Selective Activation and Accumulation of Oligoclonal Vβ-Specific T cells in Active Pulmonary Sarcoidosis

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Abstract

Sarcoidosis is a granulomatous disease in which activated T cells, responding to an unidentified stimulus, accumulate at sites of disease such as the lung. To evaluate the hypothesis that active sarcoidosis is characterized by a selective activation and expansion of a limited repertoire of T cell receptor (TCR) specific T cells, we analyzed TCR Vβ gene expression in lung and blood T cells of patients with active sarcoidosis and, for comparison, normal individuals using polymerase chain reaction amplification of 20 Vβ gene families. Analysis of normal bronchoalveolar lavage T cells revealed TCR Vβ distributions similar to that of normal blood, providing evidence for a lack of generalized skewing of the T cell repertoire in the normal, noninfected lung. Compared to normal lung and blood, subgroups of individuals with sarcoidosis demonstrated biased expression of one or more Vβ genes in either the lung or blood. Five Vβ gene families (Vβ5, Vβ8, Vβ15, Vβ16, and Vβ18) were most frequently utilized in a biased fashion by sarcoid lung or blood T cells. Furthermore, dramatic skewing of the T cell repertoire was apparent when sarcoid lung and blood T cells were expanded by short-term culture with IL-2. Sequence analysis demonstrated a bias in Vβ gene expression was usually due to expansion of select Vβ-specific clones, some of which contained a similar V(D)J junctional region motif. These observations provide evidence for a selective activation and accumulation of antigen-specific Vβ-expressing T cells in sarcoidosis. (*J. Clin. Invest.* 1994. 94:1533–1542.)
Key words: sarcoidosis • T cell repertoire • T cell receptor • bronchoalveolar lavage • polymerase chain reaction

Introduction

Sarcoidosis is a systemic disease histologically characterized by the presence of noncaseating granulomas and T lymphocytic infiltration in involved tissues such as the lung (1). At sites of inflammation, T lymphocytes are predominantly of the CD4+ phenotype, morphologically appear activated, and express cell surface markers such as interleukin 2 receptors (IL-2R) and DR class II major histocompatibility complex (MHC) molecules characteristic of T cells recently stimulated via the T cell antigen receptor (TCR)[1] pathway (1–3). Cytokines capable of augmenting an inflammatory response such as IL-2 and interferon γ are also produced by cells in the lungs of individuals with active pulmonary sarcoidosis (1). This inflammatory pattern is characteristic of immune-mediated granulomatous inflammation and suggests that sarcoidosis is caused by an immunologic response to a specific, yet unknown, antigen(s).

The T cell antigen receptor responsible for recognizing peptide antigens bound to class I or II MHC molecules on most T cells is composed of an α and a β chain (4). During T cell ontogeny, imprecise somatic rearrangement of variable (V), diversity (D), and joining (J) segments along with the addition of N-region nucleotides results in a clonally unique V(D)J junctional region of the β chain analogous to the hypervariable third complementarity determining region (CDR) of immunoglobulin genes (4, 5). This region and a similar region in the α-chain are thought to play crucial roles in defining T cell clonal specificity by coding for amino acids that interact with specific peptide–MHC ligands (4, 5). Studies of the composition of the TCR repertoire have indicated the presence of limited heterogeneity in the use of specific V, J, or V(D)J junctional residues of the α or β chain in many antigen-specific T cell immune responses and several autoimmune diseases (6–15). For a disease of unknown etiology such as sarcoidosis, studies of the TCR repertoire in affected individuals offer the potential for providing clues to the nature of the stimulus driving the accumulation of T cells at sites of granulomatous inflammation.

Previous work on sarcoidosis using an anti-Vβ8 TCR monoclonal antibody (mAb) identified marked skewing of the T cell repertoire of lung and blood T cells in a subgroup of patients with active pulmonary sarcoidosis (16). The possibility that biases for V region genes other than Vβ8 might be present in selected subsets of T cells remained untested. To test the hypothesis that active sarcoidosis is characterized by the selective accumulation of oligoclonal Vβ-specific T cells at sites of disease, we have evaluated the expression of a large number of TCR Vβ genes in the lung and blood T cells of individuals with sarcoidosis and, for comparison, normal individuals.

Methods

Study population. A diagnosis of sarcoidosis was established in 23 individuals based on clinical criteria of a compatible history and physical examination with confirmation by tissue biopsy demonstrating noncaseating granulomas and no evidence for infections or alternative diagnosis (16). 9 men and 14 women with a mean age of 38 yr participated in the study. There were 12 nonsmokers, 4 previous smokers (> 1 yr smoke free), and 5 current smokers. No patient was receiving corticosteroids at the time of study or within the previous 2 mo. All individuals had an abnormal chest x ray; 5 demonstrated hilar adenopathy alone, 16 had

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1. *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; CDR, complementarity determining region; D, diversity region; J, joining region; TCR, T cell antigen receptor; V, variable region.

Table I. Analysis of Bronchoalveolar Lavage Fluid

	Normal	Sarcoid
	$n = 10$	$n = 15$
Percent recovery	54.7±11.2	54.2±9.7
Cells/ml ($\times 10^6$)	0.22±0.10	0.45±0.27
Differential*		
Lymphocytes	13.6±6.4	44.0±18.4
Macrophages	85.1±6.6	54.8±18.3
Neutrophils	1.4±1.0	0.7±0.9
Eosinophils	0.1±0.4	0.5±1.7
CD4/CD8 ratio‡	1.8±1.0	6.8±6.5

* Differential cell count given as percent of total cell count. ‡ Ratio calculated as % CD4 divided by % CD8 as determined by FACS analysis (see Methods).

hilar adenopathy and interstitial infiltrates, and 2 had interstitial infiltrates without evidence for intrathoracic adenopathy. Pulmonary function tests revealed reduced forced vital capacity (FVC, 79±16.6% predicted), forced expiratory volume at 1 s (FEV1, 77±17.8% predicted) and diffusing capacity (DLCO, 77.5±27.9% predicted). Based on clinical presentation, chest radiograph, and pulmonary function tests, all patients had active sarcoidosis (16). Bronchoalveolar lavage was performed on 15 of these individuals at the time of their original diagnosis. The results of the lavage were consistent with active pulmonary sarcoidosis and are summarized in Table I (1, 16).

For comparison, 17 normal individuals (12 men, 5 women aged 28.5±6.0 yr) were studied. All were nonsmokers without a history of lung disease, heart disease, or other chronic medical illness. All of these individuals had normal pulmonary function tests (FVC, 102.8±13.9% predicted; FEV1, 106.1±13.9% predicted). 10 of these individuals underwent bronchoalveolar lavage. Results of the lavages are summarized in Table I. Informed consent was obtained from each patient and normal volunteer under a protocol approved by the Institutional Review Board.

Blood and lung mononuclear cell preparations. Lung mononuclear cells were obtained by bronchoalveolar lavage (BAL) as previously described (16). Cells were > 95% viable as determined by trypan blue exclusion. Cell differential counts were determined using cytocentrifugation and Diff-Quik® staining as previously described (16).

Blood mononuclear cells were isolated by centrifugation through lymphocyte separation media (LSM; Boehringer Mannheim Biochemicals, Indianapolis, IN).

Immunofluorescence analysis. Evaluation of lung and blood mononuclear cells for the proportions of CD3+, CD4+, CD8+, and Vβ8+ T cells was performed using monoclonal antibodies and flow cytometry as previously described (16). Briefly, lung and blood T cells were incubated with anti-CD3, (Leu 4), anti-CD4, (Leu 3), anti-CD8 (Leu 2; Becton Dickinson Immunocytometry Systems, Mountainview, CA) or anti-Vβ8 (5REX9H5, [17] gift of E. Reinherz, Dana-Farber Cancer Institute, Boston, MA 02115) monoclonal antibodies and stained with FITC-conjugated goat anti-mouse Ig (Tago, Inc., Burlingame, CA). After washing, cells were fixed in 1% paraformaldehyde in PBS and analyzed by flow cytometry. Mouse myeloma immunoglobulin (MOPC21) was used as a control antibody to assess nonspecific immunofluorescence. Single color immunofluorescence was carried out using a FACScan (Becton Dickinson Immunocytometry Systems) after establishing a lymphocyte gate using forward-angle and 90° light scatter analysis. The proportion of lymphocytes expressing CD3, CD4, CD8, or Vβ8 surface determinants was calculated by subtracting the control values from the values obtained using the test antibodies.

Analysis of T cell receptor Vβ gene expression. To evaluate T cell receptor Vβ gene expression, total cellular RNA from lung or blood mononuclear cell populations was isolated using either 4 M guanidine isothiocyanate and ultracentrifugation through a cesium chloride gradient or via phenol-chloroform extraction. Total RNA was resuspended in RNAse free water with an RNAse inhibitor, RNasin (Promega Biotec, Madison, WI) and quantified by UV spectrophotometry.

Complementary DNA was synthesized from 2 μg of total RNA by reverse transcription using 5 μM random hexamers (Pharmacia, Uppsala, Sweden) and 10 U/μl Maloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) in the presence of 1.0 mM each of deoxynucleotide triphosphates (Pharmacia), 0.01 M dithiothreitol, and 2 U/μl RNasin (Promega Biotec) for 1 h at 37°C.

PCR analysis of TCR Vβ gene expression was performed using a panel of 20 Vβ-specific oligonucleotide sense primers and an antisense oligonucleotide Cβ primer 5′-CGGCTGCTCAGGCAGTATC-3′, which hybridizes equally to both Cβ1 and Cβ2 gene segments (18). Oligonucleotides Vβ1 through Vβ20 were identical to those used by Choi and coworkers and are specific for one to three members of each Vβ gene family as determined from previously published sequences (19). All PCR amplifications were performed in a 50-μl reaction mix containing 0.3 mM sense and antisense primers, 0.2 mM deoxynucleotide triphosphates (Pharmacia), and 2.5 U Thermus aquaticus DNA polymerase (Perkin-Elmer Corp., Norwalk, CT). Negative controls consisting of the reaction mixture without cDNA were simultaneously run. All reactions were set up in a dedicated sterile hood using positive displacement and filter tip pipettes to avoid cross contamination. Reaction cycles were as follows: 1 min at 95°C, 2 min at 55°C, and 3 min at 72°C.

For quantification of PCR products, one-fifth of the reaction mix (10 μl) was blotted onto Zeta-Probe nylon membranes (Bio-Rad Laboratories, Richmond, CA) using a Bio-Rad Dot Blot Apparatus according to the manufacturer's recommendations. After UV cross-linking of the DNA to the nylon, the membranes were hybridized with a γ-^{32}P-labeled anti-sense Cβ primer 5′-TTCTGATGGCTCAAACAC-3′ internal to the 3′ Cβ primer. Results were visualized by autoradiography and quantified by direct scintillation counting of individual blots. To verify that the amplified products were specific for TCR genes, selected samples underwent Southern blot analysis by electrophoresis on 2% agarose gels, were transferred to Zeta-Probe nylon membranes according to the manufacturer's recommendations, and were hybridized with the same γ-^{32}P-labeled Cβ probe used for the dot-blot analysis. Amplified bands of the size expected for TCR gene products were always detected with minimal nonspecific signals.

The primers used in this study were originally used by Choi and coworkers to establish a quantitative PCR technique for evaluation of TCR Vβ repertoire (19). To confirm previous reports that these primers have similar amplification efficiencies, serial dilutions of two cDNA samples were analyzed by PCR amplifications using the Vβ and Cβ primers for 24, 27, 30, and 33 cycles (19–21). For each Vβ–Cβ amplification, a linear response was obtained with similar slopes, indicating similar primer efficiencies. A similar analysis was performed using as a template the plasmid pUCM4-4 (gift of T. H. Rabbitts, Medical Research Council, Cambridge CB2 2QH, United Kingdom [22]) containing a full-length Vβ2-Dβ-Jβ2.1-Cβ2 gene and the 5′ Cβ sense (5′GTGTTCCCA-CCCGAGGTCGCTGTGTTTGAG 3′) and 3′ anti-sense Cβ primers described above. This analysis demonstrated that approximately 3×10^5 starting copies of TCR Cβ DNA resulted in linear amplification using 24–30 cycles; this starting concentration was within the range of the amount of TCR Cβ cDNA contained in blood and lung T cell samples. For the subsequent analysis, 27 cycles was chosen for evaluation of TCR Vβ repertoire.

To assure that all samples were tested within the linear range of the PCR amplification and that similar amounts of cDNA were used, prior to analysis of Vβ expression, serial dilutions of each cDNA sample underwent PCR amplification using the 5′Cβ and 3′Cβ primers described above. Simultaneously, amplification of the plasmid pUCM4-4 was carried out in duplicate or triplicate using approximately 3×10^5 starting copies per 50 μl reaction mix. The products were quantified by dot–blot analysis as outlined above. For subsequent analysis of Vβ expression, a dilution of cDNA was used that contained amounts of

Cβ-specific cDNA comparable to the amount of pUCM4-4 DNA, based on a comparison with the pUCM4-4 PCR amplification.

Validation of quantitative PCR analysis. In order to validate the quantitative PCR analysis of T cell receptor gene expression, a comparison was performed between the proportion of Vβ8-expressing T cells determined by flow cytometric analysis using an anti-Vβ8 monoclonal antibody and the relative expression of Vβ8 genes determined by the PCR technique described above. 15 samples with a range of 1–18% Vβ8+ T cells by flow cytometry yielded a correlation of $R^2 = 0.836$ when compared to PCR analysis. The correlation between these two methods was highest when the relative usage of each Vβ-gene was expressed as a percentage of the sum of all Vβ gene transcripts detected. Thus, quantitation of Vβ gene expression throughout the study is expressed as Vβ cpm/ΣVβ cpm. The validity of this method of comparison has been previously described (20, 21, 23).

Reproducibility of this method of calculating Vβ gene expression was assessed by analyzing selected samples in duplicate if sufficient RNA was available for analysis. Results of this analysis demonstrated the relative level of each individual Vβ gene was almost always less than 3% of the total Vβ gene expression for lung and blood T cell samples. A representative example of a lung and a blood sample analyzed in duplicate are shown in Table II. Based on this analysis, a significant difference in the expression of an individual Vβ gene in lung compared to blood T cell samples was defined as ≥ 6% total Vβ gene expression.

Cell culture with interleukin 2. To determine whether sarcoid T cells bearing specific Vβ-encoded T cell receptors preferentially express functional IL-2 receptors and can proliferate in the presence of IL-2, lung and blood lymphocytes were cultured in vitro with exogenous IL-2. Lung and blood mononuclear cells were adhered to polystyrene tissue culture plates (Miles Scientific, Naperville, IL) for 15 min in a 37°C, 5% CO_2 incubator. Nonadherent cells were collected and incubated (37°C, 5% CO_2) at 1×10^6 cells/ml in RPMI 1640 with 10% heat inactivated human AB serum (Irvine Scientific, Santa Ana, CA), 0.01M Hepes buffer, penicillin 100 U/ml, streptomycin 100 U/ml, L-glutamine 2 mM and recombinant IL-2, final concentration 10 U/ml (Boehringer Mannheim Biochemicals). 50% of the media plus IL-2 was replaced on days 3, 6, and 9, and the cells harvested between days 10 and 14. RNA was extracted and T cell receptor analysis was performed as described above.

Sequence analysis of T cell receptor gene expression. In order to determine whether selected T cell receptor Vβ genes expressed by sarcoid T cell lines were derived from oligoclonal populations of T cells, the PCR products of Vβ gene amplification were directly ligated into TA Cloning® vectors (Invitrogen, San Diego, CA) using T4 polynucleotide kinase (Invitrogen or Stratagene Inc., La Jolla, CA). The ligation products were transformed into either INVaF′® competent Escherichia coli cells (Invitrogen) or XL-1 Blue Epicurean Coli® cells (Stratagene Inc.). A random selection of recombinant colonies was purified by a standard miniprep DNA method and double-stranded recombinant DNA containing inserts of the appropriate molecular weight size as determined by 2% agarose gel electrophoresis were directly sequenced by a double-stranded DNA method using the modified T7 DNA polymerase Sequenase® Version 2.0 (United States Biochem. Corp., Cleveland, OH) and the anti-sense Cβ probe (above) according to the manufacturer's recommendations.

Statistical analysis. Data are presented as mean±standard deviation of the mean. Group means were compared using the two-tailed Student's *t* test.

Results

Analysis of T cell receptor gene expression in normal lung. Evaluation of T cell receptor Vβ gene expression of T cells recovered from the epithelial lining fluid of the lungs of 10 normal individuals revealed different levels of expression of all of the 20 Vβ families that were tested (Fig. 1 *A*). As a group, the dominant Vβ gene families that were expressed included

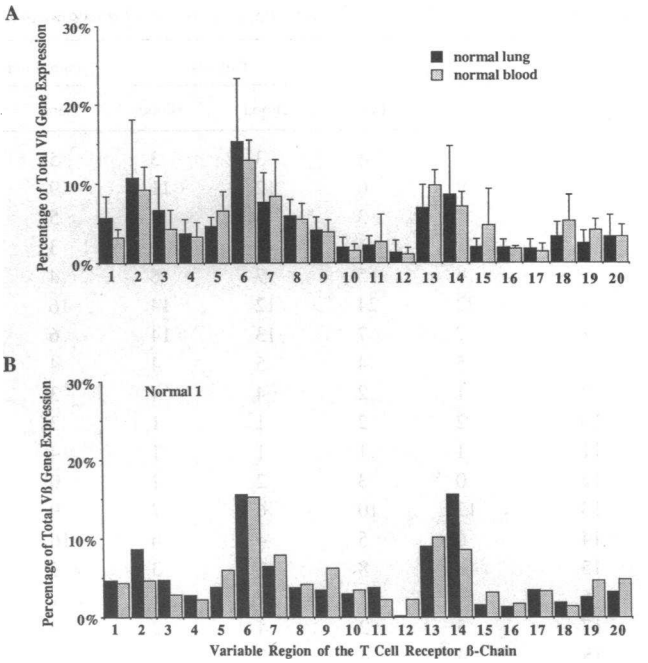

Figure 1. Analysis of T cell receptor Vβ gene expression in lung (*solid bar*) and blood (*speckled bar*) T cells of normal individuals. Data are presented as percentage of Vβ gene expression determined by quantitative PCR analysis and expressed as a percentage of the total Vβ gene expression as described in Methods. (*A*) Shown are the results of Vβ gene expression in normal lung ($n = 10$) and normal blood ($n = 10$) samples. Each bar represents the mean±SD of individual Vβ gene expression of Vβ1 through Vβ20. (*B*) Example of the distribution of T cell receptor Vβ gene expression from paired lung (*solid bar*) and blood (*speckled bar*) T cell populations of a single normal individual.

Vβ2 (11±7%), Vβ6 (15±8%), Vβ7 (8±4%), Vβ13 (7±3%), and Vβ14 (9±6%). T cell receptors Vβ10 (2±1%), Vβ12 (1±2%), Vβ16 (2±1%) and Vβ17 (2±1%) were typically expressed at very low levels relative to the other Vβ gene families. When compared to normal blood T cells, the distribution of T cell receptor gene expression was very similar, i.e., there was no statistically significant difference between the mean Vβ gene expression of lung compared to blood T cells in any of the 20 Vβ gene families.

In order to determine whether there was a relative compartmentalization of T cells expressing specific Vβ genes to the lung compared to blood, an analysis of four individually paired blood and lung T cell samples was performed. Overall, the Vβ gene distribution of lung T cells closely paralleled that of blood samples (Table II). A possible exception involved expression of Vβ14, which was expressed at significantly greater levels in the lung compared to blood (defined as ≥ 6% difference) in two of four individuals (Table II). For example, in normal individual 1, the relative expression of Vβ14 was greater in the lung (16%) than blood (9%) (Fig. 1 *B*). In one individual 3, expression of a single Vβ gene, Vβ6, in the blood was significantly higher than the expression of this Vβ gene in the corresponding lung (11% blood vs 3% lung). These observations suggest that, although differences may be occasionally noted in the expression of some Vβ genes such as Vβ14, there is little evidence for a widespread or generalized skewing of the T cell repertoire in the normal lung.

*Table II. Analysis of T Cell Vβ Gene Expression in Duplicate and Paired Lung and Blood T Cell Samples**

	Duplicate		Duplicate		Paired normal No. 1		Paired normal No. 2		Paired normal No. 3		Paired normal No. 4	
Vβ Family	Lung	Lung	Blood	Blood	Lung	Blood	Lung	Blood	Lung	Blood	Lung	Blood
1	3	4	3	3	5	4	9	4	3	3	4	5
2	9	6	12	11	9	5	8	9	14	12	9	11
3	3	3	3	3	5	3	2	2	10	11	7	6
4	3	4	8	6	3	2	3	1	6	2	5	9
5	4	5	7	6	4	6	6	10	5	7	2	3
6	22	24	12	14	16	15	8	12	3	11	6	6
7	7	7	13	14	6	8	9	12	9	8	7	10
8	5	4	5	4	4	4	6	6	7	3	6	8
9	1	2	4	4	3	6	4	4	8	3	4	4
10	2	2	1	1	3	4	1	2	4	2	3	1
11	1	1	1	1	4	2	1	2	4	2	2	2
12	0	3	2	1	0	2	1	1	1	2	4	3
13	12	10	6	7	9	10	9	7	4	8	8	5
14	6	5	4	6	16	9	15	7	6	7	13	8
15	8	8	3	3	2	3	3	6	4	6	3	2
16	1	1	1	1	1	2	1	2	2	1	2	2
17	3	2	4	2	3	3	3	1	1	1	2	2
18	4	4	3	4	2	2	5	4	5	6	6	4
19	2	2	6	5	2	5	3	4	2	3	4	3
20	4	3	2	2	3	5	3	4	2	3	3	6

* Data refer to Vβ gene expression determined by quantitative PCR analysis and expressed as a percentage of total Vβ gene expression.

Analysis of T cell receptor Vβ gene expression in sarcoid lung and blood, evidence for bias in the T cell receptor repertoire. To evaluate whether a preferential accumulation of Vβ-specific T cells occurs in the lung or blood of individuals with active pulmonary sarcoidosis, an analysis of T cell receptor Vβ gene expression was undertaken in 23 individuals with sarcoidosis, 15 of whom underwent evaluation by bronchoalveolar lavage. Biased expression of a particular Vβ gene was considered significantly different from our normal group if its relative expression exceeded the mean plus three standard deviations of the corresponding Vβ expression in normal lung or blood. Compared to normal lung, subgroups of individuals with sarcoidosis preferentially expressed a limited number of specific Vβ-gene families in their lungs (Fig. 2, Table III). In this regard, 9 of 15 individuals demonstrated a bias for Vβ5 expression, 6 individuals demonstrated a bias for Vβ15, 3 individuals demonstrated a bias for Vβ18 expression, 2 demonstrated a bias for Vβ13 and 1 individual demonstrated a bias for Vβ11. Interestingly, Vβ3, a TCR gene segment previously shown to vary markedly in its expression in normal peripheral blood, was uniformly expressed at low levels in the sarcoid lung group compared to a more heterogeneous distribution in the normal lung group (23). With this possible exception, the expression of all other Vβ genes tested was comparable in the sarcoid lung to levels of expression found on the epithelial surface of the normal lung. Analysis of the blood of individuals with sarcoidosis most frequently demonstrated biased expression of Vβ8 and Vβ16 gene families with 3 of 15 individuals demonstrating skewing in one of these Vβ genes (Table III). Seven individuals with sarcoidosis demonstrated an absence of any bias in the Vβ repertoire of the blood. Thus, when 20 Vβ gene families were tested, a limited number of select Vβ genes appeared to be preferentially used by sarcoid lung T cells and to a lesser extent, sarcoid blood T cells.

In order to assess whether a bias for T cell receptor Vβ gene expression could be associated with a compartmentalization of select T cells to the lung or blood, an evaluation of paired blood and lung samples was performed in seven individuals with active sarcoidosis (Fig. 3, Table III). This analysis revealed that the expression of specific Vβ genes was frequently expressed preferentially in either the lung or the corresponding blood. For example, the increased expression of Vβ18+ T cells in the lung of patient 1 was not associated with a concomitant increase in Vβ18 expression of the blood indicative of a significant compartmentalization of Vβ18 to the lung. Interestingly, although patient 3 had significantly increased expression of Vβ15 and Vβ18 in the lung compared to normal individuals, there was a striking compartmentalization of Vβ6 usage in the lung with more than a threefold increase in Vβ6 expression in the lung (21%) than blood (6%). In this same individual, a striking bias was also found in the blood for the expression of the Vβ8 gene family, a variable region previously shown to be expressed in increased numbers of lung and/or blood T cells in a subset of individuals with sarcoidosis (16). In this case, preferential expression of Vβ8 was simultaneously evaluated by flow cytometric analysis using an anti-Vβ8 monoclonal antibody, confirming a bias for Vβ8+ T cells (proportion of Vβ8+ T cells 20% by PCR vs 18% by FACS®). Patient 4 also demonstrated a bias for Vβ8 gene expression in the blood (20%) which was strikingly elevated only in this compartment. In patient 5, despite the absence of bias for any of the 20 Vβ genes in either lung or blood T cell populations compared to the normal lung or blood, there was a significant difference in the expression of Vβ6 gene in the lung compared to blood suggesting local compartmentalization of this Vβ subset to the lung in this individual (Fig. 3).

Since small but statistically significant differences in Vβ gene expression may be seen in the subset of CD4+ T cells

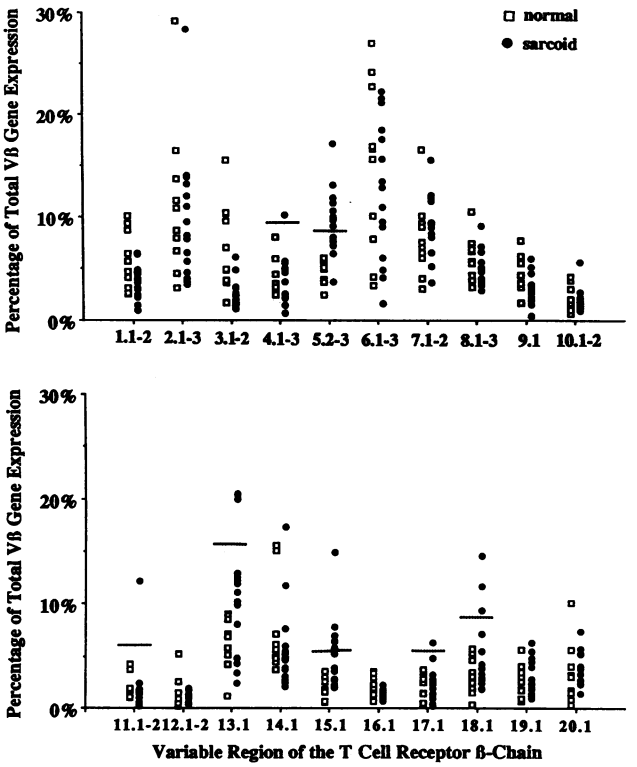

Figure 2. Analysis of T cell receptor Vβ gene expression in lung T lymphocytes of individuals with active pulmonary sarcoidosis and normal individuals. Shown are the results of Vβ gene expression in lung T cells of 10 normal individuals (*open box*) or 15 individuals with active sarcoidosis (*closed circles*). Data are presented as percentage of Vβ gene expression determined by quantitative PCR analysis as described in Methods. Dotted lines represent 3 SD above mean Vβ gene expression in normal lung and are shown for those Vβ gene families that have biased representation in at least one individual with pulmonary sarcoidosis.

*Table III. Summary of TCR Vβ Gene Biases in Individuals with Sarcoidosis**

Patient	Specimen	Vβ Bias	Patient	Specimen	Vβ Bias
1	blood	none	9	lung	5, 15
	lung	5, 15, 18			
	blood IL-2‡	13	10	lung	5
	lung IL-2‡	18	11	lung	5
2	blood	none			
	lung	5, 13	12	lung	5
	blood IL-2	none			
	lung IL-2	2	13	lung	5, 11, 18
3	blood	8	14	lung	5, 15
	lung	15, 18			
			15	lung	none
4	blood	8			
	lung	15	16	blood	none
	blood IL-2	2, 14			
	lung IL-2	6	17	blood	16
5	blood	none	18	blood	none
	lung	none			
	blood IL-2	none	19	blood	16, 17
	lung IL-2	15			
			20	blood	15
6	blood	8			
	lung	15	21	blood	3
7	blood	none	22	blood	none
	lung	none			
			23	blood	3, 16
8	lung	5, 13			

* Biased expression of an individual Vβ gene family was significant if the percentage of Vβ gene expression exceeded the mean plus three standard deviations of the corresponding Vβ gene expression in normal lung or blood. ‡ Blood IL-2 or lung IL-2 refer to T cell populations cultured in media with recombinant IL-2 for 10–14 d prior to TCR analysis. Biased expression of IL-2 samples indicates the dominant Vβ gene family whose expression was enhanced by culturing in IL-2.

compared to CD8+ T cells (24), the possibility that biased expression of specific TCR Vβ genes was a result of alterations in the underlying CD4+ to CD8+ ratio of T cells in the sarcoid lung or blood was analyzed by correlating relative Vβ gene expression with proportion of CD4+ T cells of the corresponding lung and blood T cell populations. No correlations were found in any of the Vβ genes tested (e.g., % CD4 vs Vβ5, R^2 = 0.019; % CD4 vs Vβ15, R^2 = 0.018, % CD4 vs Vβ18, R^2 = 0.004) in agreement with previous observations (18). Thus, bias in the T cell repertoire in sarcoidosis does not appear to be solely a reflection of a perturbation in the underlying proportion of CD4+ T cells found on the alveolar lining surface of the lung.

Preferential expansion of subsets of Vβ-specific T cells in sarcoid lung and blood with interleukin 2. The skewing in the T cell repertoire in uncultured sarcoid lung (and to a lesser degree, sarcoid blood) T cells suggested the possibility that even more dramatic biases might be detected in selected subsets of activated T cells, such as those expressing IL-2 receptor genes. To evaluate this hypothesis, lung and blood T cells purified by adherence to plastic were cultured in vitro for 10–14 d with recombinant IL-2 and analyzed for their pattern of Vβ gene expression. A striking preferential expansion of select Vβ-expressing T cells derived from sarcoid lung T cell populations was observed (Fig. 4, Table III). In patient 1, lung T cells expressing a bias for Vβ18 were dramatically and selectively expanded in vitro from a baseline of 15% to 52% of total Vβ gene expression. In two other individuals, culture with IL-2 resulted in the expansion of Vβ subsets that were nonbiased compared to normal lung and blood but that were expressed at high levels relative to most or all other Vβ genes. For example, in patient 4 lung T cells expressing Vβ6 were expanded in vitro from 22% to 39% of total Vβ gene expression. In patient 2, lung T cells expressing Vβ2 were expanded with culture in IL-2 from a modest 8% to 23% total Vβ gene expression. In one individual, there was a striking discordance between those Vβ genes expressed at high relative levels in the unstimulated lung T cell population compared to the expression of Vβ genes after short-term culture with IL-2. In this individual (patient 5), Vβ15 was markedly expanded by in vitro culture with IL-2 from 2% to 46% total Vβ gene expression to dominate the T cell repertoire. Preferential expression of Vβ-selective T cells was due to proliferation of these T cells since analysis of the T cell lines demonstrated increased T cell blasts as detected by an increase in the size and granularity of the lymphocytes by flow cytometric analysis.

Sarcoid blood T cells cultured with IL-2 also demonstrated

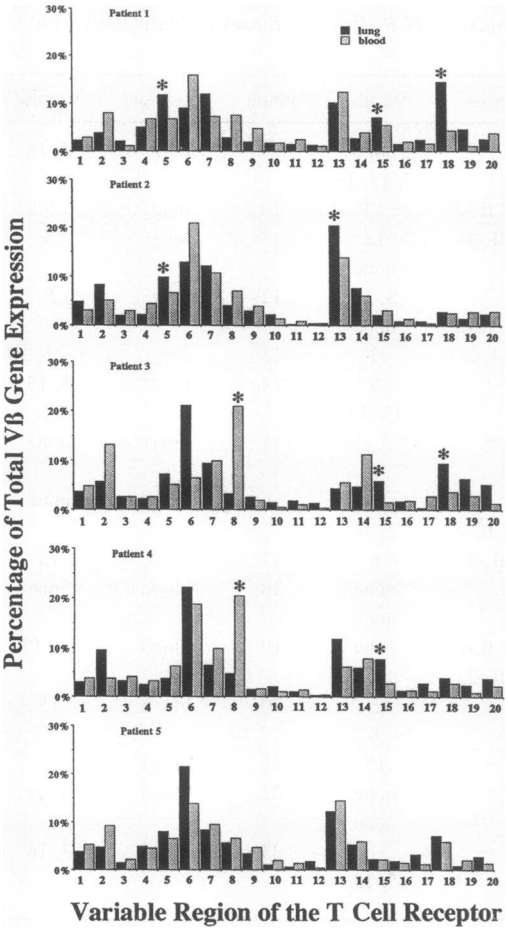

Figure 3. Bias in the T cell receptor Vβ repertoire of individuals with active pulmonary sarcoidosis. Shown are the results of the T cell receptor Vβ gene analysis of paired lung (*closed bars*) and blood (*speckled bars*) specimens of individuals with active sarcoidosis. Data are presented as percentage of Vβ gene expression determined by quantitative PCR analysis as described in Methods. Shown are data from patients 1–5. A bias in Vβ gene expression in either the lung or blood T cell populations compared to normal lung and blood is indicated by an asterisk.

a selective expansion of T cells expressing specific Vβ genes. For example, of the same four individuals, two blood T cell lines showed preferential expansion of Vβ-specific T cells. In these blood T cell lines, the skewing of the T cell repertoire involving Vβ14+ and Vβ13+ T cells was as dramatic as the biases in the T cell repertoire that were seen after culturing sarcoid lung T cells with IL-2. In the case of patient 1, Vβ13 started off at a high relative level of expression in the blood and dramatically increased with culture in IL-2. In contrast, Vβ14 gene expression in the blood of patient 4 was at a modest level before dramatically increasing after short-term culture with IL-2. Two individuals with sarcoidosis (patients 2 and 5) demonstrated little bias in the IL-2 cultured blood T cell lines, suggesting that the IL-2R+ blood T cells found in these samples were not derived from a small number of Vβ-specific T cell subsets. In contrast to the striking selective proliferation of sarcoid lung and blood T cells when cultured with IL-2, bronchoalveolar lavage and blood T cells of two normal individuals cultured under similar conditions demonstrated no evidence for selective expansion of Vβ-specific T cell subsets (data not shown). These results of normal lung T cells confirm previous observations reported by Forrester and co-workers (25).

Sequence analysis of biased T cell receptor genes in sarcoidosis. To determine if a bias in Vβ gene usage in the sarcoid lung is the result of a limited number of clonal populations of T cells, a sequence analysis was performed on selected Vβ genes of the unstimulated lung T cell populations (Table IV). In patient 1 bias in the expression of Vβ5 and Vβ18 in the lung was markedly oligoclonal with 7/12 (58%) Vβ5 and 5/5 (100%) Vβ18 sequences identical. In contrast, a sequence analysis of Vβ18 expression in the uncultured blood of this individual was largely polyclonal indicating the oligoclonality of lung Vβ18+ T cells in this patient was not due to limited clonality of the circulating Vβ18 subset. Similarly, Vβ5 and Vβ15 expression in patient 14 was dramatically oligoclonal with 6 of 8 (75%) and 11 of 13 (85%) sequences identical, respectively. In contrast, the expression of Vβ5 in patient 12 demonstrated lesser degrees of clonality (29%). Together, these results suggest that biased expression of Vβ genes in the sarcoid lung is usually due to a limited number of T cell clones. To determine whether similar degrees of clonality exist in the normal lung, a sequence analysis of several Vβ genes (Vβ5, Vβ13, and Vβ18) was performed. The results of this analysis indicate that the normal lung is typically characterized by a heterogeneous population of T cells that is either polyclonal, or at most, has a small proportion of clonally expanded T cells on the epithelial surface (Table IV).

In order to determine if the skewing in the Vβ T cell repertoire of the IL-2 expanded T cell lines was the result of an outgrowth of only a few individual T cell clones, a sequence analysis of the Vβ genes that were overrepresented in the T cell lines was performed (Table IV). Three of four lung T cell lines demonstrated marked oligoclonality of the dominantly expressed Vβ gene. For example, 7 of 8 (88%) Vβ18 gene sequences of the lung T cell line of patient 1 were identical. Sequence analysis of the lung T cell lines of patient 5 (Vβ15.1) and 2 (Vβ2.1) demonstrated that 6/8 (75%) and 7/9 (78%), respectively, of the dominantly expressed Vβ gene sequences were identical (Table IV). In individual 4 analysis of lung T cells expressing Vβ6 demonstrated that only 2 of 10 (20%) sequences were identical. In this case, the preferential expression of a specific Vβ gene in a lung T cell line was likely due to several Vβ-specific T cell clones.

Analysis of those blood T cell lines (patients 1 and 4) that demonstrated preferential expression of a limited number of Vβ genes also demonstrated striking clonality of the Vβ gene sequences similar to the findings of the lung T cell lines (Table IV). The marked clonality in Vβ gene expression in lung and blood T cell lines was not due to an inherent property of growing a heterogenous T cell population in vitro, since sequence analysis of a Vβ gene that was not preferentially expressed in an IL-2 expanded T cell line demonstrated limited evidence for oligoclonality (patient 2, blood T cell line). As a control for the sequence analysis of normal and sarcoid T cell populations, an analysis of Vβ gene expression in normal blood was performed and demonstrated polyclonality of several Vβ genes such as Vβ5 and Vβ18, consistent with analysis of other Vβ genes in previous reports (26, 27).

Despite the enormous potential for diversity in the amino acid sequences in the V(D)J junctional region, of potential interest is the fact that several of the dominant TCR sequences from biased Vβ T cell subsets contained either an arginine–glycine–arginine (RGR) or arginine–glycine–glycine–arginine (RGGR) segment beginning at position 98–100 of the Vβ chain. In

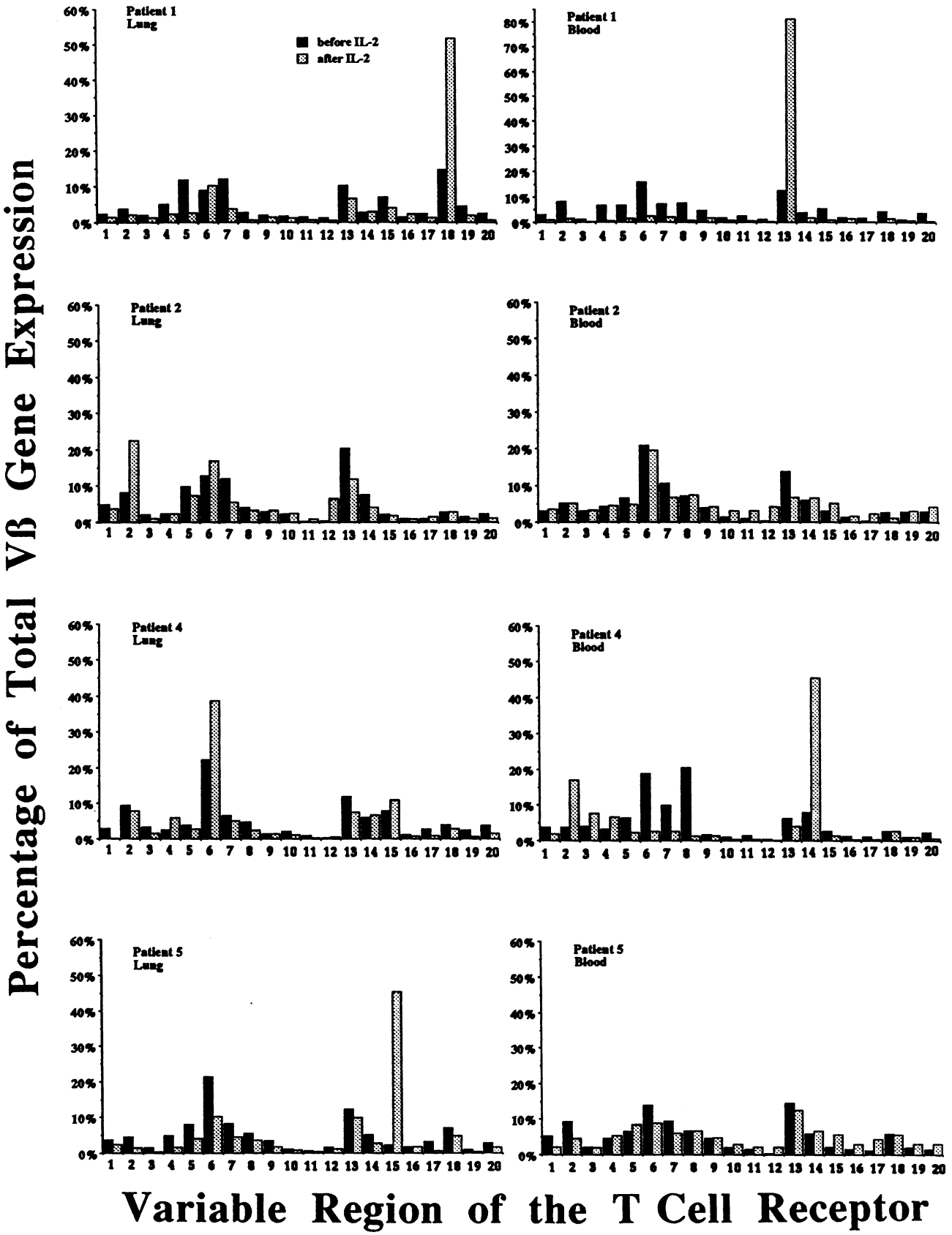

Figure 4. Expression of Vβ specific T cell receptor genes in lung and blood T cells of individuals with active pulmonary sarcoidosis before (*closed bars*) and after (*speckled bars*) culture in media containing 10 U/ml of recombinant IL-2. Data are presented as percent Vβ gene expression determined by quantitative PCR analysis outlined in Methods. Shown are paired samples from lung (*left side*) and blood (*right side*) of four individuals with sarcoidosis. Individual Vβ gene expression ranged from 0 to 80% of the total amount of Vβ genes expressed.

Table IV. Analysis of TCR Vβ Gene Sequences from Individuals with Sarcoidosis and Normal Individuals

					Sequences occurring more than once					Sequences occurring once			
	Specimen	Vβ* bias	Vβ seq.	No. seq.	No. of occurr.	% of total	Vβ	N-Dβ-N**	Jβ	No.	% of total	Jβ1 1 2 3 4 5 6	Jβ2 1 2 3 4 5 6 7
Patient													
1	Lung	5, 15, 18	5	12	7	58	5.3 CASS	DYSQR	N 2.4	1	8	0 0 0 0 0 0	0 0 0 0 0 0 1
					2	17	5.3 CASS	RGTSGGAF	Y 2.7				
					2	17	5.3 CASS	PTDTS	E 2.5				
			18	5	5	100	18.1 CASS	TIPPRGR	S 2.6	0	0	0 0 0 0 0 0	0 0 0 0 0 0 0
	Lung IL-2†	18	18	8	7	88	18.1 CASS	TIPPRGR	S 2.6	1	12	0 0 0 0 0 1	0 0 0 0 0 0 0
	Blood	None	18	9	2	22	18.1 CASSP	PAAD	S 2.1	7	78	0 1 0 0 0 2	0 0 2 0 0 1 1
	Blood IL-2†	13	13	11	9	82	13.1 CASS	TGRGRFKG	E 1.4	2	18	0 1 0 0 0 0	0 0 0 0 0 0 1
2	Lung IL-2	2	2	9	7	78	2.1 CSA	RRGQGPG	T 2.2	2	22	0 0 0 0 0 0	0 0 0 0 1 0 1
	Blood IL-2	None	13	9	2	22	13.1 CASS	CVGARL	N 1.2	3	33	0 0 0 0 1 0	0 1 1 0 0 0 0
					2	22	13.1 CASS	SQG	N1.1				
					2	22	13.1 CAS	SGQVGI	Y 2.7				
3	Lung	15, 18	6‡	8	2	25	6.3 CASS	FIRGG	E 2.7	4	50	1 0 0 0 0 0	0 0 0 0 1 0 2
					2	25	6.3 CASS	PRGLYSI	Y 2.7				
			18	10	3	30	18.1 CASS	TGTPDG	Q 1.5	5	50	2 0 0 0 0 0	2 0 0 0 1 0 0
					2	20	18.1 CASS	GGHQ	Y 2.7				
4	Lung IL-2	6	6	10	2	20	6.1 CASSL	VGGWG	D 2.3	8	80	2 0 0 0 0 0	2 2 1 0 1 0 0
	Blood IL-2	2, 14	14	9	9	100	14.1 CASSL	FRGGR	E 1.4	0	0	0 0 0 0 0 0	0 0 0 0 0 0 0
5	Lung IL-2	15	15	8	6	75	15.1 CATS	DLGSGS	E 2.1	2	25	0 0 0 0 0 0	0 0 1 0 1 0 0
12	Lung	5	5	7	2	29	5.3 CASS	VGLVV	N 2.1	5	71	0 0 0 0 0 0	2 0 1 0 1 0 1
14	Lung	5, 15	5	8	6	75	5.3 CASS	LQQGG	Y 2.1	2	17	0 0 0 1 0 0	1 0 0 0 0 0 0
			15	13	11	85	15.1 CATS	EAGG	E 2.7	2	15	0 0 0 0 0 0	0 0 0 1 0 0 1
Normal													
1	Lung	N/A	13	14	3	25	13.1 CAS	KLNYNIH	E 2.1	9	64	1 0 0 0 3 0	2 0 0 0 2 0 1
					2	17	13.1 CAS	TVPGQG	E 1.1				
		N/A	18	6	0	0	—	—	—	6	100	0 0 0 0 0 0	1 2 0 1 0 0 2
2	Lung	N/A	5	6	0	0	—	—	—	6	100	1 1 0 0 1 0	0 1 2 0 0 0 0
3	Blood	N/A	5	4	0	0	—	—	—	4	100	0 0 0 0 0 0	1 0 0 0 2 0 1
		N/A	18	9	0	0	—	—	—	9	100	1 0 0 0 0 0	2 2 0 0 0 0 4

Sequences were derived from Vβ-specific cDNA following amplification by PCR. Predicted amino acid sequences are based on the nucleotide sequences of the corresponding V(D)J junctional regions of the β-chain sequences. The sequences start at the conserved cys (C) at position 92 of the β-chain and are shown using the single letter code notation. All sequences were determined to be in frame. * Biased expression of Vβ gene expression determined by percent expression exceeding the mean plus 3 SD of the corresponding Vβ expression in normal lung or blood; N/A, not applicable. ‡ Vβ6 expression in the lung was 21% compared to 6% in the corresponding blood sample; i.e., greater than a threefold difference compartmentalized to the lung. § Lung IL-2 or blood IL-2 refer to T cell populations cultured in media with recombinant IL-2 for 10–14 d prior to TCR analysis. ** TCR sequences containing an RGR or RGGR segment are underlined. Nucleotide sequences resulting in the consensus motif were identical within each individual sample.

patient 1 both the unstimulated and IL-2 expanded lung T cell populations contained the same dominant Vβ18 sequence with an RGR segment in the junctional region (Table IV). The IL-2 expanded blood T cell line from this same patient was also markedly skewed with the dominant Vβ13 sequence containing an RGR sequence starting at position 98. In patient 4, the IL-2 expanded blood T cell population was dominated by Vβ14 expression of a TCR sequence containing a similar RGGR sequence starting at position 98. In all cases, the nucleotide sequences resulting in the consensus motif were identical within an individual sample. However, no Vβ sequences with an RGR or RGGR sequence in the V(D)J junctional region were seen in over 150 TCR sequences analyzed from T cell populations of normal individuals in the current study and previous reports (26–29). Thus, biased TCR Vβ gene expression in sarcoidosis may be associated with T cells with similar V(D)J junctional region motifs.

Discussion

Sarcoidosis serves as a model of a human disease characterized by chronic granulomatous inflammation. Using a molecular analysis of T cell receptor gene expression, the current study provides evidence that the granulomatous response preferentially involves αβ+ T cells expressing a limited number of Vβ genes. The skewing in the T cell repertoire was most pronounced in the lung, consistent with a relative compartmentalization of the immune response to tissues involved with granulomatous inflammation. The Vβ genes most frequently expressed in lung T cells from this study involved Vβ5, Vβ15, and Vβ18 gene families. Previous work had demonstrated individuals with sarcoidosis that were characterized by biases in αβ+ T cells expressing Vβ8 (16). Together, these studies indicate that biases in the sarcoid T cell repertoire are frequent and can selectively involve different Vβ gene segments.

The fact that subgroups of individuals with sarcoidosis preferentially use different Vβ genes may be the result of their specific MHC background since MHC haplotype can influence both the peripheral TCR repertoire and the use of specific Vβ genes in antigen-specific T cell responses (6, 7, 12, 20, 23). A likely example of the influence of MHC haplotype on the TCR repertoire is the striking association of biased usage of Vα2.3 in the lung and blood of DR3+ Swedish individuals with sarcoidosis (30). Alternatively, selection for specific Vβ genes in some individuals with sarcoidosis may be a function of the antigenic stimulus alone since there are examples of Vβ gene selection in an antigen-specific response independent of MHC haplotype (8). Thus, the use of different Vβ genes in sarcoidosis may reflect an individualized response to the same or different antigenic determinants.

The absence of skewing in the T cell repertoire of some sarcoid lung T cell populations could be due to T cell responses that preferentially utilized Vβ genes not tested. However, a more likely explanation for a lack of bias is that nonspecifically recruited T cells at sites of inflammation are diluting a specific T cell response. Additionally, there may be a diverse T cell response to different antigenic determinants of an initiating antigen(s) depending on the stage and duration of the disease (31).

The T cell repertoire of the normal lung, with the possible exception of Vβ14, varied little from that of normal blood. The relative absence of generalized skewing of the Vβ repertoire suggests that the normal, noninfected lung has a diverse, heterogeneous T cell population on its epithelial lining surface despite chronic exposure to microbial and environmental antigens. Whether the significantly higher expression of some Vβ-specific genes in the normal lung, for example Vβ14, could reflect prior selective stimulation by environmental antigens remains unknown. Such a possibility is enhanced by findings that infectious respiratory pathogens such as the influenza virus preferentially stimulate selective T cells expressing specific T cell receptor genes (10). Thus, it is likely that the T cell population in the normal lung could be skewed toward restricted Vβ-specific T cell subsets upon selective stimulation by potent antigenic or infectious agents.

Several observations from this study support the concept that biases in the T cell receptor repertoire in active pulmonary sarcoidosis are the result of a local antigen-driven inflammatory process. First, the fact that a limited number of specific T cell receptor genes are preferentially used in the lungs of individuals with active pulmonary sarcoidosis suggests an antigenic response. For example, skewing in the T cell repertoire analogous to that observed in sarcoidosis has been reported in chronic beryllium disease, a chronic granulomatous lung disease of known etiology that is histopathologically indistinguishable from pulmonary sarcoidosis (32). Second, although the presence of biases of Vβ-specific T cells in sarcoidosis raises the possibility of a superantigenic stimulus, sequence analysis of Vβ genes that are preferentially expressed in the sarcoid lung in situ or after IL-2 expansion demonstrated limited junctional diversity consistent with a conventional antigenic stimulus. Finally, the preferential accumulation of Vα2.3+ T cells in the lung and blood of DR3+ Swedish individuals with sarcoidosis is also consistent with stimulation by a conventional antigen (30).

The selective use of specific Vβ genes in active pulmonary sarcoidosis was illustrated by a TCR repertoire analysis of lung and blood T cells cultured in the presence of IL-2. In several instances, short-term culture with IL-2 resulted in a marked exaggeration of an already existing biased Vβ gene or the expansion of a Vβ subset that was expressed at a relatively high, though unbiased, level. However, in other instances, discordance between the initial unstimulated T cell population and the same T cell population cultured with IL-2 was observed, and at times, was quite striking. It is possible in these instances that the in situ expanded T cell population was relatively refractory to further proliferation with IL-2 or that additional growth factors were needed for proliferation. In these cases, a small subset of recently activated, unrelated Vβ-specific T cell clones may be primed for rapid expansion with IL-2 resulting in their domination of the T cell repertoire. The observation of Vβ bias in IL-2 expanded sarcoid blood T cell populations is consistent with previous studies demonstrating the presence of functional, IL-2R+ T cells in the blood compartment (3).

The possibility that the inflammatory response in sarcoidosis may be instigated and/or perpetuated by a limited set of antigens is supported by an analysis of the deduced TCR β-chain amino acid sequences of sarcoid T cell populations with biased usage of Vβ genes. The dominant Vβ genes expressed by some of these sarcoid T cell populations contained amino acid sequence similarities consisting of an RGR or RGGR sequence residing in the CDR3 region of the Vβ gene. Such similarities within the hypervariable region of the β chain suggest selective activation of these T cells by specific antigen-MHC combinations. Interestingly, a similar motif was seen in a separate study analyzing the T cell repertoire at sites of Kviem–Siltzbach reactions, a specific localized granulomatous response resulting from the intradermal injection of sarcoid tissue into an individual with sarcoidosis (manuscript in preparation). In one of the Kviem–Siltzbach reaction biopsy sites, TCR Vβ gene expression was dominated by the expression of a Vβ8.1-Jβ2.1-Cβ2 sequence which contained an RGR motif in the V(D)J junctional region. Although additional examples of this motif are likely to be found, the presence of an uncommon amino acid sequence in several of the dominant TCR sequences of selected, biased T cell populations in sarcoid lung, blood and skin, invites the speculation that these T cells may be responding to a similar antigenic determinant. The use of different Vβ genes by T cell clones with this motif could be explained by differences in the MHC haplotypes of these individuals. In keeping with this possibility, there are examples of antigen-specific T cells with conserved V(D)J junctional region amino acids located at variable lengths from conserved 3′ Vβ residues when using different Vβ genes (12).

Although there is evidence that sarcoidosis is an antigen-driven disorder, the nature of such a putative antigen remains unknown. Attractive possibilities include antigens derived from infectious agents or autoantigens. In this regard, it is intriguing that one of a panel of *M. leprae*-specific T cell clones responsive to the 65-kD heat shock polypeptide expressed a β chain with a Vβ5 gene member and a junctional region sequence with an RGGR sequence similar to the junctional region motif seen in sarcoidosis (11). Although any correlation of CDR3 region motifs to T cell specificity remains highly speculative, the hypothesis that a subset of biased sarcoid T cell clones may have specificity for either exogenous or endogenous heat shock proteins is enhanced by reports of 65-kD heat shock protein expression at sites of sarcoid granulomatous inflammation and from studies of γδ+ T cells in sarcoidosis (25, 33–36). It is conceivable that the preferential use of specific Vβ genes in sarcoidosis reflects an autoimmune process to self-antigens such as endogenous stress proteins or other autoantigens with homology to infectious agents (36). Results similar to this study showing

limited heterogeneity of T cell receptor gene expression have been described in autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (14, 15).

Regardless of the underlying stimulus, the clonal expansion of T cells with restricted T cell receptor heterogeneity isolated de novo or after short term culture with IL-2 implies the presence of functionally responsive antigen-specific T cell populations in both the blood and lungs of individuals with sarcoidosis. Analysis of these T cell clones isolated in vitro may provide evidence for their playing a central role in granulomatous inflammation by demonstrating an ability to express lymphokines important to granuloma formation. Such evidence would provide a rational basis for efforts designed to specifically target these restricted and potentially pathogenic T cells.

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