

Characterization of the Interstitial Lung and Peripheral Blood T Cell Receptor Repertoire in Cigarette Smokers

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T lymphocytes modulate the pulmonary inflammatory response. The aim of this study was to evaluate the clonality within the interstitial lung and peripheral blood T cell receptor (TCR) repertoire in smokers. Interstitial T lymphocytes were isolated from surplus tissue of 16 patients (63 ± 9 [\pm SD] yr old, 11 male) undergoing surgery due to lung cancer ($n = 15$) or emphysema. TCR clonality was assessed by PCR amplification followed by spectratyping. Nearly all TCR of interstitial lung lymphocytes showed oligoclonal bands ($CD4^+$ subset 13/16 patients, 81%; $CD8^+$ 100%) indicating a specific differentiation. Peripheral blood T lymphocytes (PBL) TCR (especially $CD4^+$) had less oligoclonal bands ($CD4^+$ 31%, $CD8^+$ 88%). Likewise, more oligoclonal bands were seen in lung TCR (total of 168 bands; 37 $CD4^+$; 131 $CD8^+$), compared with 59 bands in PBL TCR (13 $CD4^+$; 46 $CD8^+$). Intraindividual comparison revealed a more prominent difference in TCR oligoclonality between lung and blood in $CD8^+$ T cells (median of difference lung minus blood 5; interquartile range 1–10; $P = 0.002$) compared with $CD4^+$ T cells (median 2, 0–3, $P = 0.039$). Thus, TCR oligoclonality is preferentially found in the $CD8^+$ T cell subset, most distinctive in the lung. These findings indicate a specific interstitial T cell differentiation in response to local stimuli.

Keywords: smoker; lymphocytes; T cell receptor; lung; interstitial compartment

The antigen recognition structure on T lymphocytes, the T cell receptor (TCR), is responsible for helper T cell function in humoral immunity and for killer T cell function in cell-mediated immunity (1). Exposure of naive T cells to antigen leads to activation and clonal expansion (2). Thus, an individual's TCR repertoire at any point in time may be a function of recent exposure to various antigens and pathogens (2). Therefore it is important to characterize the TCR repertoire regarding to the frequency of oligoclonality as a surrogate marker for clonal expansion. In addition, some disease processes appear to alter the "normal" TCR repertoire, providing the opportunity to identify disease-specific T cell response patterns. More than a decade ago, several groups have focused on the investigation of TCR repertoires in various diseases, e.g., in autoimmune diseases such as multiple sclerosis (3, 4) and Sjögren's syndrome (5, 6), demonstrating oligoclonal T cell accumulation in inflamed lesions. Another focus of investigation is the analysis of TCR repertoires in malignancies, especially in hematologic diseases, where TCR

$V\beta$ clonotyping is currently explored as a potential diagnostic tool, analogous to serologic markers (7).

The TCR is a heterodimer made up of two glycoproteins of highly variable structure that is clonally distributed (2). Of the two types of TCR, the $\alpha\beta$ heterodimer is the most common antigen receptor on human T cells, present on over 95% of peripheral blood T cells (2). Both the α and β chain consist of variable and constant regions. Human lymphocytes expressing $\alpha\beta$ TCR may be grouped by the β chain variable region sequence into 24 families defined on the basis of a DNA sequence homology of $\geq 75\%$ and termed β variable (BV) families at the gene segment level (8). The hypervariable region of the β chain known as the complementary determining region 3 (CDR3) is the part of the TCR most intimately associated with the peptide-MHC complex involved in specific T cell recognition (9). The CDR3 mRNA transcripts from a population of T lymphocytes of mixed specificity differ not only in nucleotide sequence, but also in length. The distribution of lengths of CDR3 transcripts within a BV family may thus be used to assess the TCR repertoire within a given population (9). Restriction in CDR3 length within a particular BV segment is visualized as a dominant band depending on its intensity with respect to the rest of the bands within the TCR BV specific PCR product, also named as oligoclonal band (10).

In patients with lung diseases, investigators have analyzed the TCR repertoire in tumor-infiltrating lymphocytes in patients with non-small cell lung cancer (11), as well as in various non-malignant conditions. Bronchoalveolar lavage (BAL) fluid T cells were characterized in sarcoidosis (12, 13) and asthma (14, 15). However, tumors or the compartment accessible by BAL may not necessarily reflect events in the "normal" lung parenchyma. To date, to the best of our knowledge, there are no reports on the TCR repertoire of interstitial lung T cells, neither in healthy individuals nor in patients with lung diseases.

To clarify the T cell receptor oligoclonality in the interstitial compartment, we analyzed the TCR repertoire and the oligoclonal expansion of T cells infiltrating the peripheral lung. We evaluated lung tissue of smokers who underwent surgery due to smoking-related pulmonary disease, taken far distant from the affected site. By comparison with the TCR repertoire of peripheral blood T lymphocytes (PBL) in the same patients, we observed that interstitial lung lymphocytes show a clearly increased oligoclonality, and that this finding is most distinctive in $CD8^+$ T cells.

MATERIALS AND METHODS

Patients

Sixteen smoking patients who underwent surgery due to lung cancer ($n = 15$) or emphysema ($n = 1$) participated in the study. The median age of the patients (11 male and 5 female) was 62.5 yr (interquartile range [IQR] 56.0–71.5 yr). All patients were smokers with a median smoking history of 30 pack-years (IQR 20–40 pack-years; Table 1). An excisional subpleural biopsy from surplus tissue obtained far distant from the affected site and blood samples were taken from each patient.

(Received in original form July 27, 2004 and in revised form October 13, 2004)

This study was supported in part by the Deutsche Forschungsgemeinschaft (DFG Grant BE2020/3-1, to K.B.).

* This publication is part of Yvonne C. Walz's doctoral thesis.

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Am J Respir Cell Mol Biol Vol 32, pp 142–148, 2005

Originally Published in Press as DOI: 10.1165/rncmb.2004-0239OC on November 11, 2004
Internet address: www.atsjournals.org

TABLE 1. PATIENT CHARACTERISTICS

Patient	Age (yr)	Sex	Tumor Histology	Smoking (pack-years)	FEV ₁ (% predicted)
1	77	Male	Adenocarcinoma	14	112
2	54	Male	Squamous cell carcinoma	10	72
3	73	Male	Adenocarcinoma	40	69
4	74	Female	Squamous cell carcinoma	40	106
5	44	Female	Large cell carcinoma	20	89
6	56	Male	Adenocarcinoma	35	118
7	60	Female	Small cell lung cancer	20	92
8	56	Male	Small cell lung cancer	54	64
9	54	Female	Emphysema	30	31
10	62	Male	Squamous cell carcinoma	40	70
11	62	Male	Adeno squamous carcinoma	30	90
12	67	Female	Adenocarcinoma	20	124
13	65	Male	Squamous cell carcinoma	30	85
14	62	Male	Adenocarcinoma	40	83
15	65	Male	Adenocarcinoma	10	80
16	76	Male	Adenocarcinoma	40	73

All subjects gave written informed consent and the study was approved by the local hospital ethics committee.

Immunomagnetic Separation of CD4⁺ and CD8⁺ T Cells

Peripheral blood mononuclear cells (PBMCs) were purified from heparinized venous blood, using density gradient centrifugation by Ficoll-Hypaque (Amersham Biosciences AB, Uppsala, Sweden). After centrifugation, cells at the interface were collected, washed twice, and resuspended in phosphate-buffered saline and 0.1% bovine serum albumin. Fresh surplus tissue was mechanically and enzymatically dissociated in RPMI medium supplemented with 10% fetal bovine serum (Gibco/Invitrogen GmbH, Karlsruhe, Germany), DNAase 0.3 U/ml (Sigma, St. Louis, MO), collagenase 0.5 U/ml (Sigma), and dispase 2 mg/ml (Sigma) as described elsewhere (11, 16) and kept over night at 37°C. The cell suspension was put through a cell strainer, and purified by a Percoll density gradient centrifugation (Amersham Biosciences AB, Uppsala, Sweden).

Interstitial lung and peripheral blood T cells were isolated, respectively, by sheep red blood cell rosetting (5%) as previously described (17). T cells were further separated into CD4⁺ and CD8⁺ populations using anti-CD4- and anti-CD8-labeled immunomagnetic beads (Dyna, Great Neck, NY) according to the manufacturer's instructions: the cells were incubated with the specified beads for 20 min at 4°C on a rotating shaker. Cells bound to the beads were placed directly into Trizol Reagent (Gibco BRL, Grand Island, NY) for RNA extraction.

TCR Repertoire/CDR3 Length Analysis

The TCR repertoire was analyzed by a multiplex PCR assay as previously described (18). A combination of two or three forward primers, specific for the variable regions of different BV families, and a reverse primer specific for the constant region of the TCR β-chain were coamplified in the same reaction. The different size of the products permitted specification of the different BV families (see Table 2 for primer sequences). The cDNA synthesis was performed with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY) at 40°C for 1 h in a total volume of 20 μl (4.5 μl RNA). The reaction was stopped by heat inactivation (99°C for 5 min). For each PCR reaction, 0.5 μl of cDNA, 10 pM each forward and reverse primer, *Hot Star Taq-Polymerase* (1 U) with the supplied buffer, 2 mM MgCl₂ and 200 mM dNTPs (Perkin-Elmer, Wellesley, MA) were used in 25 μl final volume. The PCR cycle was 15 min at 95°C to activate the enzyme, then 94°C for 60 s, 55°C for 60 s, and 72°C for 1 min. After 35 cycles, a 10-min extension at 72°C was conducted.

Ten microliters of the PCR product was run on a standard 6% polyacrylamide sequencing gel. The bands were detected by silver staining (Promega, Madison, WI). The gel was scanned and band intensity was quantified with Imagequant Molecular Dynamics software by two independent investigators to improve reproducibility of the image analysis. This allowed us to evaluate the bands' intensity within each BV family. Restriction in CDR3 length was detected as a dominant band

within the cluster of bands derived from each TCR BV family. A band was defined as dominant when its intensity was ≥ 50% within a given BV family. Based on established criteria (10), restriction of the CDR3 length is highly suggestive of oligoclonality.

Determination of BV Families by Flow Cytometry

Detection of individual BV families was performed in nine patients using surplus interstitial lung and peripheral blood T cells (19). A total of 2 × 10⁵ cells were stained simultaneously with monoclonal antibodies (MAbs) directed against CD4 (energy-coupled dye) and CD8 (R-phycoerythrin-cyanin 5 [PC5]) and a set of three antibodies directed against TCR BV families according to the manufactures instructions (IOTest Beta Mark TCR Vβ Repertoire Kit; Beckman Coulter, Marseille, France). MAbs directed against TCR families were labeled with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE), and the third anti-TCR directed MAb was labeled with PE and FITC. Nineteen of the 24 BV families used in this kit were identical with primers used in TCR-spectratyping (see Table 3 for identical BV families). Flow cytometry was performed using a Coulter Epics Altra instrument and EXPO32 software. Cells were gated either on the CD4⁺ or

TABLE 2. SEQUENCES OF THE PRIMERS USED FOR PCR

Primer	Sequence	Reaction Set
BV1	5'-CAA CAG TTC CCT GAC TTG CAC-3'	A
BV18	5'-GAG TCA GGA ATG CCA AAG GAA-3'	
BV23	5'-TCA TTT CGT TTT ATG AAA AGA TGC-3'	
BV2	5'-TCA ACC ATG CAA GCC TGA CCT-3'	B
BV4	5'-CAT ATG AGA GTG GAT TTG TCA TT-3'	
BV8	5'-TAC TTT AAC AAC AAC GTT CCG-3'	
BV3	5'-TCT AGA GAG AAG AAG GAG CGC-3'	C
BV13S1	5'-GAC CAA GGA GAA GTC CCC AAT-3'	
BV5S2	5'-CCT AAC TAT AGC TCT GAG CTG-3'	
BV20	5'-TCT GAG GTG CCC CAG AAT CTC-3'	D
BV5S1	5'-TTC AGT GAG ACA CAG AGA AAC-3'	
BV6	5'-AGG CCT GAG GGA TCC GTC TC-3'	E
BV7	5'-CTG AAT GCC CCA ACA GCT CTC-3'	
BV22	5'-CAG AGA AGT CTG AAA TAT TCG A-3'	F
BV9	5'-AAA TCT CCA GAC AAA GCT CAC-3'	
BV16	5'-GAG TCT AAA CAG GAT GAG TCC-3'	G
BV11	5'-ACA GTC TCC AGA ATA AGG ACG-3'	
BV12	5'-GAC AAA GGA GAA GTC TCA GAT-3'	H
BV15	5'-GTC TCT CGA CAG GCA GCT CAC-3'	
BV13S2	5'-GTT GGT GAG GGT ACA ACT GCC-3'	I
BV14	5'-TCT CGA AAA GAG AAG AGG AAT-3'	
BV17	5'-CAC AGA TAG TAA ATG ACT TTC AG-3'	J
BV24	5'-AAA GAT TTT AAC AAT GAA GCA GAC-3'	
BV21	5'-GAT ATG AGA ATG AGG AAG CAG-3'	L
CB-R	5'-CTT CTG ATG GCT CAA ACA C-3'	

TABLE 3. FREQUENCY OF CLONAL EXPANSION

BV	Lung CD4 ⁺		Lung CD8 ⁺		Peripheral Blood CD4 ⁺		Peripheral Blood CD8 ⁺		All Compartments	
	CE (n)	CE + O (n)	CE (n)	CE + O (n)	CE (n)	CE + O (n)	CE (n)	CE + O (n)	CE (n)	CE + O (n)
1										
2										
3										
4			1	1					1	1
5S1										
5S2										
8	1	0							1	0
9										
11			2	1			2	0	4	1
12	5	0	4	1	2	0	4	1	15	2
13S1	1	1	3	0			1	0	5	1
13S2	1	0	1	0	2	0	2	1	6	1
14	1	0							1	0
16	2	0	3	0	2	0	4	0	11	0
17			1	1					1	1
18			1	1					1	1
20	1	0							1	0
22										
23	1	0	2	1			1	0	4	1
Total	13	1	18	6	6	0	14	2	51	9

Frequency (n) of clonal expansion (CE) in lung and blood for various BV families was determined by flow cytometry in a subset of patients ($n = 9$). Intraindividual comparisons with oligoclonality (O) in all compartments revealed coincidences in 9/51 cases (17.6%). For statistical analysis see Figure 5.

on the CD8⁺ population, and individual TCR families were evaluated based on T cells showing exclusive staining for either FITC or PE or double staining for both FITC and PE. Clonal expansion was defined as a result greater than the mean percentage plus three standard deviations of expression of a given BV family in CD4⁺ and CD8⁺ lymphocyte subsets from a cohort of 85 normal peripheral blood specimens as described by the manufacturer of the kit. All reagents were from Beckman/Coulter, Krefeld, Germany.

Statistical Analysis

Data description is primarily based on medians and quartiles, graphic representation on nonparametric boxplots displaying counts of oligoclonal bands. For the primary statistical analysis, the count differences of oligoclonal bands between lung and peripheral blood samples are compared intraindividually. The resulting two pairwise comparisons are based on nonparametric sign tests (P values < 0.025 indicate multiple significance according to the Bonferroni correction method). Additionally, counts of oligoclonal bands of CD4⁺ and CD8⁺ cells are compared intraindividually both between and within lung and peripheral blood samples. For these exploratory comparisons sign tests P values < 0.05 indicate local statistical significance. Interindividual comparisons of count data between patient subgroups are based on two sample Wilcoxon tests; again P values < 0.05 indicate local statistical significance. Univariate tests for significant compartment differences were based on McNemar's test for categorical data. Correlations between continuous endpoints like age and the oligoclonal band counts are estimated by means of Spearman's nonparametric correlation coefficient.

RESULTS

All 16 smoking individuals showed oligoclonality within the CD8⁺ T cell subset in the lung, with a median of 8.2 dominant bands (IQR 4.5–11.5 bands) per patient. Figure 1 shows a representative silver gel of a complete TCR analysis. Conversely, oligoclonality was found notably reduced within the CD4⁺ T cell population in the lung (13/16 patients, 81%), with a median of 2.3 clonal bands (IQR 1.3–3.0 bands) per patient (Figure 2A). In PBL oligoclonal bands were found less frequently in both CD8⁺ and CD4⁺ T cells (CD8⁺ 14/16 patients, 88%; CD4⁺ 5/16 patients, 31%). CD8⁺ and CD4⁺ T cells of PBL showed a median of 2.9 and 0.8 dominant bands (IQR 1.0–4.8 and 0.0–1.0 bands), respectively (Figure 2B). Likewise, more oligoclonal bands were

seen in the lung (total of 168 bands; 131 CD8⁺; 37 CD4⁺), compared with 59 bands in PBLs (46 CD8⁺; 13 CD4⁺).

Intraindividual comparisons between lung and blood revealed a more prominent difference in TCR oligoclonality in CD8⁺ T cells with a median difference (lung minus blood) of 5.0 counts (IQR 1.0–10.3 counts) compared with a median 1.5 bands in CD4⁺ T cells (IQR 0.0–3.0; Figure 3A). Only for the CD8⁺ T cell population this sample difference was found to be statistically significant after Bonferroni correction (CD8⁺ $P = 0.002 < 0.025$, CD4⁺ $P = 0.039$). Intraindividual comparisons between CD8⁺ and CD4⁺ cells showed a median difference (CD8⁺ minus CD4⁺) of 5.0 dominant bands (IQR 2.3–8.8, $P = 0.001$) in TCR oligoclonality in the lung compared with a median difference of 1.5 (IQR 0.0–3.8, $P = 0.022$) counts in peripheral blood (Figure 3B).

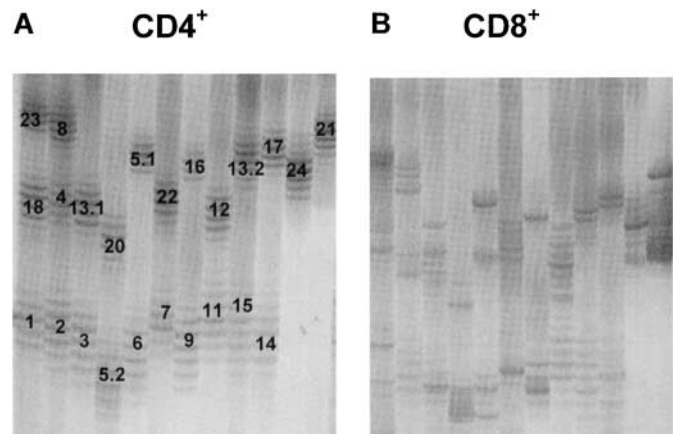


Figure 1. Representative silver gel showing a complete TCR analysis. After PCR using 24 TCR BV primers, samples were run on a 6% polyacrylamide sequencing gel, bands were detected by silver staining, scanned and digitally analyzed using the using appropriate image quantification software. Shown are reactions for 24 TCR BV families with interstitial lung samples from CD4⁺ (left panel, A) and CD8⁺ (right panel, B) T cells.

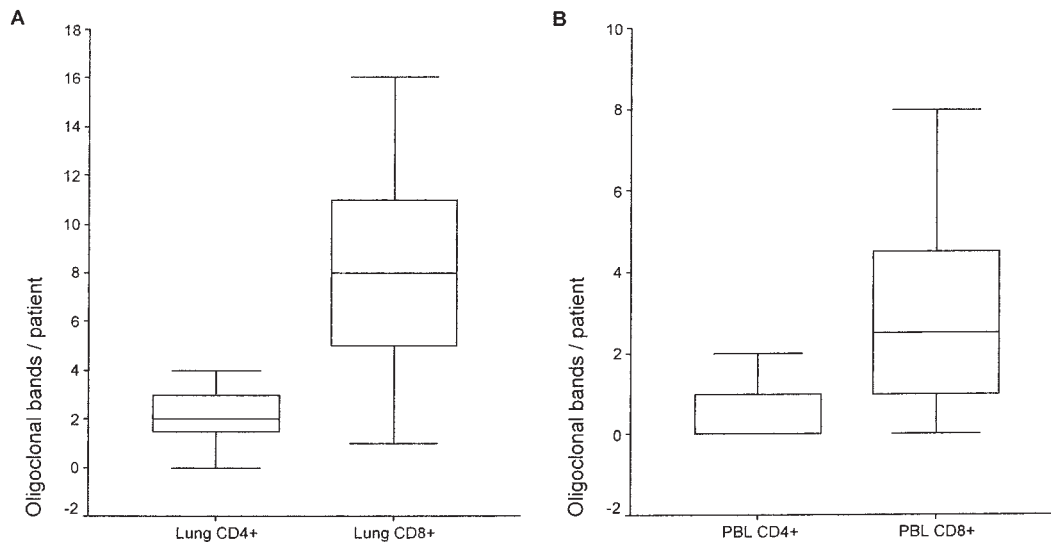


Figure 2. Oligoclonal bands per patient. *Box whisker plots* display counts of oligoclonal bands in CD4⁺ and CD8⁺ T cells in interstitial lung T lymphocytes (A) and peripheral blood T lymphocytes (B). *Horizontals* indicate the medians and 1st/3rd quartiles, *whiskers* indicate minimum and maximum.

In this patient group there was no clinically relevant correlation between oligoclonality and age of the patients, neither for CD4⁺ and CD8⁺ populations (lung and blood) nor for any of the intraindividual differences (lung minus blood or CD8⁺ minus CD4⁺); correlation coefficients with age ranged between -0.107 and 0.158 . Other clinical cofactors such as pack-years and lung function did not show a statistically significant or clinically relevant impact on the oligoclonality of the TCR in interstitial lung or peripheral blood. Patients' sex had only minor influence on TCR oligoclonality. In female patients, oligoclonal bands in CD4⁺ T cells in the peripheral blood were lower compared with males (female: no oligoclonal bands; male: median 1.0, IQR 0.0–2.0, $P = 0.090$). In addition, the difference between lung and blood in the CD4⁺ T cell population was significantly higher in female patients (female: median count difference 3.0 bands, IQR 1.5–5.0 versus male: median count difference 0.0 bands, 0.0–2.0, $P = 0.029$).

Analyzing the appearance of oligoclonal bands per each BV family member (Figure 4), oligoclonality was observed in all 24 TCR BV families in CD8⁺ interstitial lung T lymphocytes and in nearly all families (23/24 families) in CD4⁺ interstitial lung

T cells. In contrast, clonal dominance was markedly reduced in PBL (CD8⁺: 21/24 families; CD4⁺: 9/24). The difference in occurrence frequencies between lung and blood was significant only for CD4⁺ T cells ($P < 0.001$), the corresponding difference between CD8⁺ and CD4⁺ only for PBL ($P < 0.001$).

With respect to the frequency of oligoclonal TCR BV family expression, the highest counts of oligoclonal bands of a given BV family were demonstrated in the lung with a peak expression rate of 63% (Figure 4). Summarizing the four compartments, in CD8⁺ interstitial T lymphocytes oligoclonality was found most frequently in BV1 and BV18 (10/16 patients each), in CD4⁺ interstitial T cells in BV6 (6/16), in CD8⁺ PBL in BV2 and BV4 (5/16 each), and in CD4⁺ PBL in BV1, BV4 and BV21 (2/16 each).

In addition, using flow cytometry a total of 51 clonal expansions were detected in lung and blood CD4⁺ and CD8⁺ T cells (lung CD4⁺: 13 clonal expansions, CD8⁺: 18, PBL CD4⁺: 6, CD8⁺: 14; Table 3). At first sight, the results of clonal expansion seem to be well in agreement with the findings of oligoclonality, both of which were predominantly detected in CD8⁺ interstitial lung T cells. However, the results of the two analyses rarely

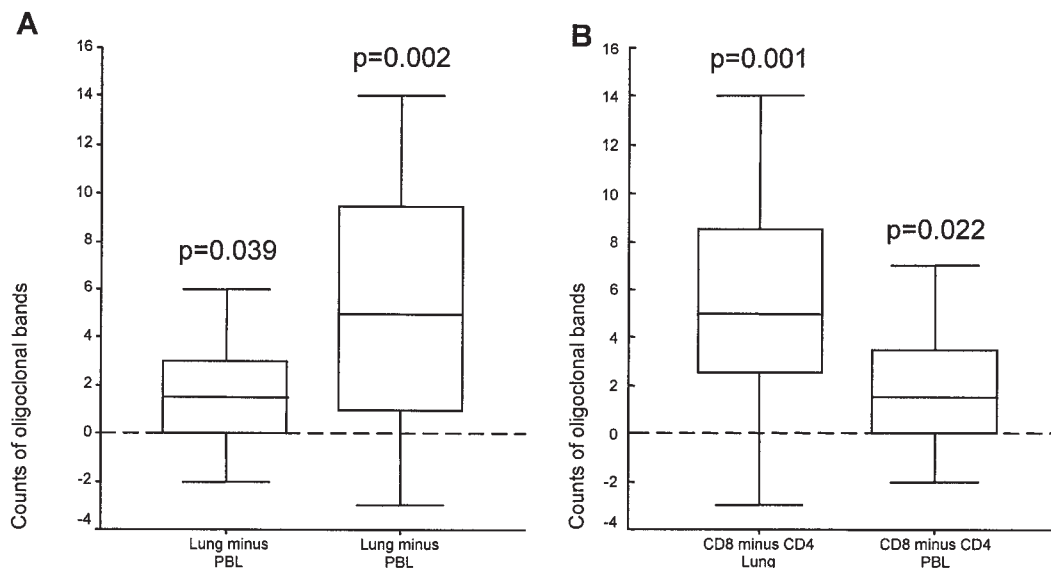


Figure 3. Intraindividual comparisons. *Box whisker plots* for the respective intraindividual comparison between lung and peripheral blood (A) and between CD8⁺ and CD4⁺ T cells (B) in counts of oligoclonal bands. *Horizontals* indicate the differences' medians and 1st/3rd quartiles, *whiskers* indicate minimum and maximum of observed count differences.

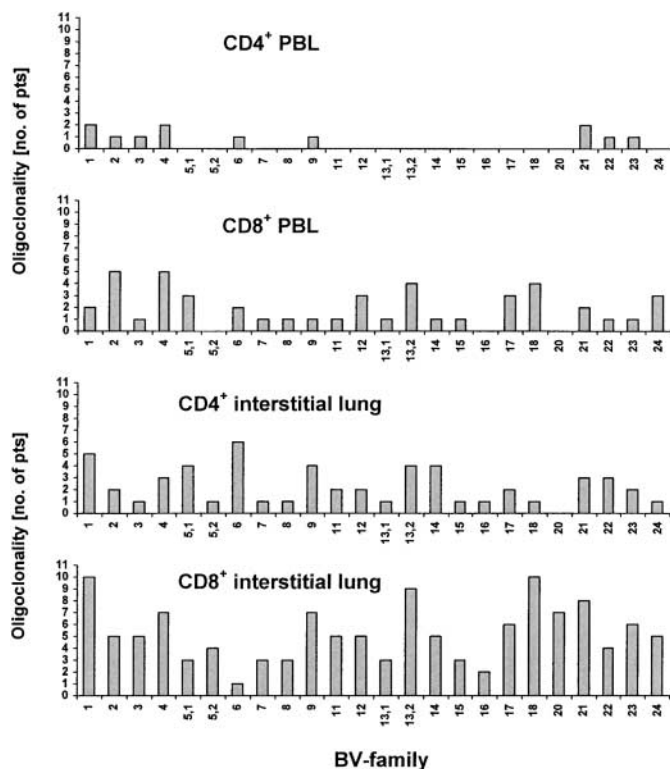


Figure 4. Frequency of oligocloneality. Bar graph representing the distribution of the frequency of oligocloneality across 24 different BV families in smokers following multiplex PCR for CDR3 length. Number of patients with oligoclonal bands in CD4⁺ cells und CD8⁺ cells of peripheral blood lymphocytes (A, B) as well as interstitial lung lymphocytes (C, D) are displayed for each BV family.

match: only in 9 out of 51 observations clonal expansion and oligocloneality were present (Table 3). Intraindividual analyses revealed a median concordance of one BV family per patient (Figure 5) and a discordance of four BV families per patient ($P = 0.039$; Figure 5), confirming that there is no correlation between oligocloneality and clonal expansion of a given BV family.

DISCUSSION

In the present study the T cell receptor repertoire of CD4⁺ and CD8⁺ lymphocytes both in lung interstitial tissue and peripheral blood of smokers was characterized. Oligocloneality was demonstrated in nearly all TCR of interstitial lung lymphocytes. In contrast, PBL (especially CD4⁺) had less oligoclonal bands. Oligoclonal expansion was preferentially found in the CD8⁺ T cell subset, in particular in the lung.

Only few investigators have tried to characterize the TCR repertoire of interstitial T lymphocytes (13). In sarcoidosis, using BAL cells and transbronchial biopsy material, enzymatic digestion of the small tissue pieces resulted in very low lymphocyte numbers, allowing only analyses of a limited number of BV families. These analyses demonstrated that the immune reaction in sarcoidosis was strongly compartmentalized and that interstitium and alveolar region differ in the composition of the T cell subpopulation (13). However, bronchoalveolar T cells may only reflect a small part of the lung T cell pool (13–15, 20–25).

The lung interstitium is the largest compartment, including $\sim 10^{10}$ T cells (16). To perform a complete TCR repertoire analysis of interstitial T lymphocytes adequate amounts of mate-

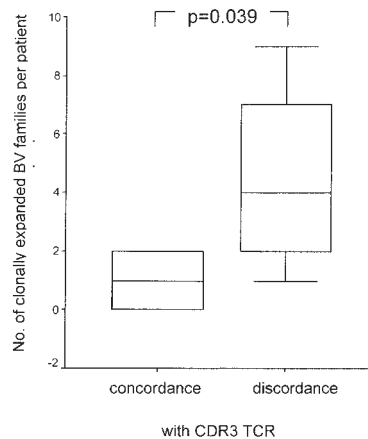


Figure 5. Clonal expansion and oligocloneality of BV families. Box whisker plots display numbers of BV families per patient detected as clonally expanded that does (left) and does not (right) coincide with oligocloneality of the particular BV family. Horizontal lines indicate the medians and 1st/3rd quartiles, whiskers indicate minimum and maximum.

rial must be used with a minimum of 5×10^4 cells per BV family (9). Hence, transbronchial biopsies contain not enough T cells (13). We used surplus tissue from patients undergoing lung resection with a weight of ~ 10 g yielding at least 10 million T cells per sample (data not shown). To maximize the yield of interstitial T lymphocytes, only peripheral, subpleural tissue was used. Naturally, this technique cannot exclude a minimal contamination with lymphocytes stemming from other compartments. The contamination of the interstitial T cell pool by peripheral T cells originating from the pulmonary vasculature is, as measured by contaminating erythrocytes, below 1%. A complete separation of the bronchoalveolar and interstitial compartments is currently impossible, even using advanced microdissection techniques. However, the optimized purification process followed in this study including several washing steps before enzymatic digestion of the tissue samples together with the absolute numerical difference between interstitial and bronchoalveolar compartment in the order of two magnitudes ensure a homogenous interstitial T cell population with negligible peripheral and bronchoalveolar contamination. This raises the question whether the subpleural location of tissue samples may influence TCR repertoire analyses. Although this possibility cannot completely be excluded, at least BV gene expression patterns in bronchoalveolar T cells obtained from three separate lobes of the lung of a single patient were similar (20).

This approach allowed for the first time the analysis and characterization of a complete TCR repertoire of interstitial lung T lymphocytes, providing a basis for further investigations of this compartment in specific lung diseases. Interpretation of the data of this study is limited by the lack of healthy, nonsmoking control subjects. However, the majority of patients undergoing lung surgery involving resection of significant quantities of lung tissue are smokers presenting with lung cancer or emphysema. In contrast, the majority of pulmonary nodules in nonsmoking patients are benign. In most cases, upon intraoperative confirmation of benignity, the volume of adjacent tissue resected with the nodule is limited as much as possible, to avoid unnecessary functional losses. For these reasons, the analysis of the interstitial lung T lymphocyte population was started in smokers, with peripheral blood cells included as intraindividual controls. CDR3 length analysis to evaluate oligocloneality of the TCR repertoire was chosen because CDR3 length distribution may be determined rapidly and in a statistically robust manner (9).

The analysis of interstitial T cells of smokers is of great interest given the increased evidence for a major role of T lymphocytes, especially CD8⁺ T cells, in smokers with airway inflammation who develop chronic obstructive pulmonary disease and/or lung

cancer (26–29). These findings suggest that tissue injury is dependent on T lymphocyte abnormality and might be of critical importance for the pathogenesis of smoking-related diseases.

About a decade ago the TCR repertoire was characterized predominantly in peripheral blood T cells of healthy individuals (10, 20, 21, 30, 31). All investigators showed that oligoclonality is more common in CD8⁺ than in CD4⁺ PBL. Likewise, a substantial individual variation in BV segment frequencies in PBL of normal individuals was reported (30). The functional significance of clonal dominance in the CD8⁺ compartment is as yet unclear. Extreme clonality within the peripheral T cell compartment has generally been interpreted as due to malignant or premalignant transformation of such cells (10). However, recent studies have shown that quite restricted clonotypes may arise in the course of some antigen-specific T cell responses, perhaps as a result of chronic subclinical virus infection (31).

There are several studies that compare the TCR repertoire of lung lymphocytes obtained by bronchoalveolar lavage with peripheral blood lymphocytes (13–15, 20–22, 24). In healthy non-smokers, in terms of clonal composition the TCR repertoire in the lung was largely as heterogeneous as in peripheral blood (20, 21). In addition, lung T lymphocytes expressed all the BV gene families of the TCR that were also expressed by peripheral blood T cells. Both the T cell clones of the lower respiratory tract and of peripheral blood showed predominantly polyclonality (21) and BV gene families were expressed at similar levels (20). This is in clear contrast to our analyses of interstitial T cells in smokers, where all patients showed oligoclonality not corresponding to findings in peripheral blood.

In patients with pulmonary sarcoidosis, clonality of bronchoalveolar T cells is significantly greater than that of PBL (22). This indicates that in sarcoidosis T cell accumulation in the lung is not a passive event reflecting only influx of peripheral blood T cells, but rather an accumulation and expansion in response to as yet unknown stimuli. Sarcoid T cell clones use a TCR BV gene repertoire consisting of all BV1 to BV24 subfamilies, and not just a limited number of BV families (22). Thus, based on the results of the present study, on the TCR receptor level sarcoidosis and cigarette smoking result in a very similar pattern. Potential explanations are that stimulated, expanded T cells use a wider range of BV subfamilies, and that not all T cells accumulating in sarcoid or smokers lungs are specific to disease-related antigens.

As in sarcoidosis, T cells in BAL fluid of patients with idiopathic pulmonary fibrosis expand oligoclonally in the lung compared with healthy subjects, also suggesting antigen stimulation of these cells (25). A similar pattern was demonstrated in a patient with chronic eosinophilic pneumonitis, where BAL T cells showed an expanded clonality compared with PBL (24). The lung TCR repertoire in individuals with asthma was shown to be broadly representative of blood T cells, with population differences that may result from response to persistent exposure to airborne antigens common to normal and atopic individuals (15). In this investigation oligoclonal TCR BV expansion appeared to be primarily lung specific, but independent of atopic asthma. In contrast to the results of the present study, there were no significant differences in TCR repertoire between CD4⁺ and CD8⁺ T cell subpopulations, and all TCR BV gene families in blood T cells were polyclonal (15). Another study has shown that some TCR BV gene segments were expressed to different degrees in BAL and PBL in nonsmokers with asthma (14). This is in line with the findings in smokers in the present study showing clear differences between the TCR repertoire in PBL and interstitial lung cells.

Various authors tried to link certain BV subgroups with specific diseases (10, 11, 13, 14, 32, 33). Broadly speaking, there is

low evidence for disease-specific BV subgroup oligoclonality, even in lung malignancies. In patients with non-small lung cancer, tumor infiltrating T cells and PBLs displayed a diverse TCR repertoire, with virtually all BV specifications being expressed (11). However, associations have been postulated for asthma (BV3, 5S2, 6S1–3) (14), sarcoidosis (BV5, 6) (13), and chronic Chagas' disease (BV7 in patients with arrhythmia) (32). With respect to the frequency of certain BV families in our patient population, we found no uniform distribution, neither in PBL and lung nor in CD4⁺ and CD8⁺ T cells (Figure 4). A potential hypothesis to explain these findings involves various triggers responsible for oligoclonal expansion of T cells in smokers.

In principle, antigen-specific T cell responses may be described either based on the quality of T cells as defined by the TCR structure using CDR3 length distribution or based on the quantity of T cell responses as defined by the number of responding T cells within the CD4/CD8 population using quantitative measurements of individual BV families. In a recent study an improved assessment of T cell receptor BV repertoire by combination of TCR CDR3 spectratyping with flow cytometry based TCR BV frequency analysis is described (34). Using a similar approach, the results presented in this study show no correlation between oligoclonality and clonal expansion of a given BV family as assessed by flow cytometry analyses. These findings again confirm the difference between oligoclonality and clonal expansion and highlight that immunostimulatory effects on (interstitial lung) T cells may lead independently to development of oligoclonality as well as to expansion of certain polyclonal BV families.

In summary, in a population of smoking patients intraindividual comparisons between lung and blood revealed a more prominent difference in TCR oligoclonality in CD8⁺ T cells compared with CD4⁺ T cells. These results underline the broad diversity in the patterns of CD8⁺ T cell oligoclonality, with probably complex environmental interference (10, 20). It is tempting to speculate that cigarette smoking may be an important contributing factor promoting oligoclonal expansion of interstitial T lymphocytes. Heavy smokers are more susceptible to subclinical chronic viral and bacterial infections, at least partially explaining an oligoclonal expansion of T cell receptors. Another potential explanation for the increased oligoclonality in CD8⁺ T cells is the age of our patients. A natural accumulation of clonal populations of CD8⁺ T cells with aging is well accepted (17). Taken in the context of previous studies, the findings of the present study add further evidence to the concept of alterations in the T cell populations in smokers, potentially contributing to the development of lung specific disease processes such as chronic obstructive pulmonary disease. This is even more so because all patients investigated in this study developed some form of lung disease consequent to cigarette smoking.

Conflict of Interest Statement: S.K. has no declared conflicts of interest; R.W. has no declared conflicts of interest; Y.C.W. has no declared conflicts of interest; K.B. has no declared conflicts of interest; E.M. has no declared conflicts of interest; F.K. has no declared conflicts of interest; and R.B. has no declared conflicts of interest.

Acknowledgments: The authors thank Dr. Cedrik Britten, III, Medical Department, for his excellent immunological advice, and Jörg Schreiner for his precise technical assistance.

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