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Lung and blood T-cell receptor repertoire in extrinsic allergic alveolitis

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ABSTRACT: Patients with extrinsic allergic alveolitis (EAA) have accumulations of T-lymphocytes in their lungs. CD8+ lung T-cells, in particular, have been implicated in the pathogenesis of EAA. The objective of the present study was to analyse the T-cell receptor (TCR) Vα and Vβ gene usage of CD4+ and CD8+ lung and peripheral blood lymphocytes (PBLs) before and after treatment.

Twelve patients with clinical signs of extrinsic allergic alveolitis were studied at disease onset, and nine of the 12 were also studied after treatment and clinical recovery. Lung cells, obtained by bronchoalveolar lavage (BAL), and paired PBL samples were analysed by flow cytometry using a panel of anti-TCR V monoclonal antibodies.

The changes in TCR V gene usage were most pronounced in BAL CD8+ cells, as compared to the BAL CD4+, PBL CD8+ and PBL CD4+ subsets. At disease onset, 10 of the 12 patients had lung restricted expansions of CD8+ T-cells using a particular Vα or Vβ gene segment, and 8 of the 12 patients had CD8+ T-cell expansions in PBL. For the patients in whom a follow-up was possible, a majority of the expansions in the lungs were normalized, whereas most of the expansions in PBL remained. An over-representation of human leucocyte antigen (HLA)-DR2 (15) was detected, particularly among patients with farmer's lung.

An increased selected T-cell receptor V gene usage may follow specific interactions between T-cells and antigens. In extrinsic allergic alveolitis, we determined that such expansions occur most frequently in the lung CD8+ T-cells. Since most expansions of lung CD8+ T-cells normalized with clinical improvement, these are further implicated in the pathogenesis of extrinsic allergic alveolitis.
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Extrinsic allergic alveolitis (EAA), or hypersensitivity pneumonitis (HP), comprises a group of related inflammatory interstitial lung diseases caused by the inhalation of organic dusts and small molecular weight chemical compounds. Well-known aetiological agents include actinomycetes in decaying, mouldy grain (farmer's lung) and avian serum proteins (bird fancier's disease). Patients with acute disease typically present with self-limited episodes of fever, chills and dyspnoea, 4–6 h after antigen exposure, which is usually intermittent but intense. In contrast, the chronic/subacute forms of allergic alveolitis are associated with an insidious onset. Treatment includes cessation of exposure and, in severe cases, steroid therapy to prevent progression to fibrosis. Routine laboratory tests include serology to detect precipitating antibodies (immunoglobulin G (IgG)) against the antigen. Chest radiography typically shows reticulonodular interstitial infiltrates, whilst pulmonary function tests commonly reveal a restrictive dysfunction. EAA, particularly the chronic form, is characterized histopathologically by mononuclear cell infiltrates and granuloma formation. Bronchoalveolar lavage (BAL) fluid typically shows lymphocytosis [1], with a dominance of CD8+ T-cells at the onset [2]. The pathogenesis and clinical features of EAA have recently been reviewed [3].

Evidence of an important role for T-cells in the pathogenesis of allergic alveolitis (reviewed in [4]) includes: 1) antigen-reactive T-cells with an activated phenotype that are present in the blood and lung [5–7]; 2) histopathological features that are typical of the T-cell mediated delayed-type hypersensitivity reaction [8]; and 3) in an animal model, the possibility of transferring the disease to healthy animals by adoptive transfer of T-cells [9]. T-lymphocytes use their T-cell receptors (TCRs) to recognize a combination of antigenic peptide plus major histocompatibility complex (MHC) molecules (in man, human leucocyte antigen (HLA)) on the surface of antigen-presenting cells. The most common type of TCR consists of one α- and one β-polypeptide chain, each comprising constant and variable parts, the latter interacting with the antigen [10]. The enormous diversity of TCRs is created by the process of genomic recombination, wherein a limited number of TCR segments can be randomly combined. The TCR α and β variable parts are, thus, made up of variable (V), joining (J) and, for the β-chain, diversity (D) segments [11]. There exist in

man about 32 Vα and 26 Vβ functional segment families [12]. One method of studying the TCR repertoire is to employ TCR V-specific antibodies for analysing the expression of different Vα and Vβ segments. The normal expression in peripheral blood of different individual TCR V gene segments is in the range of 1–8%.

Our objective was to study the role of T-cells in the pathogenesis of EAA by analysing the TCR repertoire in the lung as compared to that in the blood, in the acute phase and after recovery following steroid therapy.

Materials and methods

Subjects

Paired samples of PBLs and lung T-cells acquired by BAL were obtained from 12 patients (aged 30–67 yrs, median age 52 yrs; 2 females and 10 males) with acute hypersensitivity pneumonitis. The median time from cessation of exposure to the allergen to the first investigation was 1 day (range 1–28 days). Seven of the patients had never smoked and five were ex-smokers.

Nine patients had farmer's lung disease and three had bird fancier's disease. Signs of clinically active allergic alveolitis and determination of clinical improvement were judged from the results of chest radiography, lung function tests (spirometry, transfer factor), blood gas measurements and the presentation of symptoms. Serology was performed to detect precipitating antibodies against relevant antigens. PBL and BAL follow-up samples were also obtained from nine patients after treatment. The patients were treated with steroids *per os*. After the first investigation, exposure to the allergens ceased completely or was minimized. The median treatment time was 4 months (range 2–12 months, including patient No. 9, who suffered a relapse and was also receiving steroids at follow-up), and the median time from cessation of treatment to the second investigation was 4 months (range 0–12 months). The median total time between the two investigations was 8 months (range 5.5–15 months). The study was approved by the Ethics Committees of the Karolinska Institute and Umeå University Hospital.

BAL procedure and handling of cells

BAL was performed as described previously [13]. Briefly, after premedication with morphine and scopolamine, bronchoscopy was carried out with a flexible fibreoptic bronchoscope under local anaesthesia with 2% lignocaine. The bronchoscope was wedged in a bronchus in the middle lobe, and sterile saline solution at 37°C was instilled in six aliquots of 50 mL. After each instillation, the fluid was gently aspirated.

Immunofluorescence and flow cytometry

Anti-TCR Vα2.3-, Vβ3-, Vβ5.1-, Vβ5.2/5.3-, Vβ5.3-, Vβ6.7-, Vβ8.1-, Vβ12.1-specific monoclonal antibodies (moAbs) were obtained from T-Cell Sciences (Cambridge, MA, USA). The anti-TCR Vβ2-, Vβ13.6-, Vβ14-, Vβ17-, Vβ21.3-, and Vβ22-specific MoAbs were purchased from Immunotech (Luminy, France), and the anti-Vα 12.1 moAb from Serotec (Oxford, UK). Anti-CD4 MoAbs were conjugated either with PerCP (Becton Dickinson, Mountain View, CA, USA) or with RPE-Cy5 (Dakopatts, Glostrup, Denmark). Phycoerythrin (PE)-conjugated anti-CD8 moAb and fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin were obtained from Dakopatts. Normal mouse serum (NMS) from Balb/c mice was used as negative control at a dilution of 1:500 in phosphate-buffered saline (PBS). The OKT3 (CD3) hybridoma used for positive controls was acquired from the American Type Culture Collection (Rockville, MD, USA).

The staining procedure has been described in detail previously [14]. Briefly, cells were incubated with unlabelled TCR V-specific MoAb and washed twice. FITC-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin were added for detection of bound antibodies. NMS, diluted 1:500, was used to block remaining rabbit anti-mouse immunoglobulin before adding the PerCP (or RPE-Cy5)-conjugated anti-CD4 and PE-conjugated anti-CD8 MoAbs. Cells were analysed in a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA). Lymphocytes were gated by forward and side scatters. NMS was used as a negative control (in all cases <1%). Our panel of TCR V-specific moAbs covers approximately 50% of the CD4+ αβ T-cells, and 40% of the CD8+ cells, in normal peripheral blood.

Definition of T-cell expansion

Reference values for MoAb reactivities were established from TCR V analyses on peripheral blood lymphocytes in 79 healthy Scandinavian blood donors. The median number of analyses for each specific TCR MoAb was 72 (range 36–79) for CD4+ and 64 (range 34–70) for CD8+ cells [15]. Since the normal TCR repertoire in BAL has not been extensively studied, these reference values were also used for BAL. Studies that further justify this are referred to in the Discussion section.

T-cell expansions were defined as those that exhibited at least three times higher anti-TCR V MoAb reactivity compared to the corresponding median reactivity in PBLs from healthy blood donors, or any value >15%. This definition supposes that TCR V gene segments are normally expressed at different levels. The subject of T-cell expansions in health and disease has recently been reviewed [15].

Delta value

To determine in which cell population the mean change in TCR V gene segment usage was highest, a delta-value was computed (for each of the subjects PBL, CD4+, PBL CD8+, BAL CD4+ and BAL CD8+) as the median of the absolute values of the change in TCR Vα/Vβ MoAb reactivity before and after treatment.

HLA typing

HLA class I (HLA-A, B and C) were determined by the microlymphocytotoxicity technique. Class II (HLA-DR) typing was performed on deoxyribonucleic acid (DNA), using polymerase chain reaction (PCR) techniques and amplification with sequence-specific primers [16].

Statistics

Since it is not established with certainty that the parameters compared were normally distributed, and since the number of samples was small, nonparametric statistics were selected. The Mann-Whitney nonparametric test was used to compare the total number of BAL cells, percentage of lymphocytes and of CD3+, CD4+ and CD8+ cells between samples from the lung and blood, and also for the delta-values. Wilcoxon's signed rank test was used to compare values before and after treatment. A p-value of less than 0.05 was considered significant.

Results

Clinical parameters

Patient characteristics and clinical information are represented in table 1. Nine patients had farmer's lung disease and three had bird fancier's disease. The patients developed fever, chills and dyspnoea a couple of hours after exposure, and had chest radiographs with widely distributed micronodular shadowing. Thus, all patients fulfilled the three major criteria (history of exposure, symptoms and chest radiographic appearance) for diagnosis, established by TERHO [17]. In addition, 11 patients had basal crepitant rales audible on auscultation of the lungs, 11 had an impairment of the transfer factor of the lungs, 7 had a decreased arterial oxygen tension at rest, and six patients had a restrictive ventilatory defect found at spirometry. Regarding the remaining minor criteria, lung biopsy specimens were not routinely obtained, and provocation tests were not performed. Diagnosis of EAA was confirmed since all patients fulfilled at least two of the minor criteria (with the only exception of patient No. 12, for whom lung function tests were not performed at the initial investigation). The results of the serological investigations also support the diagnosis of EAA. Six patients had precipitating antibodies against *Termactinomyces vulgaris*, two had antibodies both against *Micropolyspora faeni* and *T. vulgaris*, and three patients had antibodies to bird antigens.

All patients were symptom-free at follow-up, with the exception of patient No. 9, who was the only patient still receiving treatment at this time. The chest radiographic appearances had also normalized, except for patient No. 9. The results of the lung function tests, (mean±SD, with follow-up values in parenthesis) were as follows: vital capacity (VC) 80±14 (106±14) % predicted; total lung capacity (TLC) 84±14 (97±12) % pred; forced expiratory volume in one second (FEV_1) 78±20 (107±13) % pred; and transfer factor of the lungs for carbon monoxide ($T_{L,CO}$) 53±11 (82±10) % pred; the changes being significant ($p<0.05$) for all parameters. These data for the individual patients are illustrated in figure 1. Indeed, all follow-up patients improved their values with the exception of patient No. 9.

BAL and PBLs

The total number of cells per millilitre recovered BAL fluid, expressed as median values (10th–90th percentile), before and after treatment were 0.43 (0.06–1.02) × 10^6, and 0.40 (0.05–0.67) × 10^6, respectively (NS). The proportion of lymphocytes in the BAL fluid was 71 (37–80)% before and 58 (25–69)% after treatment ($p<0.01$). The relative numbers of CD3+ (*i.e.* T-cells), CD4+/CD3+ and CD8+/CD3+ cells are presented in table 2. No significant changes in these latter parameters occurred either in BAL or PBLs. However, a comparison between BAL and PBLs illustrates that, after treatment, there were significantly relatively more CD4+/CD3+ cells ($p<0.05$) and fewer CD8+/CD3+ cells ($p<0.05$) in BAL than in PBLs.

The CD4+/CD8+ ratios in PBLs and BAL, before and after treatment, demonstrated a heterogenous picture. The median value (10th–90th percentile) of the CD4/CD8 ratio for PBLs before treatment was 2.8 (1.3–5.6) *versus* 3.1 (1.0–3.8) after treatment (NS). Corresponding values for BAL were 4.0 (0.7–11.8) and 6.1 (1.2–25.9) (NS). Before treatment, the difference in CD4+/CD8+ ratio between PBLs and BAL was not significant, but after treatment the ratio was significantly higher in BAL than in PBL ($p<0.05$).

Table 1. – Patient characteristics, EAA type, number of minor clinical criteria, treatment time and time from treatment to follow-up

Pt No.	Age yrs	Sex	HLA class I	HLA class II	Smoker	EAA type	Minor criteria#	Treatment months	Interval months‡
1	52	M	A 10, 24 (9); B 7, 8	DR 2 (15), 3 (17)	LNSM	FL	3	4	3
2	49	M	A 1, 3; B7, 8	DR 2 (15), 3 (17)	LNSM	FL	4	4	4
3	67	M	A 2, 3; B 5, 7	DR 2 (15), 3 (17)	EXSM (ND)	FL	2	3	9
4	59	M	ND	DR 2 (15), 6 (13)	EXSM (10)	FL	3	12	NFU
5	53	M	A 2, 31 (19); B7, 27	DR 2 (15)	LNSM	FL	4	5	5
6	53	F	A 1, 2; B 18, 40	DR 2 (15)	LNSM	FL	5	12	NFU
7	60	M	A 3, 24 (9) B 27, 35	DR 1	EXSM (1)	FL	2	5	3
8	50	M	A 3, 32 (19); B 7, 27	DR 1, 8	LNSM	BF	2	12	NFU
9	62	M	A 11; B 5, 44 (12)	DR 5 (11), 7	LNSM	FL	4	5	12
10	44	M	A 31 (19); B 18, 27	DR 2 (15), 8	EXSM (4)	BF	3	2	5
11	34	M	A 24 (9), 32 (19); B 7, 40	DR 2 (15), 6 (13)	LNSM	FL	4	4	1, 5
12	30	F	A 2; B 8, 27	DR 2 (16), 3 (17)	EXSM (9)	BF	ND	3	12

Pt: patient; M: male; F: female; HLA: human leucocyte antigen; LNSM: lifetime nonsmoker; EXSM: ex-smoker, with number of years since cessation of smoking in parenthesis; FL: farmer's lung disease; BF: bird fancier's disease; EAA: extrinsic allergic alveolitis; ND: not determined; NFU: no follow-up. #: number of minor criteria for the diagnosis, as established by TERHO [17]; briefly, these are: 1) basal crepitant rales; 2) impairment of the transfer factor; 3) decreased arterial blood oxygen saturation; 4) a restrictive ventilatory defect; 5) histological changes compatible with EAA. All patients fulfilled all the major criteria (history of exposure, typical symptoms and chest radiographic appearance). ‡: time between cessation of treatment and second investigation.

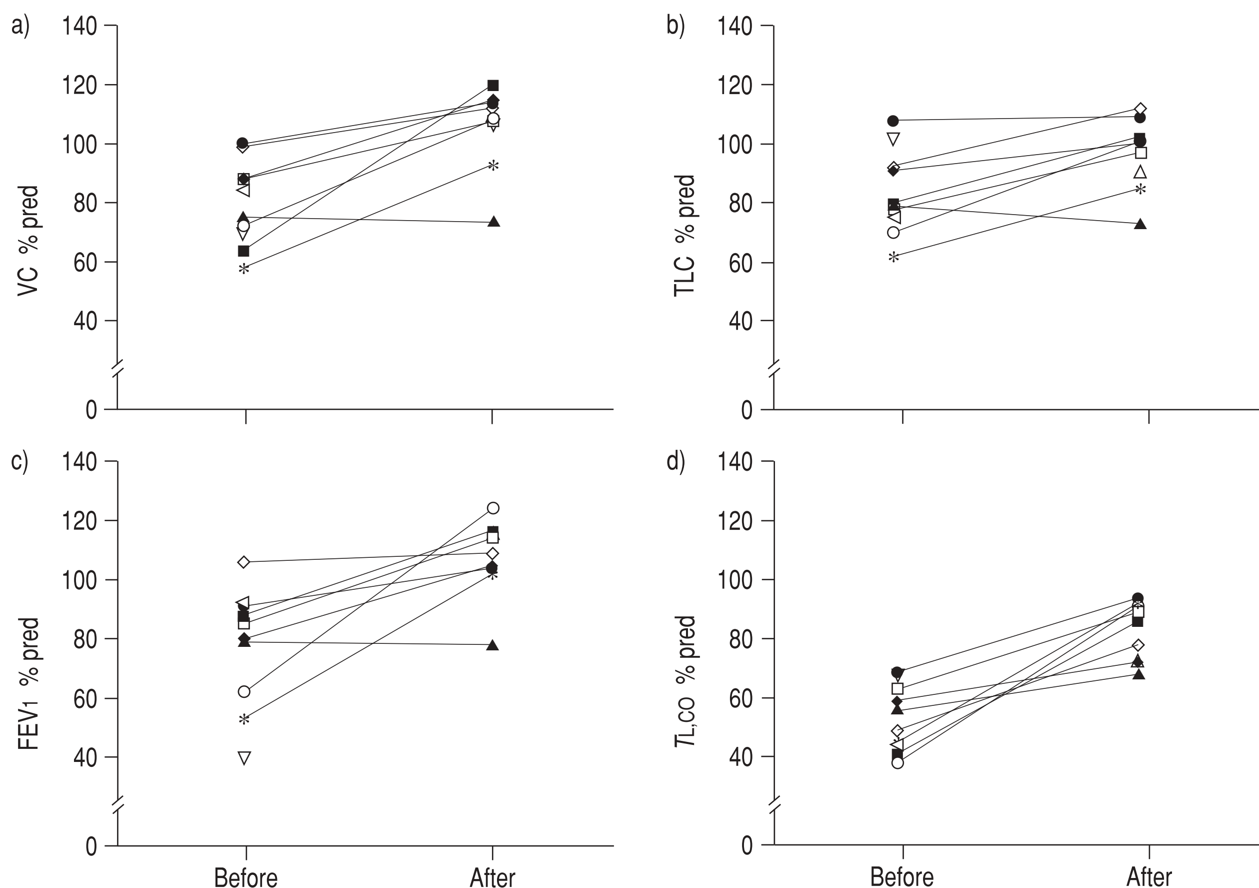

Fig. 1. – Lung function tests performed before and after treatment. a) vital capacity (VC); b) total lung capacity (TLC); c) forced expiratory volume in one second (FEV1); d) transfer factor of the lungs for carbon monoxide (*T*L,CO). All results are expressed as percentage of predicted. Note that patient No. 9 was the only one showing decreased VC, TLC and FEV1. Pt: patient. —□— : Pt No. 1; —○—: Pt No. 2; —△—: Pt No. 3; —◁— : Pt No. 4; —■— : Pt No. 5; —◆— : Pt No. 6; —●— : Pt No. 7; —▽— : Pt No. 8; —▲— : Pt No. 9; —◇— : Pt No. 10; —*— : Pt No. 11.

Table 2. – Relative occurrence of major lymphocyte subsets in BAL and PBL cells

	BAL		PBL	
	Before	After	Before	After
CD3+ %	86 (45–95)	91 (46–96)	64 (31–81)	70 (51–80)
CD4+/CD3+ %	76 (39–88)	86 (52–95)	71 (52–81)	72 (47–79)
CD8+/CD3+ %	19 (7–52)	14 (5–43)	26 (14–42)	23 (20–48)

Lymphocytes were gated by forward and side scatter, and the values for the percentage of CD3+, CD4+/CD3+ and CD8+/CD3+ cells calculated. Median values are presented, and 10th–90th percentiles in parenthesis. BAL: bronchoalveolar lavage; PBL: peripheral blood lymphocyte.

TCR V gene usage

The total number of expansions before treatment was calculated for the following cell populations: PBL CD4+, PBL CD8+, BAL CD4+ and BAL CD8+ (table 3). T-cell expansions occurred most often in the CD8+ subsets, both in PBL and BAL cells. Large expansions (>20%) were only recorded in the CD8+ subsets. In BAL CD8+ cells, six such dramatic expansions (>20%) were determined compared to three in the PBL CD8+ cells.

TCR V gene usage of BAL and PBL CD8+ cells, respectively, is depicted in table 4. Expansions were generally more dramatic in the lung than in the blood, *e.g.*: in patient No. 1, 25% of the BAL CD8+ cells expressed Vβ2, in patient No. 2, 32% expressed Vβ 13.6; and in patient No. 6, 29% expressed Vα 12.1 and 48% Vβ 21.3. The number of CD8+ T-cell expansions in individual patients varied between zero and five (table 5).

In three patients, expansions of the same TCR V gene segment occurred both in BAL CD8+ and PBL CD8+ cells, so called "tandem expansions". These occurred in patient No. 4 (Vβ 5.1), in patient No. 6 (Vα 12.1 and

Table 3. – Number of analyses and number of T-cell expansions in different cell populations before treatment

Cell population	Analyses n	Expansions n
PBL CD4+	148	2
BAL CD4+	156	5
PBL CD8+	145	11
BAL CD8+	155	15

T-cell expansions were defined as those that exhibited at least three times higher anti-TCR V MoAb reactivity compared to the corresponding median reactivity in PBLs from healthy blood donors, or any value >15%. PBL: peripheral blood lymphocytes; BAL: bronchoalveolar lavage; TCR: T-cell receptor; MoAb: monoclonal antibody.

Table 4. – TCR V gene usage by BAL and PBL CD8+ T-cells before treatment

Pt. No.	Vα2.3	Vα12.1	Vβ2	Vβ3	Vβ5.1	Vβ5.2–3	Vβ5.3	Vβ6.7	Vβ8.1	Vβ12	Vβ13.6	Vβ17	Vβ21.3	Vβ22
1	6.2/**9.3**	8.6/6.7	**24.6**/8.7	1.6/3.6	2.0/1.4	4.5/1.2	0.4/0.5	3.9/1.1	1.2/1.2	0.6/0.8	0.6/1.7	1.1/1.0	ND/ND	ND/ND
2	0.6/2.0	0.5/2.7	3.6/5.3	1.8/2.7	1.6/3.3	1.4/3.6	0.4/1.6	1.0/1.9	2.3/2.7	0.4/1.0	**32.0**/0.8	0.9/4.4	ND/ND	ND/ND
3	2.3/ND	**23.4**/ND	5.2/ND	2.0/13.7	ND/ND	3.3/ND	ND/ND	1.0/ND	0.7/ND	1.2/2.3	1.9/ND	4.0/ND	ND/ND	ND/ND
4	2.3/0.3	3.8/**44.2**	2.0/1.9	2.1/0.8	**16.1/8.8**	1.4/1.4	0.5/0.7	1.3/0.4	6.1/0.4	0.4/0.7	0.7/0.9	0.4/0.3	1.4/0.7	2.8/1.1
5	5.2/4.2	3.1/3.8	5.2/5.0	6.1/13.5	1.5/3.0	2.2/ND	0.9/0.9	0.8/1.1	**20.0**/4.6	0.6/0.6	0.9/**10.5**	7.4/3.6	ND/ND	ND/ND
6	1.7/2.4	**29.4/19.2**	5.2/8.0	3.5/5.4	3.0/4.3	2.6/1.7	0.3/0.9	1.0/2.5	0.9/1.8	**4.1**/1.8	0.9/1.8	2.9/4.2	**48.3/24.2**	3.6/3.5
7	1.8/4.5	3.7/10.0	3.8/9.2	0.2/0.6	2.1/2.4	2.0/2.0	1.0/1.3	3.6/0.2	3.2/9.7	1.7/13.9	1.3/1.7	2.7/3.1	2.8/0.9	2.9/2.7
8	2.4/**10.1**	**17.0**/1.6	**15.6**/4.4	5.0/**25.4**	1.2/1.9	3.7/1.8	0.4/0.1	ND/ND	9.7/7.5	4.7/1.8	2.4/0.2	5.3/10.0	1.3/0.3	0.4/0.8
9	3.4/4.2	8.6/6.6	12.7/5.9	8.3/7.6	2.1/2.6	5.9/2.6	1.6/1.7	ND/ND	1.4/3.2	0.8/0.9	0.3/ND	5.0/7.0	3.7/3.0	3.2/2.9
10	6.4/1.9	7.6/8.1	3.3/3.4	2.7/3.3	2.4/1.7	1.5/6.2	0.4/**6.2**	ND/ND	7.9/5.1	0.8/2.4	**4.9**/1.1	6.0/5.9	3.6/1.6	**6.2**/4.2
11	2.0/1.0	6.3/4.3	7.4/2.1	10.6/2.0	**10.7/9.5**	1.0/1.5	0.4/ND	2.1/2.5	3.2/**17.9**	1.1/0.6	0.5/1.1	2.1/13.3	8.2/2.5	0.6/2.0
12	4.4/2.5	8.8/4.9	3.2/9.4	1.7/3.7	1.5/2.0	1.4/4.1	ND/ND	1.6/0.9	3.0/5.2	2.0/0.9	2.0/1.2	**19.0**/4.7	2.3/3.7	3.7/4.1

Values are expressed as BAL/PBL. Expansions are bold. Pt: patient. TCR: T-cell receptor; BAL: bronchoalveolar lavage; PBL: peripheral blood lymphocytes.

Vβ 21), and in patient No. 11 (Vβ 5.1). Parallel expansions of the same TCR V-segment both in BAL CD8+ and BAL CD4+ occurred in two patients. In patient No. 2. Vβ 13.6 made up 32% of BAL CD8+ cells and 5.7% of BAL CD4+ cells. In patient No. 8, Vα 12.1 constituted 17% of BAL CD8+ and 9.5% of BAL CD4+, while Vβ 12 made up 4.7% of BAL CD8+ and 18% of BAL CD4+ cells.

Follow-up

It was possible to obtain samples from nine patients after treatment. The most pronounced changes in TCR V gene segment usage occurred in the BAL CD8+ compartment, as determined by the delta-value calculation (fig. 2). The median change in TCR Vα/Vβ moAb reactivity in BAL CD8+ was 1.6%, significantly higher than the changes in BAL CD4+ (0.8%; $p<0.001$), PBL CD8+ (0.7%; $p<0.001$) and PBL CD4+ (0.5%; $p<0.001$). Furthermore, the changes in BAL CD4+ were larger than in PBL CD4+ ($p<0.01$) and those in PBL CD8+ larger than in PBL CD4+ ($p<0.05$).

The expansions among CD8+ cells that could be followed-up are depicted in figure 3. It is apparent that the majority of BAL CD8+ expansions normalized with treatment in contrast to PBL CD8+ expansions, most of which remained abnormal. Some new small magnitude expansions were observed after treatment (4 out of 5 <6%, one 17%) in BAL CD8+, whilst the two new expansions in PBL CD8+ only constituted a minor change from a value in the upper reference range before treatment.

Table 5. – Occurrence of T-cell expansions

Expansion n	PBL CD4+ n	BAL CD4+ n	PBL CD8+ n	BAL CD8+ n
0	10	9	5	2
1	2	2	3	7
2	0	0	4	1
3	0	1	0	2

Values show the number of patients who had a given number of expansions in the respective subsets. PBL: peripheral blood lymphocytes; BAL: bronchoalveolar lavage.

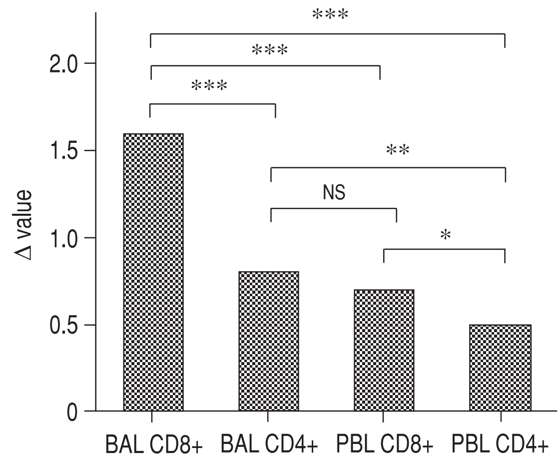

Fig. 2. – Delta values for median absolute change in anti-TCR V MoAb reactivity in different cell populations before and after treatment. TCR: T-cell receptor; MoAb: monoclonal antibody; BAL: bronchoalveolar lavage; PBL: peripheral blood lymphocytes; NS: nonsignificant. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$, compared to other cell populations as indicated.

For the "tandem expansions" in patient No. 11, a follow-up sample was available, and they were found to persist virtually unchanged (Vβ 5.1 11.4% in BAL CD8+ and 10.5% in PBL CD8+). The "parallel expansions" in patient No. 2 were also followed-up. The number of Vβ13.6+ cells had decreased from 32 to 11% of BAL CD8+ (however, still an expansion), whilst they decreased from 5.7 to 2.8% of BAL CD4+ (no longer an expansion). In patient No. 3, the Vβ 5.3 usage was only analysed on follow-up, but at this time cells expressing this TCR V gene segment made up 4.8% of BAL CD8+ and 3.2% of BAL CD4+, constituting expansions.

HLA typing

The result of the typing for HLA class I and class II is included in table 1. Notably, 67% (8 out of 12) of the patients were HLA-DR2(15)+, compared to 30% in a reference Scandinavian population (Chi squared = 6.6; NS, after correction for multiple comparisons) [18]. Of the nine patients with farmer's lung, seven were DR2(15)+ (Chi squared = 7.9; NS). No obvious correlation between HLA class and TCR V gene expansions was seen.

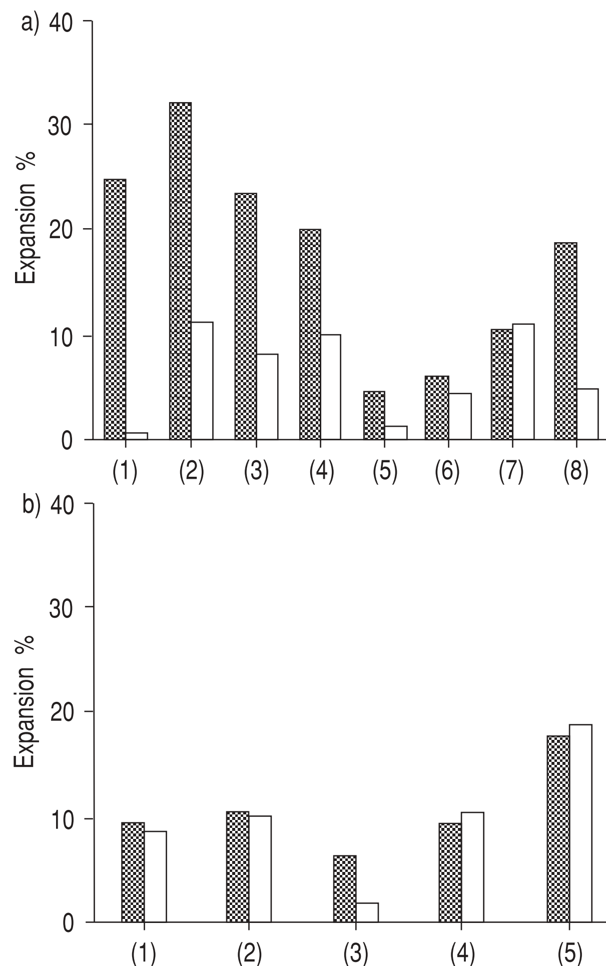

Fig. 3. – a) BAL CD8+ expansions before (▩) and after (□) treatment in: (1) patient No. 1 Vβ 2; (2) patient No. 2 Vβ 13.6; (3) patient No. 3 Vα 12.1; (4) patient No. 5 Vβ 8.1; (5) patient No. 10 Vβ 13.6; (6) patient No. 10 Vβ 22; (7) patient No. 11 Vβ 5.1; and (8) patient No. 12 Vβ 17. b) PBL CD8+ expansions before (▩) and after (□) treatment in: (1) patient No. 1 Vα 2.3; (2) patient No. 5 Vβ 13.6; (3) patient No. 10 Vβ 5.3; (4) patient No. 11 Vβ 5.1; and (5) patient No. 11 Vβ 8.1. T-cell expansions were defined as those that exhibited at least three times higher anti-TCR V MoAb reactivity compared to the corresponding median reactivity in PBLs from healthy blood donors, or any value >15%. For definitions see legend to table 2.

Discussion

The results of this study provide evidence that patients with EAA frequently have large, lung-limited, expansions of CD8+ T-cells using a particular Vα or Vβ gene segment, and that these expansions in most cases normalize with clinical improvement. A recent study of TCR V usage in EAA patients before treatment also reported TCR V gene expansions [19], but CD4+ and CD8+ T-cells were not analysed separately, and patients were not studied after treatment. In the present study, although no expansions of similar magnitude of BAL CD4+ T-cells were observed, this does not exclude an important role for CD4+ cells in the pathogenesis of EAA. Whether the CD8+ T-cells are disease-promoting, neutral or have a protective role cannot be judged from these data. It is of interest that, in the mouse, CD8+ T-cells were recently demonstrated to play a crucial role in the development of airway hyperresponsiveness [20].

As is apparent from figure 1, all patients except one showed an improved lung function on follow-up. Only patient No. 9 was unusual, in that despite some improvement with regard to symptoms, his VC, TLC and FEV1 decreased. Interestingly, this was one of the two patients without detectable BAL CD8+ expansions. Overall, no correlations could be determined between the tendency to exhibit lung CD8+ expansions and the type of EAA, clinical presentation, lung function alterations or number and differentials of BAL cells. Since the number of patients was small, however, no firm conclusions can be drawn from these observations. Regarding the frequency of expanded T-cells, 10 of the 12 patients had 1–3 TCR Vα or Vβ expansions in the BAL CD8+ subset. Considering that the antibody panel employed covered only approximately 40% of the CD8+ TCR repertoire, it seems likely that more than one V gene segment was expanded in each patient.

The relationship between TCR repertoires in the lung and blood in normal and pathological conditions has not been extensively investigated. One study based on quantitative PCR analysis of normal BAL T-cells revealed a TCR Vβ distribution similar to that of normal blood [21]. A recent study, employing PCR and heteroduplex analysis of the clonal composition of T-cell populations in healthy nonsmokers, demonstrated that the lung TCR repertoire was largely as heterogeneous as that of peripheral blood. A few T-cell clones were simultaneously expanded in blood and lungs in all individuals studied [22]. An investigation of patients with allergic asthma, but in a symptomless condition, also revealed a similar TCR Vα/Vβ usage among PBL and BAL lymphocytes, as determined by fluorescence-activated cell sorter (FACS) analysis (J. Wahlström *et al.*, manuscript in preparation).

However, even though these studies seem to suggest that the normal lung TCR repertoire mirrors that of the blood, solid quantitative data regarding the frequency and magnitude of BAL T-cell expansions in healthy controls are still lacking. In sarcoidosis, we previously demonstrated

that CD4+ cells bearing the TCR Vα 2.3 gene segment accumulate in the lungs of patients of HLA type DR3(17), DQw2 [23]. These expansions correlated with clinical disease activity. No preferential TCR Vβ usage by sarcoidosis BAL cells could be detected; even the expanded Vα2.3+ cells tended to have an oligoclonal Vβ usage, with no clear preference [24]. In the present group of EAA patients, no CD4+ Vα2.3+ BAL expansions were recorded, even in the HLA DR3(17),DQw2+ patients (n=4), further supporting the notion that such Vα2.3+ expansions are sarcoidosis-specific.

It is conceivable that perturbations in PBL TCR V gene usage may influence the BAL repertoire, and vice versa. The origin of the four "tandem expansions" in patients Nos. 4, 6 and 11 cannot be determined from the present data, but dramatic Vα2.3+ expansions in BAL of sarcoidosis patients did not "spill over" to PBLs in those patients [14]. In CD8+ PBLs, clonal expansions that are stable over time have been reported to exist in apparently healthy individuals [15, 25, 26].

Expansions of T-cells using a restricted set of Vα/Vβ genes may react with and proliferate in response to: 1) foreign antigen + major histocompatibility complex (MHC) [27]; 2) autoantigens, such as heat-shock proteins [28]; and 3) a superantigen; reviewed in [29]. If the clonality of the expanded BAL T-cells were to be studied (which was not possible in the present study because of lack of material), the results could indicate the nature of the antigen involved. A conventional peptide antigen presented in the context of an MHC-molecule will stimulate responsive T-cells to proliferate in a clonal or oligoclonal fashion. In contrast, a superantigen binds to the TCR Vβ chain outside the conventional antigen-binding cleft and stimulates T-cells in a polyclonal manner, with only the Vβ segment(s) to which the superantigen binds in common. In sarcoidosis, our analysis of the lung accumulated CD4+ Vα2.3+ cells demonstrated a restricted α-chain usage, with different nucleotide sequences coding for the same amino acids in the antigen-contacting CDR3 region [24]. This finding strongly indicates that recognition of a conventional peptide antigen is responsible for the expansion of these cells.

Since a superantigen can stimulate both CD4+ and CD8+ T-cells bearing the same Vβ gene segment [29], "parallel expansions" in these two subsets might be expected. Parallel expansions were recorded in the lungs of two patients, and may indicate a role for superantigens in some cases of EAA. No correlations to the sources of antigen were noted (bird and mould, respectively).

The finding of T-cell expansions using different V gene segments may have several, nonexclusive, reasons. Firstly, the same antigen may contain multiple epitopes, stimulating different T-cell clones. Secondly, since the TCR recognizes the combination of antigenic peptide and MHC molecule, the same protein may yield peptides which are differentially presented by different MHC molecules, thus stimulating T-cells with different V gene usages. As the offending antigens, as well as the MHC haplotypes (particularly MHC class I, interacting with CD8+ T-cells), differed between the patients, the existence of different TCR V expansions is hardly surprising, although a preference for Vα12.1+ expansions was noted.

An over-representation of HLA-DR2(15) was noted, particularly among patients with farmer's lung. Interestingly, this HLA haplotype is also heavily over-represented in sarcoidosis patients with chronic progressive disease (M. Berlin, manuscript in preparation). In this context, it should be noted that the diagnosis of EAA was firmly established in the present study, since patients fulfilled all three major, and at least two minor criteria for diagnosis, as established by TERHO [17]. Previous investigations of HLA-DR distribution in patients with EAA have failed to demonstrate a reproducible difference from unaffected individuals, but in contrast to ours, most of those data were obtained from patients with bird fancier's disease [30, 31]. The over-representation of HLA-DR2(15) in this small patient group must be interpreted with caution, however, but a larger study is now under way (Olerup *et al*, unpublished data). The HLA type may be disease-associated, either through the antigen-presenting properties of the HLA molecule leading to T-cell activation and inflammation, or *via* close linkage of a particular HLA locus to a disease-susceptibility gene. In the mouse, an important role for MHC class II genes in controlling susceptibility to extrinsic allergic alveolitis was recently demonstrated [32]. Regarding HLA-A, B and C loci, we detected no obvious over-representation of any HLA class I type, which is in agreement with earlier reports [33].

The heterogeneity of BAL CD4+/CD8+ ratios, with most T-cells being CD4+ in the majority of patients, may seem puzzling. Most earlier EAA studies, reviewed in [34], report a decreased CD4+/CD8+ ratio compared to PBLs, although ratios that were unchanged [35] or increased [36] have also been noted. The present patient group had a very acute disease, however, and in such conditions a BAL CD4+ predominance has previously been reported [37]. Wide differences in ratios between different types of EAA have also been reported [38]. Even in a single EAA type, farmer's lung, CORMIER *et al.* [39] reported that some patients had a very low ratio, whereas others had a high one, which was also a finding in the present study. Factors affecting the CD4+/CD8+ ratio may be the nature and/or dose of antigen, type of exposure, treatment, or smoking, which was shown to increase the ratio [38]. In this small patient group, no correlations could be determined between clinical parameters and CD4+/CD8+ ratios. Our finding of a significantly higher CD4+/CD8+ ratio in BAL compared to PBLs after treatment and recovery may reflect a clearance from the lung of CD8+ cells, which play an important role in the acute phase.

In conclusion, an increased selected T-cell receptor V gene usage may follow specific interactions between T-cells and antigens. In extrinsic allergic alveolitis, we determined a high frequency of expansions, correlating with clinical activity, that occur primarily in the lung CD8+ T-cells, implicating these cells in the pathogenesis of the disease.

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