	-27	180	77	-9	-27				
	7	6798	+	07	BL	02	BL	BL	16	17	20	11	1	15	16	13	3	0	2	3	5	1	0	20	9	11	7	8	14	135	110	0	4	135	110	0	4	135	110	0	4				
2	6762	+	06	07	0612	BL	BL	5	15	8	0	0	2	3	0	10	0	0	2	11	2	0	0	7	0	0	4	0	6	130	59	0	3	6	130	59	0	3							
3	6793	+	07	5021	BL	BL	BL	0	10	4	2	4	0</																																

TABLE 4. Best-fit log-linear models selected by nested classification analyses of HLA status, BCG scar status, disease status, and TCR V β expression

Model ^a	TCR V β family	Likelihood ratio	
		Chi square	Probability
1 (TH, TD)	1	15.04	0.1306
	5	13.12	0.2171
	9	13.79	0.1830
	12	13.23	0.2110
	13	20.88	0.0219
2 (TD, H, B)	4	12.52	0.2518
	6	12.28	0.2666
	8	12.07	0.2806
	10	8.64	0.5661
	11	6.62	0.4693
	14	12.80	0.2350
	18	16.51	0.0860
	19	14.15	0.1663
3 (TH, B, D)	7	8.65	0.5661
	19	14.15	0.1663
4 (T, H, BD)	2	12.74	0.2384
	3	14.58	0.1481
	15	14.20	0.1641
	16	9.16	0.5174
	17	19.47	0.0347
	20	7.60	0.6680
	22	9.90	0.4496

^a T, TCR V β expression (positive or negative); H, high-risk HLA (DRB1*1501, DRB1*08, DOB1*0601) status (positive or negative); B, BCG scar status (positive or negative); D, disease status (healthy or diseased). Best-fit log-linear models were selected by using the BMPD statistical software (13).

TCR V β families were independent of HLA status, BCG status, and disease status, although there were interactions between BCG status and disease status). There was no correlation between TCR V β expression and other parameters, including age, sex, caste, and cytokine expression.

DISCUSSION

In the present study we identified the BCG scar status- and HLA class II high-risk allele status-dependent PPD-specific expression of various TCR V β families in patients and controls. The controls used more TCR V β families, while the patients used only a few TCR V β families. Furthermore, the majority of the responders among patients belonged to the BCG scar-negative HLA high-risk allele group.

In the present study carried out in an area in southern India where tuberculosis is endemic, we identified expression of many TCR V β families in both controls and patients even in the absence of antigen (repertoire). Exposure to various mycobacterial antigens in the environment, atypical mycobacteria, and infection per se may have been the cause of this expression. The expression of TCR V β families 4, 6, 8 to 12, and 14 in the presence of PPD in significantly more controls than patients (Fig. 3) indicated that there was antigen-specific usage of these TCR V β families presumably involved in health status. Most of the TCR usage (except usage of family 12) fit model 2 (i.e., TD interaction).

The lack of expression of many TCR V β families and the restricted usage in patients may have been the result of infection per se. The restricted TCR V β usage in the peripheral blood of the patients in the present study suggests that there is

a focused immune response to some selected epitopes of mycobacterial antigens or downregulation of TCR gene expression due to active suppression. Restricted TCR V β expression has been reported in multiple sclerosis, human immunodeficiency virus infection, rheumatoid arthritis, and Epstein-Barr virus infection (10, 14, 21, 33). AIDS patients that use one to three TCR V β families during primary infection deteriorate rapidly, while other patients that use more TCR V β families deteriorate slowly (26). Patients with chronic hepatitis B show underexpression of TCR V β families 14 and 15 and expansion of TCR V β family 7 (5). Reactivation of PPD-specific Th1 lymphocytes with PPD resulted in a concentration-dependent hyporesponsiveness due to an increase in apoptosis of $\alpha\beta$, $\gamma\delta$ CD4⁺, and $\alpha\beta$ CD8⁺ cells (30). It is possible that the cells become unresponsive after signaling through their TCR (25). Upon contact with antigen, cells may downregulate their TCR and coreceptors. This may be a consequence of activation under chronic stimulation conditions leading to anergy, a cellular state in which a lymphocyte is alive but fails to exhibit certain functional responses (25). This may be the mechanism of failure of immune surveillance and the resultant disease progression in chronic infections.

Limited TCR diversity with an antigen or infection can be attributed to selection and expansion of certain antigen-specific T cells that become unresponsive in patients (15, 20). The present study showed that this refractory status in patients was determined by BCG scar status and HLA high-risk allele status (Table 3). The downregulation observed might have been due to the enormous amount of *M. tuberculosis* antigens available in the system due to infection. This possibility was supported by the observation that more TCR V β families were used by BCG scar-negative and HLA high-risk allele-positive patients that were treated for >50 days than by patients that were treated for <50 days ($P = 0.0256$). The perturbation in the TCR repertoire and distribution thus seems to be a common feature of various chronic infectious diseases (5, 10, 14, 28, 32, 33).

Investigators in the field of immunology are confronted with dilemmas concerning the site and tissue used for sampling and the antigens that are used. Cells and tissues obtained from the focus of infection may be representative of the disease status and may be the result of the localized immune response. On the other hand, samples from the peripheral blood may indicate the immune surveillance status of the individual. In many previous studies on TCR V β expression in various disease states the workers have examined the TCR V β repertoire at the site of disease. In tuberculosis patients, Ohmen et al. identified selective expansion of TCR V β family 8 in the pleural fluid but not in the peripheral blood (19). In tuberculoid leprosy patients, T cells bearing TCR V β family 6 are overrepresented in lesions but not in the peripheral blood (34, 35). *M. bovis* hsp65 peptide-specific T-cell clones express more TCR V β families 5.1 and J α 9 (15, 31). Previous studies of cytokine expression in the peripheral blood of tuberculosis patients and endemic controls have shown that there is PPD-specific enhancement or suppression of gamma interferon and IL-10 following 48 h of culture (7). The reason for the observed PPD-recalled expression of many TCR V β families in the peripheral blood of more endemic controls than patients in the present study can thus be attributed to the constant exposure to typical

and atypical mycobacteria in the endemic environment and the resultant memory. Such exposure has been suggested to be responsible for the spectrum of immune reactivity in endemic controls (3, 12, 22). The exposure and cross-reactivity might have resulted in expansion and persistence of a defined T-cell memory pool, performing immune surveillance. A broader antigen, such as PPD, was thus capable of recalling this global memory (all memory T cells directed towards various epitopes of PPD shared with *M. tuberculosis*), leading to a better understanding of the factors involved in determining the prevailing adaptive immune status, as indicated in the present study, although we are unaware of the epitope specificity (since it was not the purpose of the study). The use recombinant *M. tuberculosis* antigens and peptides in various subgroups as reported in this paper may lead to identification of exact epitopes involved in pathogenesis and resistance.

The present study demonstrated that it is possible to identify PPD-recalled memory in the peripheral blood at the TCR V β usage level and account for the disease status (Table 4). Model 1 of the nested classification analysis revealed that usage of PPD-specific TCR V β families 1, 5, 9, 12, and 13 along with the HLA class II high-risk alleles was involved in the disease process, while model 2 suggested that TCR V β families 4, 6, 8, 10, 11, 14, and 18 were also involved in the disease process, although an HLA interaction could not be identified in the cohort used. Model 4 revealed that BCG scar status interacted with the disease status in the presence of TCR V β 2, 3, 15, 16, 17, 20, and 22, as listed in Table 4. Thus, the disease status was essentially linked to specific TCR V β usage (models 1 and 2, 12 TCR V β families) and HLA class II high-risk allele status (model 1, five TCR V β families). Previous studies have shown that HLA-DR2 status, as well as HLA non-DR2, BCG scar-negative status and IL-10 expression, are associated with pulmonary tuberculosis (4, 7, 24). In the present study we identified a role for TCR V β usage in tuberculosis susceptibility and protection operating in the context of HLA high-risk allele status and within the parameters of BCG vaccination and environmental exposure.

ACKNOWLEDGMENTS

Financial support from the Department of Biotechnology, Government of India, New Delhi (grant BT/PRO281/Med/09/057/96), the Commission of European Communities, Brussels, Belgium (fixed contribution contract C/1-CT93-0079), The Wellcome Trust, London, United Kingdom (grant 061237/Z/00/Z/HH/KO), and the Centre for Scientific and Industrial Research, India (grant CSIR-SRF, Roll no. 112066992) is acknowledged.

We gratefully acknowledge the gift of PPD-RT23 from the BCG vaccine laboratory, Guindy, Chennai, India. Permission from Director of Rural and Public Health, Government of Tamil Nadu (H.Dis.104968/TB/1/97), to carry out this study is acknowledged.

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Editor: S. H. E. Kaufmann