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

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Purified protein derivative (PPD) RT23-recalled T-cell receptor (TCR) Vβ 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 Vβ families was assessed by using family-specific primers. PPD-specific expression (usage) of TCR Vβ 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 Vβ families 2, 3, 7, 13, and 17. The majority of the patients did not show usage of most of the TCR Vβ families, and this was attributed to T-cell downregulation. A four-way nested classification analysis revealed that TCR Vβ 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 Vβ families readily recalled by PPD, while the disease status of the patients was attributed to TCR Vβ 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 Vβ 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 tuberculosis (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 CO_2 incubator at 37°C in the presence of 5% CO_2 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′ AAGAGAGAGCAAAAGGAAACATTCTTGAAC 3′	
TCRBV2...............5′ GCTCCAAGGCCACATACGAGCAAGGCGTCG 3′	
TCRBV3...............5′ AAAATGAAAGAAAAAGGAGATATTCCTGAG 3′	
TCRBV4...............5′ CTGAGGCCACATATGAGAGTGGATTTGTCA 3′	
TCRBV5A.............5′ ATACTTCAGTGAGACACAGAGAAACA3′	
TCRBV5B.............5′TTCCCTAACTATAGCTCTGAGCTG3′	
TCRBV6...............5′CTCAGGTGTGATCCAATTTC3′	
TCRBV7...............5′ ATAAATGAAAGTGTGCCAAGTCGCTTCTCS 3′	
TCRBV8...............5′ AACGTTCCGATAGATGATTCAGGGATGCCC 3′	
TCRBV9...............5′ CATTATAAATGAAACAGTTCCAAATCGCTT 3′	
TCRBV10.............5′ CTTATTCAGAAAGCAGAAATAATCAATGAG 3′	
TCRBV11.............5′ TCCACAGAGAAGGGAGATCTTTCCTCTGAG 3′	
TCRBV12.............5′ CTGAGATGTCACCAGACTGAGAACCACCGC 3′	
TCRBV13.............5′CAAGGAGAAGTCCCCAAT3′	
TCRBV14.............5′ GTGACTGATAAGGGAGATGTTCCTGAAGGG 3′	
TCRBV15.............5′ GATATAAACAAAGGAGAGATCTCTGATGGA 3′	
TCRBV16.............5′ CATGATAATCTTTATCGACGTGTTATGGGA 3′	
TCRBV17.............5′GATATAAACAAAGGAGAGATCTCTGATGGA3′	
TCRBV18.............5′ CATCTGTCTTCTGGGGGGCAGGTCTCTCAAA 3′	
TCRBV19.............5′GCACAAGAAGCGATTCTCATCTCAATGCCC3′	
TCRBV20.............5′ TCTAATATTCATCAATGGCCAGCGACCCT 3′	
TCRBV21.............5′ GATTCACAGTTGCCTAAGGAT 3′	
TCRBV22.............5′ ATGCAGAGCGATAAAGGAAG 3′	
TCRBV23.............5′ ATCTCAGAGAAGTCTGAAAT 3′	
TCRBV24.............5′ GATTTTAACAATGAAGCAGA 3′	
5′TCRBC.............5′ CCGAGGTCGCTGTGTTTGAGCCAT 3′	
Reverse primer	
3′TCRBC[b] ....5′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, Pamisford, 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 $MgCl_2$, 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,
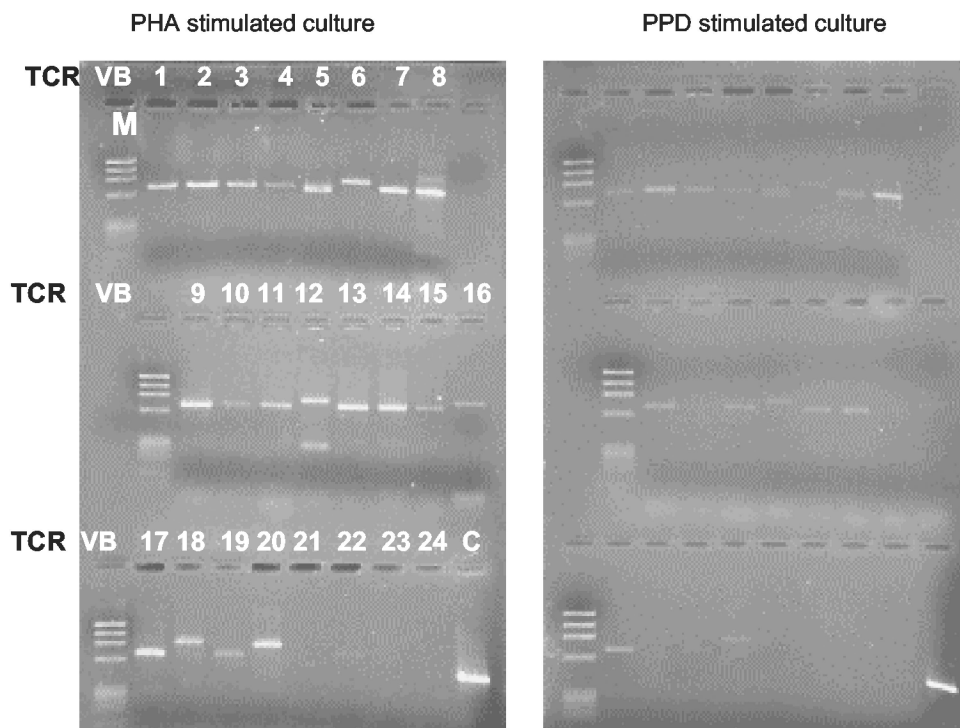
FIG. 1. Agarose gels showing expression of various TCR Vβ families in PHA- or PPD-stimulated, 48-h whole-blood cultures from a healthy control. M, marker φX-*Hae*III.

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 Vβ 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 Vβ families was studied in both cDNAs, and the concordance in TCR Vβ expression in the two aliquots of the 10 samples was analyzed. Except for TCR Vβ families 5, 11, 14, 18, and 19, all of the TCR Vβ families showed more than 80% concordance. The lower concordance in some of the TCR Vβ families may have been due to difficult primers under the conditions used. The quantitative expression data for various TCR Vβ families obtained with the two cDNA aliquots of the 10 samples were concordant. Repeat assays for 11 TCR Vβ families gave correlation coefficients of >0.8 ($P = 0.003$ to $P = 0.0001$), and repeat assays for six TCR Vβ 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 Vβ 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 Vβ 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 Vβ 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 Vβ expression in controls and patients. The numbers of controls and patients expressing various TCR Vβ 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 Vβ families and that the number of individuals expressing a particular TCR Vβ family did not differ significantly between controls and patients except for TCR Vβ family 17 ($P = 0.048$) (Fig. 3A). In the presence of PPD as many as 65% of the controls expressed selected TCR Vβ families. Furthermore, more controls than patients expressed many TCR Vβ families; eight TCR Vβ families (TCR Vβ 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 Vβ expression data in responders (the individuals who used a TCR Vβ family) was also performed (data not shown). The TCR Vβ expression levels were significantly higher in responding controls than in responding patients for TCR Vβ 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 Vβ 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 Vβ family expression by controls indi-
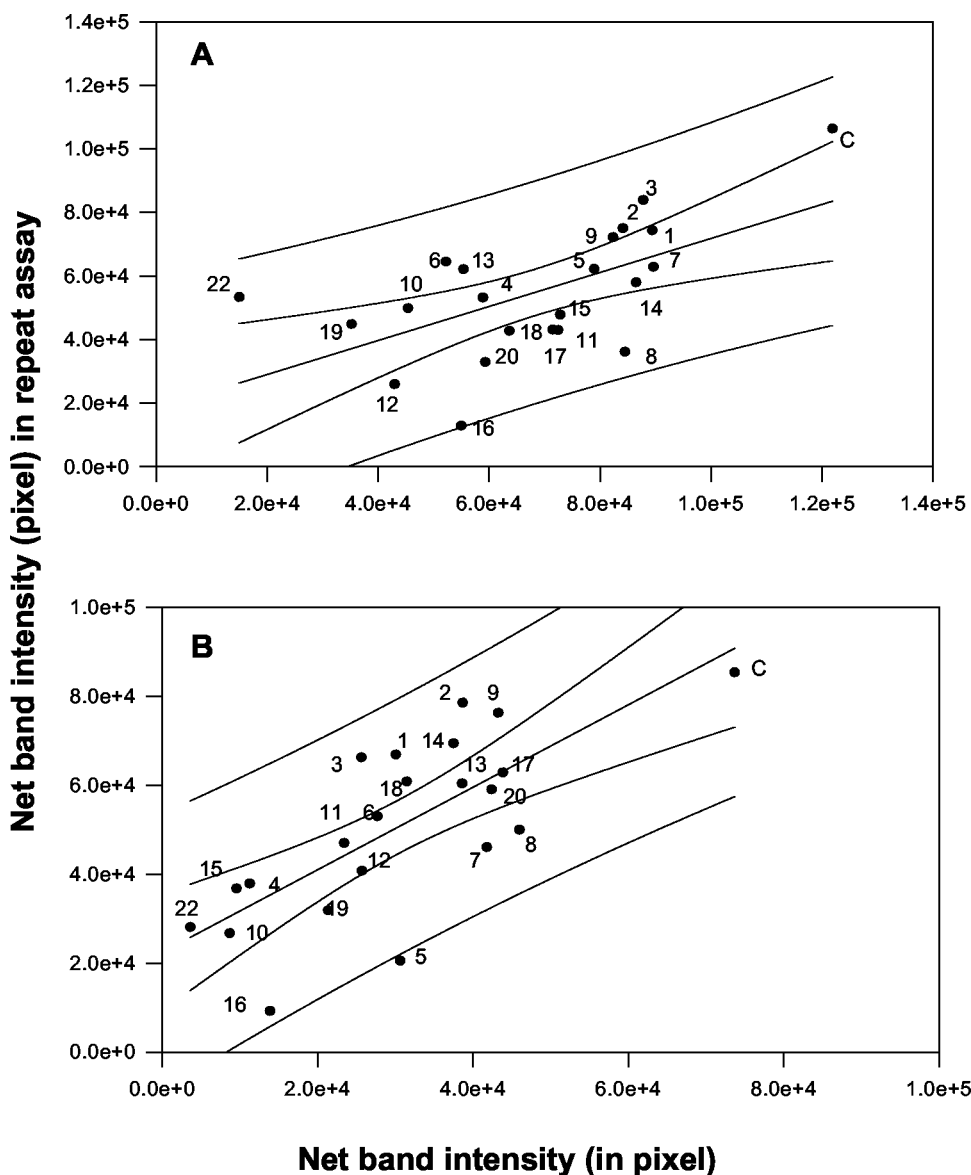
FIG. 2. Concordance of repeat assays of TCR Vβ expression in response to PHA in two different individuals (A and B). Whole-blood cultures were set up in quadruplicate with 0.4 μg of PHA and harvested after 48 h by pooling two wells. RNAs were extracted, cDNAs were synthesized from both aliquots and pooled, 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-
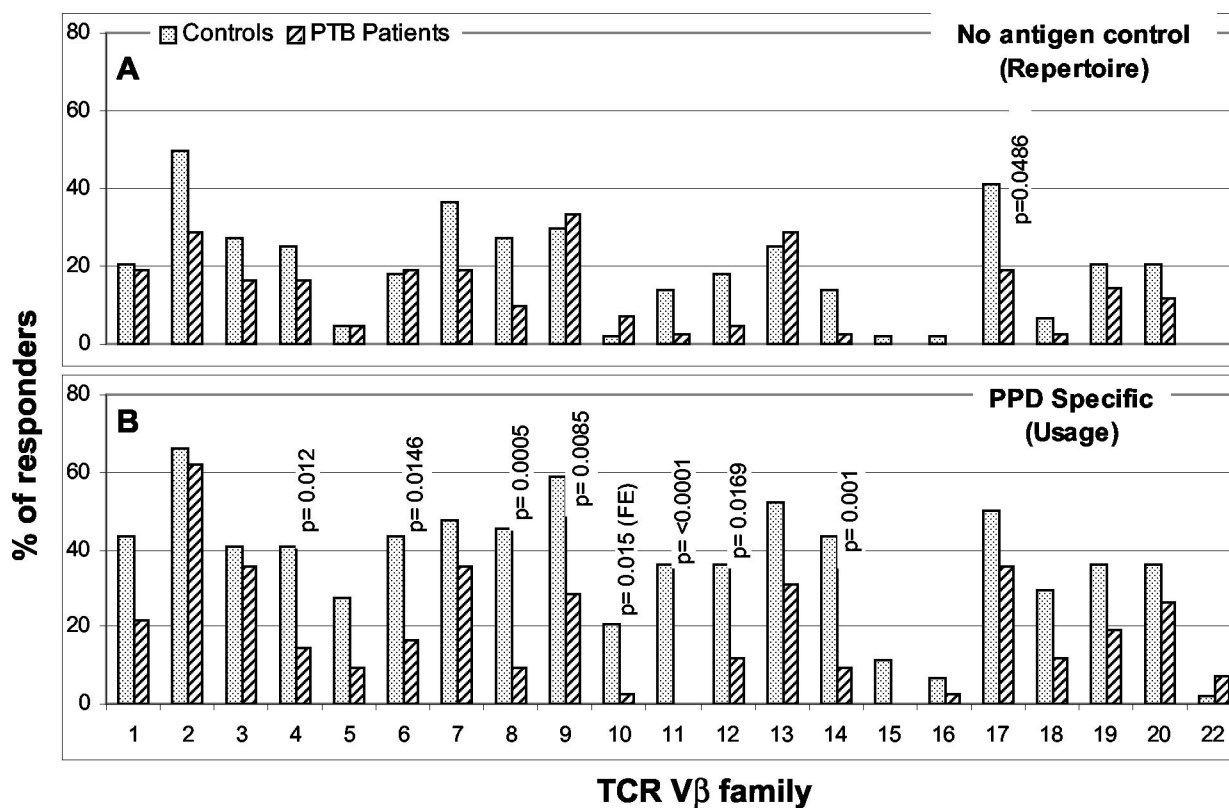
FIG. 3. TCR Vβ responders in healthy controls and pulmonary tuberculosis patients. The data indicate the percentage of responders in each TCR Vβ family. (A) No-antigen control; (B) antigen-specific usage. *P* values were obtained based on a chi-square analysis with the Yates correction.

specific expression of various TCR Vβ 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 Vβ family in a group was determined and defined as the number of responders. The total number of TCR Vβ families used by each individual was also determined. Among the controls, the total number of responders and the total number of TCR Vβ 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 Vβ 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 Vβ families.

In order to determine whether chemotherapy has any effect on TCR unresponsiveness in patients, we compared the number of TCR Vβ families expressed in patients treated for ≤50 days (*n* = 19) and the number of TCR Vβ families expressed in patients treated for >50 days (*n* = 14). This analysis revealed that there was recovery from TCR Vβ 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 Vβ families, 3 were in the group that was treated for ≤50 days and expressed more than five TCR Vβ families, none was in the group that was treated for >50 days

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

Nested classification analysis of HLA status, BCG status, TCR Vβ 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 Vβ family usage status [T] [positive versus negative]) were assessed in a nested classification analysis (13). The number of individuals expressing a particular TCR Vβ 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 Vβ families 1, 5, 9, 12, and 13 fit model 1 (TH, TD) well (i.e., interaction of the TCR Vβ families with HLA status and with disease status). TCR Vβ families 4, 6, 8, 10, 11, 14, and 18 fit model 2 (TD, H, B) well, implying that there were interactions between the TCR Vβ families and disease status, which were independent of HLA status and BCG scar status. TCR Vβ families 7 and 19 fit model 3 (TH, B, D) well (i.e., interaction between the TCR Vβ families and HLA status, independent of BCG status and disease status). TCR Vβ families 2, 3, 15, 16, 17, 20, and 22 fit model 4 (T, H, BD) well (i.e.,

TABLE 2. HLA class II alleles, BCG scar status, PPD-specific expression of various TCR Vβ families, and cytokine expression data for various groups of controls

Group[a]	Sample no.	Identification no.	BCG scar status	DRB1*	DRB1*	DQB1*	DQB1*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	No. of TCR Vβ families expressed	Gamma interferon	IL-4	IL-10
a	1	6828	−	15011	10	0601	0501	35	66	47	49	10	52	47	26	57	42	27	17	49	42	30	0	45	36	43	50	30	20	151	0	0
	2	6312	−	15011	1602	0001	0502	19	47	41	22	13	25	25	22	24	2	−11	3	30	29	3	6	31	12	5	1	4	14	131	0	0
	3	6828	−	15011	01	0001	0501	8	23	5	5	0	3	19	19	11	6	0	18	10	9	1	0	0	14	5	7	3	14	23	0	0
	4	5984	−	04	15021	0302	0302	6	14	1	−4	0	−6	22	40	43	20	6	−8	10	9	0	42	0	14	19	7	3	13	169	12	0
	5	6813	−	15011	15021	0001	BL	15	35	27	21	24	31	0	5	12	1	3	13	15	37	3	8	14	6	19	12	0	12	86	15	20
	6	6811	−	08	06	0612	0612	10	19	2	5	7	24	12	0	12	−1	3	0	12	8	0	6	15	7	2	0	0	12	26	17	−20
	7	6815	−	15011	01	0001	0501	8	18	21	10	3	0	19	19	13	13	−2	0	3	12	5	11	7	6	1	24	12	12	128	39	1
	8	3137	−	08	0301	0501	0501	3	7	6	0	14	3	2	9	7	−2	1	−2	8	10	3	−18	11	0	6	0	9	9	48	6	7
	9	6812	−	08	10	0001	0501	6	15	10	−1	0	2	2	4	5	9	5	8	0	1	5	0	3	0	10	2	7	11	96	4	−6
	10	6832	−	15011	06	0001	0603	23	13	4	5	4	27	18	9	9	3	0	1	13	0	2	0	−6	0	0	0		7	148	6	−6
	11	6803	−	04	1401	0001	02	17	6	4	4	0	4	0	0	4	4	0	0	0	11	0	0	4	0	0	0		6	121	0	−3
	12	6837	−	15011	06	02	0612	0	5	0	0	9	0	5	0	0	5	0	0	0	3	0	0	0	0	0	0		3	75	0	0
	13	6831	−	15011	10	0001	0612	−2	10	0	−16	0	9	−1	−18	0	−12	−23	−4	−10	−12	0	−15	0	0	0	8		3	182	8	−58
	14	6838	−	15011	10	0001	0602	0	−11	3	0	0	−1	−23	−7	−46	0	−4	−4	9	4	0	0	0	0	0	0		1	121	0	−6
	15	6823	−	15011	03	0001	02	−3	−15	0	0	0	−4	−4	−4	2	−1	−12	−5	0	−2	0	0	−6	0	−6	−7		0	135	0	−10
b	1	6819	−	04	10	0001	0501	21	48	37	12	13	12	38	41	41	3	13	6	11	1	0	18	6	4	15	6	4	15	138	−8	−12
	2	5985	−	03	BL	0604	0612	4	22	23	11	12	15	14	16	16	4	5	4	12	0	6	13	8	11	4	8	0	15	373	15	28
	3	6743	−	03	09	02	0305	2	7	27	1	0	1	0	1	11	5	1	12	0	0	1	0	0	0	11	8	3	8	119	−65	−68
	4	6825	−	06	BL	0301	0612	0	8	2	0	2	0	2	9	9	0	6	0	4	0	0	0	6	0	6	6	0	5	52	0	0
	5	2014	−	04	BL	0302	BL	12	0	0	0	2	2	1	0	5	0	0	0	0	0	0	0	0	0	0	2	2	3	99	0	0
	6	6821	−	1401	BL	0601	0301	0	0	−27	0	0	8	1	−1	2	0	17	2	0	0	0	−16	−1	−4	−4	3	1	2	90	8	0
	7	6809	−	03	07	0602	02	−157	−128	−140	−16	−10	−32	−145	−134	−195	0	−18	−23	−77	−65	0	−19	−20	−75	−66	0		0	105	26	−10
c	1	6829	+	15011	07	0001	02	28	51	65	25	10	21	36	26	48	7	12	22	28	14	6	0	74	14	14	11	19	19	99	0	−39
	2	5518	+	08	04	0503	0302	19	32	18	15	15	15	19	38	22	12	17	16	20	22	0	13	23	0	14	14	17	17	50	0	−5
	3	6806	+	08	08	0503	02	11	28	19	12	26	10	7	9	19	3	14	6	28	0	0	10	0	9	4	9	16	16	57	0	−38
	4	6816	+	04	BL	0001	0602	2	26	24	15	53	25	25	8	11	12	3	12	6	11	3	0	14	−1	10	9	14	14	107	7	−2
	5	5917	+	15011	03	02	BL	6	28	0	4	0	12	9	20	12	15	0	10	15	11	2	2	25	2	10	−1		13	68	4	−8
	6	6836	+	08	07	0503	03032	4	−8	8	6	27	5	12	14	13	10	15	7	10	6	4	14	23	0	4	0	12	12	127	4	−8
	7	6323	+	15011	06	0001	0612	10	16	10	5	0	25	8	13	10	8	13	20	15	1	0	7	7	0	0	60	10	10	126	0	0
	8	6834	+	15011	04	0502	0302	0	0	0	5	0	0	0	29	20	0	0	21	13	1	2	0	9	1	6	0	7	7	73	0	0
	9	6835	+	15011	1502	0001	0603	5	5	0	6	7	−3	−3	0	7	3	0	1	7	1	0	1	0	0	6	0	6	6	92	0	−24
	10	5910	+	04	15021	0001	0302	7	13	5	0	0	7	−2	−3	12	0	1	2	7	3	0	−7	0	1	0	6	5	5	82	0	−4
	11	6824	+	08	15021	0001	02	1	2	0	0	10	0	6	1	19	2	1	19	4	1	2	0	2	1	3	4	4	5	53	0	0
	12	5918	+	15011	09	0001	BL	0	1	0	5	8	0	0	0	12	0	0	0	0	0	3	0	4	1	3	3	3	3	198	40	32
	13	6814	+	04	15021	0001	0302	0	8	0	0	0	19	0	0	15	0	0	0	0	0	0	2	0	3	0	10	0	3	105	7	3
	14	6830	+	03	1401	0001	02	5	−3	1	0	−1	0	3	0	0	0	0	−2	0	0	0	0	−1	0	0	0	1	1	125	0	−1
	15	6805	+	15011	01	0001	0501	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0	1	1	1	197	0	0
	16	5982	+	15011	09	0601	BL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	−19	−20	0	0	−66	0	0	−64	0	−35
d	1	6822	+	1401	03032	02	4	45	−8	18	20	9	35	22	−2	9	9	6	34	16	7	1	0	34	14	19	25	15	15	133	35	−21
	2	6804	+	07	01	03032	0501	58	82	4	7	0	50	4	4	32	38	5	0	0	0	0	15	0	3	8	3	3	3	105	−6	−5
	3	6810	+	06	BL	0612	0501	−2	0	−8	1	0	0	0	8	−1	0	0	5	0	6	6	8	0	6	0	6	2	6	94	0	−2
	4	6833	+	07	02	02	0501	0	0	−7	0	0	4	0	0	0	0	0	0	3	0	0	0	0	0	3	3	2	2	133	35	−30
	5	6807	+	04	01	0302	0501	0	0	4	13	0	0	12	0	0	4	0	0	4	2	0	0	0	0	0	0	2	5	11	0	−1
	6	6820	+	06	07	0603	02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	168	0	−35

[a] Group a, BCG scar negative with HLA high-risk alleles; group b, BCG scar negative with HLA no-risk alleles; group c, BCG scar positive with HLA high-risk alleles; group d, BCG scar positive with HLA no-risk alleles.

[b] HLA DRB1*1501, DRB1*08, and DQB1*0601 are high-risk alleles (24).

[c] In group a the numbers of responders for TCR Vβ families 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 22 were 9, 13, 9, 5, 4, 6, 8, 7, 8, 4, 6, 5, 8, 7, 2, 2, 7, 7, 7, 6, and 1, respectively; in group b the numbers of responders for these families were 8, 9, 6, 8, 6, 9, 8, 8, 12, 3, 5, 8, 9, 8, 6, 9, 6, 8, 6, 9, 8, 8, 12, 3, 5, 8, 9, respectively; in group c the numbers of responders for these families were 9, 13, 9, 5, 4, 6, 8, 7, 8, 4, 6, 5, 8, 7, 2, 2, 7, 7, 7, 6, and 1, respectively; and in group d the numbers of responders for these families were 1, 5, 3, 2, 2, 3, 2, 2, 5, 1, 3, 1, 5, 4, 0, 0, 4, 1, 2, 2, and 0, respectively.

[d] Data from reference 7.

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.	Identification no.	BCG scar status	DRB1*	DRB1*	DQB1*	DQB1*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	No. of TCR Vβ families expressed	No. of treatment days	Gamma interferon	IL-4	IL-10
a	1	6758	−	15011	09	0601	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	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	15	25	27	9	14	−10	21	12	2	−3	3	3	3	4	4	4	8	13	6	8	8	12	130	70	12	1
	4	6792	−	15011	04	0601	0302	7	23	1	1	1	8	2	2	10	0	3	10	8	7	3	3	8	4	5	10	0	10	53	15	6	−7
	5	6784	−	15011	15021	0601	0604	3	18	15	10	6	8	5	0	13	−3	0	0	−2	0	7	0	12	1	−9	0	0	8	165	135	6	−35
	6	6775	−	07	15021	0601	03032	11	17	16	0	0	4	0	4	2	0	0	0	8	3	0	0	9	0	0	0	0	7	62	65	−5	−4
	7	6782	−	15011	01	0602	0501	4	15	7	4	0	0	5	3	11	0	0	0	7	0	0	0	11	0	0	7	0	6	NA[e]	57	4	−9
	8	6781	−	15011	03	0601	02	6	18	5	4	0	0	6	3	13	0	0	8	0	0	0	0	8	0	8	12	0	6	34	213	10	14
	9	6772	−	08	03	0503	02	4	15	2	3	0	−1	−3	−3	−35	4	−2	8	0	−2	0	0	41	0	8	12	0	6	66	52	10	20
	10	6790	−	15011	BL	0601	BL	1	3	0	1	0	0	9	0	−1	0	0	0	6	0	0	0	0	0	9	16	0	4	30	26	0	0
	11	6779	−	15011	04	03032	0602	0	20	3	4	3	4	5	0	0	0	0	0	13	0	0	0	8	0	0	0	0	3	35	112	1	−27
	12	6767	−	08	BL	0304	BL	−6	22	1	0	0	0	20	0	2	−7	0	8	0	0	0	0	0	0	4	0	0	3	NA	130	21	−7
	13	6755	−	15011	11	0601	BL	0	10	6	4	3	1	0	1	0	0	−3	0	2	0	0	0	8	0	−11	0	0	2	36	22	2	−3
	14	6769	−	11	15021	0601	BL	0	1	−2	0	5	0	0	0	0	0	0	0	7	0	0	0	−5	0	0	0	0	2	NA	8	0	−29
	15	6786	−	15011	06	0602	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
	16	6757	−	08	06	0503	BL	0	0	0	0	0	0	0	0	0	0	0	0	−6	0	0	0	0	0	0	0	0	0	50	126	21	−109
	17	6763	−	08	12	0604	BL	−5	−13	−7	−7	−8	−8	−5	−26	−24	0	−6	−13	0	−6	0	0	−16	−5	0	0	0	0	5	156	0	−40
b	1	6756	−	BL	01	0604	0501	6	9	6	4	0	0	2	3	11	0	0	10	2	0	0	0	0	0	4	2	2	5	17	432	0	−4
	2	6780	−	06	01	0612	0501	0	5	7	4	0	12	12	0	1	1	0	0	0	0	0	4	2	0	9	9	0	5	36	121	4	17
	3	6789	−	BL	BL	06	03	0	31	0	0	0	−1	0	0	3	0	0	0	−9	0	0	0	0	0	5	6	4	4	1	103	7	1
	4	6766	−	03	03	0604	BL	0	9	0	0	0	0	0	2	6	0	0	0	−1	0	0	6	4	4	0	2	0	2	180	24	0	−30
	5	6771	−	11	01	0503	03032	0	6	2	0	0	0	0	−3	0	0	0	−6	−7	−3	0	0	−18	0	0	−3	0	2	90	14	6	72
	6	6785	−	06	01	0503	BL	−1	−1	−6	−8	0	−5	−3	0	−16	−2	0	0	−16	0	0	0	−3	0	0	0	0	0	60	21	0	−44
	7	6773	−	07	12	02	0501	−1	0	−1	−12	0	−8	−6	−8	−11	0	0	0	−4	0	0	0	0	−1	−3	−3	0	0	210	52	0	−15
	8	6760	−	04	11	0301	BL	0	0	0	0	0	0	0	0	0	0	−2	0	0	0	0	0	0	0	−3	−6	0	0	17	281	1	−6
	9	6770	−	03	07	02	0612	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	184	0	2
	10	6752	−	12	07	0301	BL	−6	−9	−10	−10	0	−7	−9	−3	−2	0	−2	0	−1	0	0	0	−6	0	0	−7	0	0	5	134	30	−11
	11	6764	−	03	BL	02	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
	12	6787	−	BL	06	0612	BL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA			
c	1	6799	+	15011	03	0601	02	4	7	5	6	6	−5	0	0	0	1	1	0	5	0	0	0	9	2	0	0	0	5	35	82	0	0
	2	6800	+	07	15021	0601	02	0	4	0	0	7	0	0	0	11	0	0	0	−4	−3	0	0	8	1	5	0	0	4	NA	87	0	0
	3	6794	+	15011	BL	0601	0610	−11	5	0	−2	0	0	6	1	0	−11	0	0	9	0	0	0	−1	4	4	6	0	3	36	137	0	−20
	4	6797	+	15011	04	0601	0302	0	8	0	0	0	0	19	0	0	0	0	0	0	0	0	0	0	0	0	2	0	3	NA	149	13	7
	5	6791	+	15011	BL	0601	0502	−6	10	0	−3	−8	4	4	0	12	0	0	4	−11	−4	0	0	−1	0	3	0	0	2	34	280	0	0
	6	6776	+	15011	08	0601	0604	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	180	77	−9	−27
d	1	6798	+	07	BL	02	BL	16	17	20	11	1	15	16	13	3	0	2	3	5	5	1	0	20	9	11	7	8	14	135	110	0	4
	2	6762	+	06	07	0612	BL	5	15	8	0	0	2	3	0	10	0	0	2	11	2	0	0	7	0	4	4	0	6	130	59	0	3
	3	6793	+	07	5021			0	10	4	2	4	0	4	3	2	0	0	2	11	2	2	0	6	6	4	0	0	3	22	526	27	0
	4	6754	+			0305	0610	4	17	1	7	0	6	0	0	−2	0	0	0	0	0	0	0	4	8	0	0	0	3	NA	77	27	35
	5	6788	+	06	07	0503	0302	0	14	0	2	2	6	6	−2	−2	0	0	2	2	0	0	0	−1	0	−3	−2	0	3	55	393	37	40
	6	6748	+	07		06	02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	75	304	0	0
	7	6765	+	04	06	0602	0305	0	2	3	0	0	−2	0	0	0	0	0	0	−1	−4	0	0	0	0	0	5	0	0	NA	155	0	−50

[a] Group a, BCG scar negative with HLA high-risk alleles; group b, BCG scar negative with HLA no-risk alleles; group c, BCG scar positive with HLA high-risk alleles; group d, BCG scar positive with HLA no-risk alleles.

[b] HLA DRB1*1501, DRB1*08, and DQB1*0601 are high-risk alleles (24).

[c] In group a the numbers of responders for TCR Vβ families 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, and 22 were 6, 12, 8, 4, 3, 4, 9, 3, 7, 1, 0, 4, 8, 3, 0, 0, 10, 3, 6, 7, and 2, respectively; in group b the numbers of responders for these families were 1, 5, 3, 0, 0, 1, 2, 0, 0, 0, 1, 0, 0, 0, 2, and 0, respectively; in group c the number of responders for these families were 0, 4, 1, 1, 1, 0, 2, 0, 0, 0, 2, and 0, respectively; and in group d the numbers of responders for these families were 2, 5, 3, 1, 0, 2, 2, 1, 1, 0, 0, 3, 1, 0, 0, 3, 2, 1, 1, and 1, respectively.

[d] Data from reference 7.

[e] NA, not available.

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

Model[a]	TCR Vβ 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
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 Vβ expression (positive or negative); H, high-risk HLA (DRB1*1501, DRB1*08, DQB1*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 Vβ 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 Vβ 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 Vβ families in patients and controls. The controls used more TCR Vβ families, while the patients used only a few TCR Vβ 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 Vβ 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 Vβ 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 Vβ 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 Vβ families and the restricted usage in patients may have been the result of infection per se. The restricted TCR Vβ 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 Vβ 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 Vβ families during primary infection deteriorate rapidly, while other patients that use more TCR Vβ families deteriorate slowly (26). Patients with chronic hepatitis B show underexpression of TCR Vβ families 14 and 15 and expansion of TCR Vβ family 7 (5). Reactivation of PPD-specific Th1 lymphocytes with PPD resulted in a concentration-dependent hyporesponsiveness due to an increase in apoptosis of αβ, γδ CD4$^+$, and αβ 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 Vβ 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 Vβ expression in various disease states the workers have examined the TCR Vβ repertoire at the site of disease. In tuberculosis patients, Ohmen et al. identified selective expansion of TCR Vβ family 8 in the pleural fluid but not in the peripheral blood (19). In tuberculoid leprosy patients, T cells bearing TCR Vβ 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 Vβ families 5.1 and Jα 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 Vβ 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 Vβ usage level and account for the disease status (Table 4). Model 1 of the nested classification analysis revealed that usage of PPD-specific TCR Vβ 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 Vβ 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 Vβ 2, 3, 15, 16, 17, 20, and 22, as listed in Table 4. Thus, the disease status was essentially linked to specific TCR Vβ usage (models 1 and 2, 12 TCR Vβ families) and HLA class II high-risk allele status (model 1, five TCR Vβ 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 Vβ 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

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