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Clonal CD8⁺ TCR-V β expanded populations with effector memory phenotype in Churg Strauss Syndrome

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KEYWORDS

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T-lymphocyte;
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Abstract Churg Strauss Syndrome (CSS) is a systemic vasculitis in which oligoclonal T cell expansions might be involved in the pathogenesis. Combined analysis of TCR-V β expression profile by flow cytometry and of TCR gene rearrangement by heteroduplex PCR was used to detect and characterize T cell expansions in 8 CSS patients, 10 asthmatics and 42 healthy subjects. In all CSS patients one or two V β families were expanded among CD8⁺ cells, with an effector memory phenotype apt to populate tissues and inflammatory sites. Heteroduplex PCR showed the presence of one or more clonal TCR rearrangements, which reveals monoclonal or oligoclonal T cells subpopulations. After purification with a V β specific monoclonal antibody, each CD8⁺/V β ⁺ expanded family showed a single TCR rearrangement, clearly suggestive of monoclonality. All CD8⁺ expansions were detectable throughout the disease course. TCR-V β expanded or deleted populations were not observed in asthmatic patients. Clonal CD8⁺/V β ⁺ T cell expansions might be useful as a disease marker.

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Introduction

Churg Strauss Syndrome (CSS) is characterized by pulmonary and systemic small-vessel necrotizing vasculitis, vascular and extra-vascular granulomas, eosinophilia and tissue infiltration by eosinophils, occurring in patients with asthma and often allergic chronic rhinosinusitis with nasal polyps [1]. A central pathogenic role for T lymphocytes in vasculitides is suggested by different observations. First, T cells are commonly present in biopsies of active lesions, both vasculitic and granulomatous [2,3]. Second, T cell lines from CSS patients, generated *in vitro* by polyclonal stimulation, are polarized towards a Th2 phenotype (IL-4 and IL-13), which may explain the eosinophilia, the hallmark of the disease. The same cells have also been shown to release significant amounts of IFN γ [4], which may be related to the granuloma formation. Third, immunosuppressive therapy targeting lymphocytes is useful in CSS patients, strengthening the concept of CSS as a T cell driven disease [5]. Moreover, anti myeloperoxidase (MPO) autoantibodies (p-ANCA) may play a pathogenic role in a subgroup of patients (about 38%) [6,7] and T cells from p-ANCA positive patients have been shown to proliferate in response to MPO, suggesting their helper function in the production of autoantibodies [8]. Allergen desensitization [9], vaccination, and/or infections, resulting in lymphocyte activation, have been implicated in triggering some cases of CSS [10,11].

Lymphocyte activation by Superantigens (SAg) has been suggested to occur in Kawasaki disease, polyarteritis nodosa and microscopic polyangiitis [12,13]. SAg stimulation of T cells bearing specific V β T cell receptors (TCR) results in the polyclonal expansion of selected V β families in CD4⁺ and CD8⁺ cells, followed by T cell restricted deletion [14].

Antigen stimulation, on the other hand, may cause oligoclonal T cell expansions, that have been actually found in CSS patients by immunoscope technique, but it is not known whether these expansions are CD4⁺ or CD8⁺ [15]. Flow cytometry analysis, using antibodies to conventional TCR β -chain variable region families (TCR-V β), can be used for assessment of T cell expansions, even if sensitivity of this technique for detection of clonality is lower than analysis of TCR rearrangement by molecular biology [16]. One advantage of flow cytometry is that TCR-V β antibodies can be combined to other T cell markers, allowing a detailed analysis of such expansions in terms of CD4 and CD8 subsets and also of naive-memory differentiation [17-19]. It would be particularly important to know the immunophenotype of expanded clonal T cell population,

since persistent clonal expansions of CD8⁺ memory T cells have been shown to populate inflammatory sites [20,21].

The aim of the study was to confirm the presence of clonal T cell expansions in peripheral blood samples from 8 CSS patients and to characterize them by flow cytometry and by molecular analysis of TCR rearrangement [22,23].

Materials and methods

Subjects

Eight patients with proved CSS diagnosed according to criteria of the American College of Rheumatology (ACR) [24], were included in the study. All patients had asthma, hypereosinophilia, rhinosinusitis and clinical manifestations consistent with systemic vasculitis, with ($n=4$) or without ($n=4$) histologic evidence [25]. P-ANCA antibodies were positive in 5/8 patients. Clinical data of the patients are summarized in Table 1. At the time of the study 6/8 patients were considered to be in remission, that is the absence of disease activity for at least 6 months according to Birmingham Vasculitis Activity Score (BVAS) item list [26] with the exception of asthma or neurologic and renal sequelae. They were all receiving low dose prednisone (<15 mg/die) for asthma. The remaining two patients (#7, #8) had active disease and were still receiving high dose corticosteroids. All eight patients were receiving regular inhaled therapy with corticosteroids combined to β_2 agonists. No patient had laboratory evidence of CMV and EBV reactivation. In each patient blood samples were obtained in 2-6 occasions throughout 3-19 months of follow up.

Ten asthmatic patients (age range 25-70, M/F 5/5) served as pathological control group. They had severe ($n=7$) or moderate ($n=3$) asthma, with persistent rhinitis and/or chronic rhinosinusitis and mild eosinophilia (range 500-1300 cells/mm³). All were receiving regular inhaled therapy with corticosteroids combined to β_2 agonists and 7/10 patients were receiving low dose prednisone (<15 mg/die). Forty-two healthy subjects were used as normal controls (see below). All subjects gave their informed consent to participate to the study which was approved by local ethical committee.

Immunophenotypic analysis

Peripheral blood mononuclear cells (PBMC) were separated from whole blood by density gradient centrifugation (Lymphoprep,

Table 1 Demographical and clinical characteristics of patients with Churg Strauss Syndrome

Patient #	Sex	Age	p-ANCA	Eosinophils n/mm ³ (%) [*]	Involved organs	Treatment
1	M	59	–	14,060 (37)	PNS, gallbladder, ileum	CCS, CYC, AZA
2	F	55	–	22,230 (59)	PNS	CCS, AZA
3	M	57	+	6920 (30)	PNS, lung (DAH), kidney	CCS, CYC, MTX
4	M	67	+	9990 (37)	PNS, lung, kidney	CCS, CYC
5	M	55	+	15,630 (50)	PNS, skin, kidney	CCS, CYC
6	M	60	+	5500 (26)	PNS, skin	CCS, AZA, MTX
7	M	61	+	28,000 (70)	PNS, lung	CCS, CYC
8	F	85	–	17,000 (50)	PNS, heart, skin	CCS, MTX

^{*}Values reported at diagnosis.

Abbreviations: peripheral nervous system PNS; diffuse alveolar hemorrhage DAH; Corticosteroids CCS; cyclophosphamide CYC; azathioprine AZA; methotrexate MTX.

Nycomed, Roskilde, Denmark) and analyzed by 4-colour flow cytometry, using a panel of monoclonal antibodies (MoAb) to 24 different TCR-V β families (IOTest Beta Mark TCR-V β -Repertoire Kit, Beckman Coulter, Miami, FL). This panel is expected to cover about 70% of all the normal human TCR-V β repertoire of CD3⁺ T cells [27]. Each TCR-V β MoAb was conjugated with Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE) or with FITC plus PE. Four-color flow cytometry was completed using MoAb to CD4 (peridinin chlorophyll-a protein labelled, PerCp, Caltag Laboratories, Burlingame, CA) or CD8 (allophycocyanin labelled, APC, Immunotech, Marseille, France). The percentage of positive cells for each V β family was determined by gating on CD8⁺ and CD4⁺ cells (at least 20,000 events were gated). A V β family was considered expanded or deleted when the percentage of V β positive cells was three SD higher or lower than reference values given by Beckman Coulter, respectively [13]. As additional control, we tested V β families in CD4⁺ and CD8⁺ cells from 42 age-matched healthy subjects (mean age 63, range 45-82). In this cohort, mean $\pm$ SD of each V β family was not significantly different from mean $\pm$ SD of the reference values from Beckman (Table 2). PBMC were further analysed by flow cytometry to quantify naive and memory subsets in the expanded families. Four-color flow cytometry analysis was performed with a single V β MoAb (FITC-conjugated) and the following MoAbs: CD62L-PE (Immunotech), CD28-PE (Immu-

notech), CCR7-PE (R&D Systems, Minneapolis, MN), CD45RA Tricolor labelled (TC) (Caltag) and CD8-APC (Immunotech). In details, CD45RA⁺/CD62L⁺, CD45RA⁺/CCR7⁺ and CD45RA⁺/CD28⁺ cells were considered naive T lymphocytes; CD45RA⁺/CD62L⁺, CD45RA⁺/CCR7⁺ and CD45RA⁺/CD28⁺ cells were considered central memory lymphocytes (T_{CM}); CD62L⁺, CCR7⁺ and CD28⁺ cells, either CD45RA⁺ or CD45RA⁺, were considered effector memory lymphocytes (T_{EM}) [17-19]. Naive and memory subsets of the expanded V β families were compared to normal controls ($n=10$). Each antibody was checked against an appropriate isotype-control antibody.

A two steps magnetic cell sorting (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was used to purify expanded V β ⁺ cells in 5 patients. PBMC were separated into CD4⁺ and CD4⁺ fractions by direct labeling with CD4 magnetic microbeads (Miltenyi). The CD4⁺ fraction was then stained with the relevant FITC-conjugated anti-V β MoAb and further separated into V β ⁺ and V β ⁺ fractions by labeling with anti-FITC magnetic microbeads. Purity of V β ⁺ purified cells was always $\geq 90\%$.

Heteroduplex PCR analysis of TCR- γ gene rearrangement

Analysis of TCR- γ rearrangement by heteroduplex PCR is routinely used to assess clonality of T cells (irrespective of

Table 2 Mean values of TCR-V β repertoire in healthy subjects and asthmatic controls compared to reference values

TCR-V β Family #	CD4 ⁺						CD8 ⁺					
	Control group ($n=42$)		Asthmatic controls ($n=10$)		Reference value* ($n=85$)		Control group ($n=42$)		Asthmatic controls ($n=10$)		Reference value* ($n=85$)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
V β 1	3.22	0.99	3.24	0.65	3.32	1.50	4.42	2.58	4.41	2.67	4.24	1.62
V β 2	9.38	1.81	9.65	1.64	9.36	1.44	5.06	3.04	6.61	2.48	5.43	1.89
V β 3	3.76	2.10	3.66	1.81	4.37	2.29	3.52	3.58	3.38	2.15	4.44	3.13
V β 4	1.55	0.52	2.25	0.44	2.03	0.37	1.33	0.97	2.04	0.98	1.9	0.80
V β 5.1	5.95	1.33	6.1	1.94	6.71	1.15	2.45	1.51	3.37	2.46	3.22	1.62
V β 5.2	1.21	0.48	1.2	0.37	1.33	0.38	0.97	0.82	1.08	0.59	1.12	0.57
V β 5.3	1.26	0.38	1.16	0.44	1.09	0.30	1.05	0.92	0.93	0.52	0.92	0.39
V β 7.1	1.57	0.61	2.36	0.88	1.93	0.62	2.77	1.72	2.92	1.39	3.39	1.23
V β 7.2	0.97	0.62	1.07	1.19	1.12	0.75	2.07	2.58	1.58	1.24	2.44	2.34
V β 8	3.96	1.23	4.47	1.15	4.81	0.77	2.39	1.51	2.93	1.07	4.06	2.06
V β 9	3.85	1.29	3.04	0.59	4.07	1.19	2.91	1.68	2.40	1.46	3.47	1.52
V β 11	1.03	0.52	0.67	0.22	0.87	0.26	0.87	0.80	0.98	0.74	0.92	0.46
V β 12	1.82	0.53	1.79	0.46	1.82	0.38	1.16	0.79	1.01	0.54	1.29	0.56
V β 13.1	3.96	1.24	4.17	1.18	4.03	0.98	2.96	1.70	3.40	1.43	3.42	0.91
V β 13.2	2.97	1.10	2.9	1.77	2.81	1.18	2.22	1.42	3.04	2.12	3.34	1.80
V β 13.6	1.77	0.47	1.6	0.60	1.86	0.49	1.15	0.85	1.21	0.75	1.6	0.70
V β 14	1.68	0.98	2.54	0.89	2.59	0.65	1.54	1.99	4.74	2.82	5.74	2.55
V β 16	1.09	0.42	1.13	0.58	0.95	0.26	0.75	0.62	0.98	0.50	0.8	0.45
V β 17	5.21	1.12	5.46	1.08	5.46	1.02	3.74	1.87	4.37	1.96	5.06	1.97
V β 18	1.44	0.81	1.34	0.75	1.92	0.46	0.48	0.43	0.43	0.31	0.57	0.41
V β 20	1.64	1.09	2.05	0.79	2.6	1.12	1.21	1.23	1.87	0.92	2.31	1.50
V β 21.3	2.16	0.47	2.31	0.41	2.46	0.53	2.37	1.53	1.66	0.55	2.39	0.93
V β 22	3.75	0.98	4.31	1.79	4.26	1.11	2.57	1.53	3.16	1.12	3.17	1.19
V β 23	0.76	0.32	0.52	0.50	0.48	0.25	1.57	1.07	0.90	0.45	1.34	0.96

*Values are referred to controls provided by the IOTest Beta Mark TCR-V β -Repertoire Kit, Beckman Coulter, Miami, FL (85 subjects were tested).

their surface expression of TCR- $\alpha\beta$ or TCR- $\gamma\delta$), because of the limited genomic complexity of TCR- γ and TCR- δ compared to TCR- α and TCR- β loci. Mono/oligoclonal or polyclonal cell populations may be identified by this method [23].

DNA was extracted from primary cells or from paraffin-embedded tissue using the QIAamp DNA Blood Mini Kit or QIAamp DNA Micro Kit (Qiagen, GmbH, Hilden, Germany). PCR was used to amplify the portion of the rearranged TCR- γ chain gene starting from primers recognizing conserved sequences of variable (V) and joining (J) region segments:

V γ I 5'-TACATCCACTGGTACCTACACCA, a consensus oligo recognizing all rearrangeable members of the first V γ family (V γ 1-8);

V γ II, specific for V γ 9 (5'-TCATACAGTTCCTGGTGTCC);

V γ III, specific for V γ 10/11 (5'-TCATTCAGTGGTACCGGCAG-AAACCAAA);

J γ 1/2, specific for J γ 1 and J γ 2 (5'-CCCGTCGACTACCTTGGA-AATGTTGTATTCTTC) and

J γ 1/P2, specific for J γ 1 and J γ 2 (5'-CCAGGTGAAGTTACTAT-GAG) (16).

Each DNA sample was analysed with six different combinations of primers. Each reaction was set up with 200 ng of genomic DNA using the following conditions: 10 pM of each primer, 20 μ M of each dNTP, 1.5 mM MgCl₂, 200 mM of Tris-HCl (pH 7.4), 500 mM of KCl (10 $\times$), and 0.5 U of Taq Polymerase (Invitrogen, Carlsbad, CA) in a total volume of 20 μ L. Jurkat, a T cell acute lymphoblastic leukemia cell line was used as positive control [28]. PCR products were first visualized on a 1.5% agarose gel with the aim of documenting the successful amplification.

Fifteen microliters of each amplified product was heated at 94 °C for 5 min, rapidly cooled to 50 °C, maintained for 1 h and then transferred on ice for heteroduplex analysis. This denaturation-renaturation step gives rise to homoduplexes in the PRC products of clonally rearranged TCR genes, whereas in the case of polyclonal cells heteroduplexes with heterogeneous junctions are formed. Heteroduplex patterns were visualized on a 10% non denaturing polyacrylamide gel after ethidium bromide staining (Biorad Precast Gel System, Hercules, CA). Samples with one to two distinct bands within the appropriate size range (from 220 to 300 bp) were defined as monoclonal, whereas samples showing a smear were considered polyclonal. Samples with three or more distinct bands were defined as oligoclonal [29]. Selected PBMC from 20/42 healthy controls (see Immunophenotypic analysis) and 10 asthmatics patients were also used as controls for TCR- γ gene rearrangement analysis.

Results

TCR-V β flow cytometry analysis

In all patients one or two V β expanded families among CD8+ cells were observed, resulting in a total of 14 different expanded families (Table 3). In one case the expanded population represented more than 20% of total circulating CD8+ cells (patient #7). We did not observe a preferential expansion of a single CD8+/V β +, being V β 13.1, V β 1, V β 13.6 and V β 18 the most common V β families involved (i.e., each of these families was detected in 2 cases). V β expansions were observed also in CD4+ cells in 4/8 patients, but the degree of

Table 3 TCR-V β expansions and deletions in CD8+ and CD4+ T cells

Patient #	CD8+				CD4+			
	Expansion		Deletion		Expansion		Deletion	
	V β family	% of total CD8+	V β family	% of total CD8+	V β family	% of total CD4+	V β family	% of total CD4+
1	V β 4	12	-	-	-	-	-	-
2	V β 20	11.5	-	-	-	-	-	-
3	V β 5.3	3.5	V β 4	0.0	-	-	V β 4	0.0
3	V β 3	17.8	-	-	-	-	-	-
4	V β 18	2.7	V β 13.6	0.1	-	-	-	-
4	V β 13.1	10.6	-	-	-	-	-	-
5	V β 18	6.6	V β 21.3	0.0	V β 4	3.3	-	-
5	V β 1	16.5	V β 4	0.0	V β 18	4.0	-	-
5	V β 16	4	V β 7.2	0.0	V β 22	9.0	-	-
6	V β 23	10.6	V β 20	0.0	V β 12	3.5	V β 7.2	0.0
7	V β 13.6	7.5	-	-	V β 14	7.1	-	-
7	V β 13.1	22.5	-	-	V β 23	2.9	V β 4	0.6
8	V β 1	9.5	-	-	V β 13.2	12.5	V β 2	4.6
8	V β 13.6	6.7	-	-	V β 13.6	10	V β 5.1	1.0
					V β 16	2.7	V β 8	1.0
					V β 12	3.7	V β 13.1	1.0
					V β 21.3	4.5	-	-

A V β family was considered expanded or deleted when the percentage of V β positive cells was three SD higher or lower than reference values, respectively.

the expansion was lower than in CD8⁺ cells (Table 3). In 5/8 patients a deletion of one or two V β families was found among CD8⁺ cells, resulting in a total of 5 different deleted families, whereas in 4/8 patients one or more deletion was found among CD4⁺ cells. V β 4 was the most commonly deleted family both in CD8⁺ and in CD4⁺ cells (Table 3). V β expanded or deleted families were not observed in asthmatic patients ($n=10$) and in healthy controls ($n=42$). Mean values and SD of both asthmatic patients and healthy controls are reported in Table 2.

Immunophenotyping of V β expanded T cells

The naive/memory phenotype of V β expanded families was determined in 7/8 patients by 4 color staining with CD62L ($n=7$), CCR7 ($n=4$) or CD28 ($n=7$) combined to CD45RA, CD8 and the relevant V β [17-19]. Values were compared to those obtained with PBMC from healthy controls stained for the

same V β family ($n=10$). In all patients the V β expanded T cells showed a shift from naive to memory phenotype (T_{EM}) compared to healthy controls (Fig. 1). In details, the percent of naive cells within each expanded V β population was always 2 standard deviations below the mean value observed in healthy donors. A significant expansion (more than the mean value of controls plus 2 SD) of T_{EM} cells was observed in all 7 patients, in 3 patients within the CD45RA⁻ population and in 4 patients within the CD45RA⁺ population (Table 4). When looking at total CD8⁺ cells, a shift from naive to memory cells was observed in CSS patients as compared to healthy controls (Fig. 1 and data not shown).

Analysis of T cells clonality by TCR- γ gene rearrangement

Molecular analysis of clonality by heteroduplex PCR on TCR-V γ genes was initially performed on PBMC collected at the same

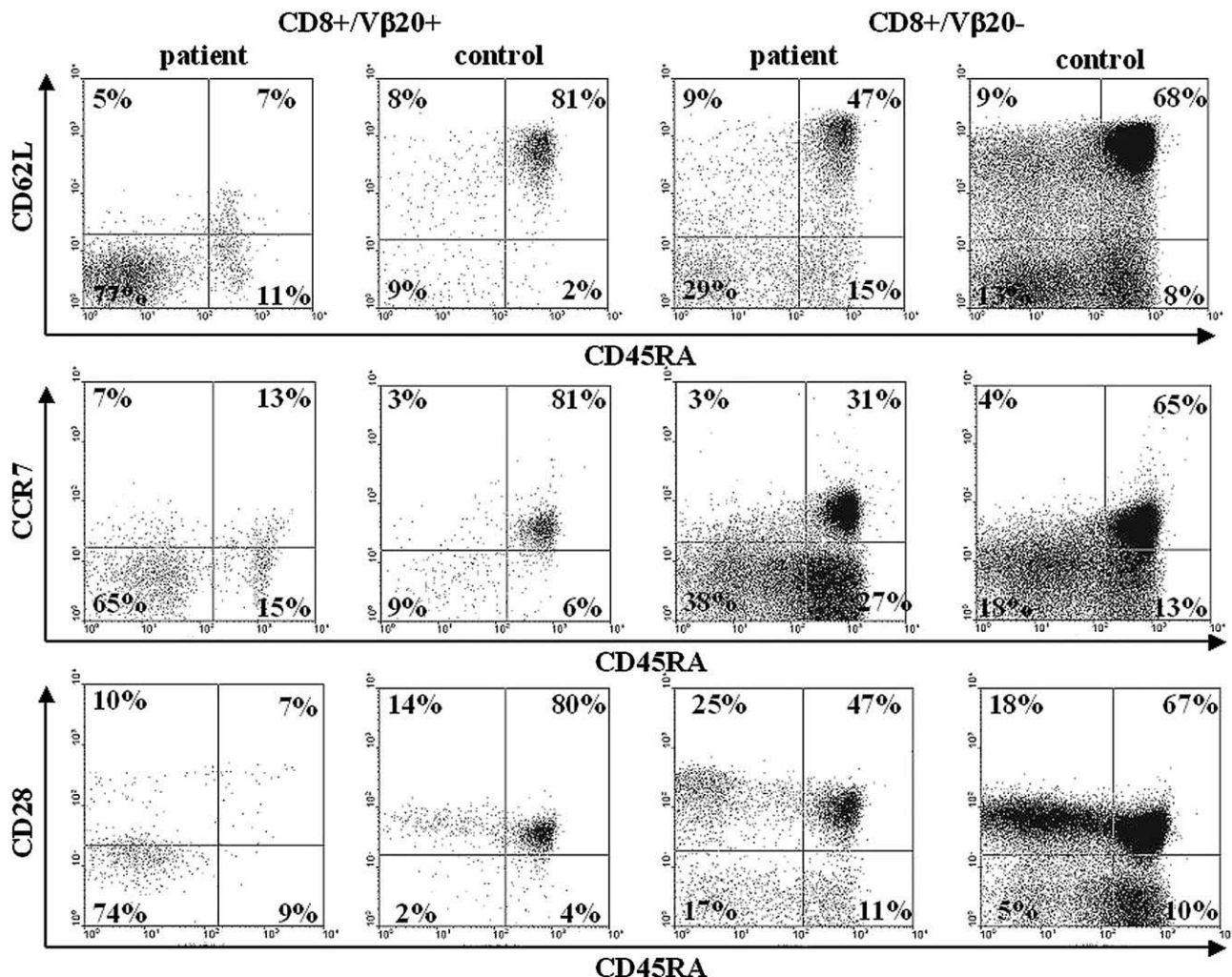


Figure 1 Analysis of naive/memory phenotype by staining with anti-CD62L, anti-CCR7 and anti-CD28 combined to anti-CD45RA. Upper pane: CD62L and CD45RA expression by CD8⁺/V β 20⁺ and CD8⁺/V β 20⁻ gated cells from patient #1 and from one healthy subject. Middle pane: CCR7 and CD45RA expression by CD8⁺/V β 20⁺ and CD8⁺/V β 20⁻ gated cells from patient #1 and from one healthy subject. Lower pane: CD28 and CD45RA expression by CD8⁺/V β 20⁺ and CD8⁺/V β 20⁻ gated cells from patient #1 and from one healthy subject.

Table 4 Naive and memory phenotype of CD8+/Vβ+ expanded families

Patient #	Expanded	Naive				Central memory				Effector memory			
		CD62L+	CCR7+	CD45RA+	(%)	CD62L+	CCR7+	CD45RA+	(%)	CD62L+	CCR7+	CD45RA+	(%)
1	Vβ20	7 (65±15)	13 (64±17)	7 (66±17)	5 (10±5)	7 (7±5)	10 (23±8)	77 (15±7)	65 (20±11)	74 (5±4)	11 (9±8)	15 (7±8)	9 (6±7)
2	Vβ5.3	6 (36±15)	ND	11 (35±10)	5 (19±8)	ND	28 (53±18)	85 (42±17)	ND	57 (9±3)	4 (3±1)	ND	4 (3±1)
3	Vβ3	5 (55±18)	9 (45±14)	8 (46±16)	3 (14±7)	7 (8±5)	4 (23±13)	9 (22±13)	20 (31±14)	5 (15±17)	83 (8±7)	64 (15±17)	83 (14±15)
4	Vβ13.1	7 (70±17)	ND	16 (69±17)	7 (11±10)	ND	13 (6±4)	22 (11±7)	ND	20 (19±8)	64 (7±8)	ND	51 (8±9)
5	Vβ1	13 (55±21)	11 (54±19)	10 (52±20)	3 (11±7)	2 (5±3)	4 (24±10)	39 (24±15)	40 (31±16)	44 (22±14)	45 (9±10)	47 (14±11)	46 (12±14)
6	Vβ23	3 (54±17)	ND	5 (61±20)	1 (12±8)	ND	6 (24±17)	88 (23±12)	ND	83 (6±4)	8 (11±9)	ND	6 (9±4)
8	Vβ1	10 (55±22)	11 (53±18)	7 (52±20)	4 (11±7)	9 (5±3)	4 (24±14)	37 (24±15)	37 (31±16)	38 (12±14)	49 (9±10)	43 (14±12)	51 (12±17)

Characters in bold indicate a significant variation in comparison to the normal counterpart. A Vβ subpopulation was considered increased or decreased when above or below mean ± 2 SD of the corresponding Vβ subpopulation from healthy controls ($N = 10$), respectively. Mean $\pm$ SD values of healthy controls are reported in brackets. ND = not determined.

Table 5 Molecular analysis of TCR-V-Jγ clonality in peripheral blood and in Vβ+ purified CD8+ cells

Patient	Expansion - Vβ family involved	Whole PBMC - V-Jγ family involved	Clonality -	
			Vβ+ fraction	Vβ- fraction
1	Vβ20	Vγ9 Jγ1/2	Vγ9 Jγ1/2	none
2	Vβ5.3	Vγ10-11 Jγ1/2	ND	ND
3	Vβ3	Vγ1-8 Jγ1/2	Vγ1-8 Jp1/P2	Vγ1-8 Jγ1/2
		Vγ1-8 Jp1/P2	Vγ1-8 Jp1/P2	Vγ9 Jγ1/2
		Vγ9 Jγ1/2		
4	Vβ13.1	Vγ1-8 Jγ1/2	Vγ1-8 Jγ1/2	Vγ1-8 Jp1/P2
		Vγ1-8 Jp1/P2		Vγ10-11 Jγ1/2
		Vγ10-11 Jγ1/2		Vγ10-11 Jp1/P2
		Vγ10-11 Jp1/P2		
5	Vβ1	Vγ1-8 Jγ1/2	Vγ1-8 Jγ1/2	Vγ1-8 Jp1/P2
6	Vβ23	Vγ1-8 Jp1/P2	ND	ND
7	Vβ13.1	Vγ1-8 Jp1/P2	ND	ND
		Vγ10-11 Jp1/P2		
8	Vβ1	Vγ1-8 Jγ1/2	Vγ1-8 Jγ1/2	Vγ1-8 Jp1/P2
		Vγ1-8 Jp1/P2		

ND = not determined.

time of the first flow cytometry analysis. All patients showed a positive PCR amplification of at least one TCR-V-Jγ family, without a preferential selection (Table 5). In 5/8 patients the presence of 2-4 discrete bands suggested that more than one clonal expansion occurred within the same patient. All 20 healthy control samples and 10 asthmatic patients resulted negative for clonal V-Jγ rearrangement.

In 5/8 patients clonality was also assessed in the purified expanded CD8+/Vβ+ population (see Materials and methods) where only one of the bands identified in whole PBMC was detected, while the other bands were detected in the CD8+ Vβ- fraction (Table 5 and Fig. 2). Therefore the expanded CD8+/Vβ+ populations detected by flow cytometry correspond to the clonal restrictions by molecular analysis. Sample availability did not allow for purifying all expanded Vβ families of each patient.

During follow up, a patient affected by chronic rhinosinusitis with nasal polyps underwent functional endoscopic sinus surgery (patient #1). PCR amplification of the biopsy sample showed the same V-Jγ clonal rearrangement previously detected in PBMC.

Flow cytometry and molecular analysis in the follow up

Flow cytometric quantification of expanded CD8+/Vβ+ families was repeated in each patient at different time points throughout a median follow up period of 14 months (range 3-19). In 7/8 cases the most relevant Vβ expansions were always detected. In one patient (#4), a transient

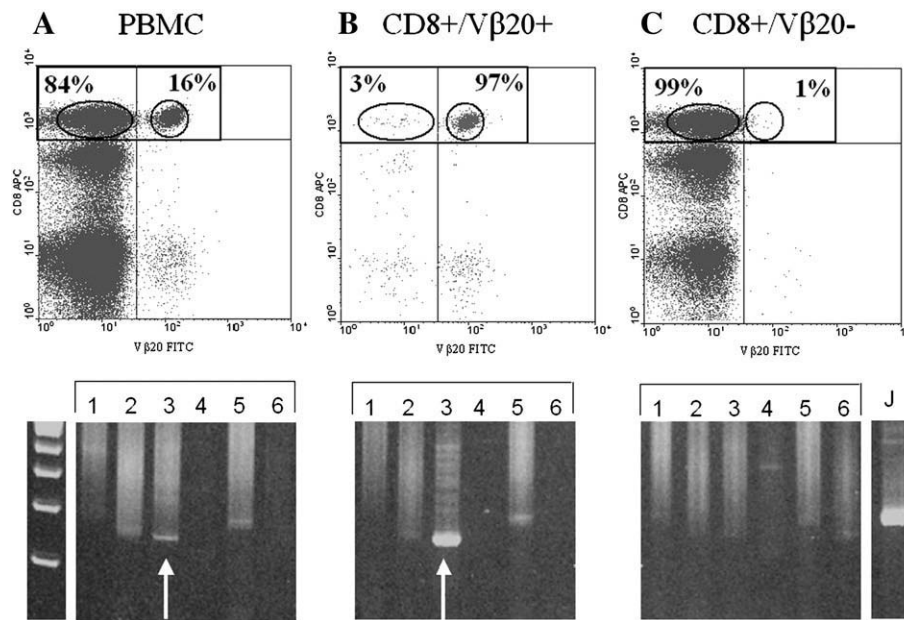


Figure 2 Purification of a V β expanded family (upper panel) and analysis of its TCR rearrangement (lower panel). Upper panel shows flow cytometry analysis of patient #1 (A = whole PBMC, B = CD8⁺/V β 20⁺ purified cells, C = CD8⁺/V β 20⁻ purified cells). Lower panel shows the heteroduplex PCR analysis of TCR-V-J γ of the same patient (A = whole PBMC, B = CD8⁺/V β 20⁺ purified cells, C = CD8⁺/V β 20⁻ purified cells). Each lane corresponds to the following primer mix: lane 1, V γ ₁₋₈ and J γ _{1/2}; lane 2, V γ ₁₋₈ and J γ _{1/P2}; lane 3, V γ ₉ and J γ _{1/2}; lane 4, V γ ₉ and J γ _{1/P2}; lane 5, V γ _{10/11} and J γ _{1/2}; lane 6, V γ _{10/11} and J γ _{1/P2}. One discrete band in the V γ ₉-J γ _{1/2} amplification (white arrow) was detected in whole PBMC and in the CD8⁺/V β 20⁺ purified fraction. J: Jurkat cell line used as positive control.

normalization of the expression of V β 13.1 was observed (Fig. 3). In all cases, including the latter, the TCR-V-J γ clonal pattern was always detected at the different time points (data not shown).

Discussion

In this study, one or more V β families were found numerically expanded by flow cytometry in all CSS patients analysed, both in clinical remission and during active treatment for the disease. These expansions were mostly represented by CD8⁺ lymphocytes with effector memory phenotype. Each CD8⁺/V β ⁺ expansion is the result of the proliferation of a clonal

T cell bearing a defined TCR, as detected by analysis of TCR-V-J γ rearrangement. These data are more consistent with a persistent antigenic stimulation, which has been reported to occur in some cases of CSS and other autoimmune diseases [9-11,30,31], than with a superantigenic stimulus, as reported in other vasculitides [12,13].

The CD8⁺/V β ⁺ expanded populations have an effector memory (T_{EM}) phenotype, as evidenced by flow cytometric analysis of CD45RA expression combined to CD62L, CCR7 or CD28 [17,18]. T_{CM} are antigen-experienced cells which constitutively express CD62L and CCR7, two surface molecules necessary for cellular extravasation and migration to peripheral lymph nodes. On the other hand, T_{EM} are antigen-experienced T cells which have low expression of CD62L and CCR7 [19] and have the propensity to populate peripheral tissues as well as inflammatory sites [21]. On these bases, we can speculate that in CSS circulating CD8⁺/V β ⁺ T_{EM} are antigen driven cells which may infiltrate target organs and participate to the inflammatory cascade of both vasculitic and granulomatous lesions. In Wegener's granulomatosis, for example, the driving force of both granuloma formation and autoimmunity has been identified in T_{EM} cells [32]. In agreement with this hypothesis, we found that a sinus biopsy sample from one patient was infiltrated by T cells bearing the same clonal TCR-V-J γ rearrangement which was previously detected in his peripheral CD8⁺/V β ⁺ T_{EM} lymphocytes. A more detailed analysis of T cells infiltrating damaged tissues in CSS patients is clearly needed to sustain this hypothesis.

The molecular analysis of TCR- γ in PBMC shows the presence of one or more clonal TCR rearrangements, which reveals monoclonal or oligoclonal T cell subpopulations. After

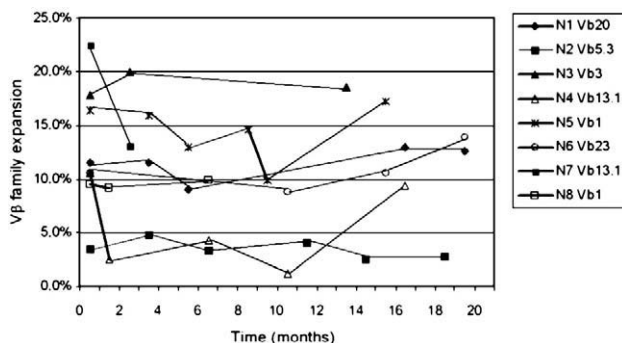


Figure 3 Percent CD8⁺/V β ⁺ expanded populations during the follow up of CSS patients. Each line marked with different symbols refers to one patient.

purification with V β specific MoAb, each CD8+/V β + expanded family shows a single TCR rearrangement indicating a monoclonal pattern. The finding of other clonal bands in the CD8+/V β - fraction suggests the existence of V β restricted populations that can be either or not quantitatively expanded. This finding may in part explain the difference between our flow cytometry data and previous findings of recurrent V β 21 oligoclonal expansions detected by immunoscope [15]. Additionally, different HLA types between the two series of patients might stimulate different TCR-V β families [33].

Diagnosis of CSS may be difficult because the syndrome arises as a common association between asthma, rhinosinusitis and eosinophilia. Asthma itself may be associated with occasional pulmonary infiltrates (i.e. mucus plugging, atelectasis) and corticosteroids dependency, so that a clear diagnosis of CSS might not be made until evidence of vasculitis in one or more organs develops. Unfortunately, there are no validated serological markers of the disease. Promising markers of CSS seem to be serum levels of vascular endothelial growth factor [34] and high-mobility group box-1 [35]. We suggest that the detection of clonal expanded peripheral CD8+ T cell populations (assessed by flow cytometry combined to analysis of TCR- γ gene rearrangement, two methods routinely used in immunopathology laboratories) might be useful in the differential diagnosis of CSS, as suggested by the negative findings in our asthmatic control patients.

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