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Immunophenotype and TCR-Vβ repertoire of peripheral blood T-cells in acute infectious mononucleosis

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Abstract

Although a number of studies on the phenotypic changes that occur after T-cell activation have already been published, the specific immunophenotypic features of T-lymphocytes and the frequency at which TCR-variable region (TCR-V) restricted T-cell expansions occur "in vivo" during acute viral infection still remains to be established. We report on the immunophenotype and TCR-V repertoire of peripheral blood T-cells from 28 patients with acute infectious mononucleosis. Immunophenotypic studies were performed by flow cytometry using direct immunofluorescence techniques and stain-and-then-lyse sample preparation protocols with three- and four-colour combinations of monoclonal antibodies directed against a large panel of T- and NK-cell associated markers, activation- and adhesion-related molecules and TCR-Vβ, -Vγ and -Vδ families. Nearly all patients (27/28) showed a massive expansion of CD8$^+$/TCR$\alpha\beta^+$ T cells, the majority (>90%) of which displayed an immunophenotype compatible with T-cell activation: CD2^{+high}, CD7^{+low}, CD11a^{+high}, CD38^{+high}, HLA-DR^{+high}, CD28$^{+/-low}$, CD45RO^{+high}, CD45RA$^{-/+low}$, CD11b$^{-/+low}$, CD11c$^{+/-low}$, CD16$^-$, CD56$^-$, CD57$^-$, CD62L$^-$, CD94$^-$, CD158a$^-$, CD161$^-$, NKB1$^-$. Additionally, the levels of both CD3 and CD5 were slightly decreased compared to those found in normal individuals. Late-activation antigens, such as CD57, were found in small proportions of CD8$^+$/TCR$\alpha\beta^+$ T-cells. Increased numbers of CD4$^+$/TCR$\alpha\beta^+$ T-cells, TCR$\gamma\delta^+$ T-cells and NK-cells were also noticed in 17, 16 and 13 of the 28 cases studied, respectively. Evidence for activation of CD4$^+$/TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ T-cells relied on changes similar to those described for CD8$^+$/TCR$\alpha\beta^+$ although less pronounced, except for higher levels of both CD5 and CD28 in the absence of reactivity for CD11c on CD4$^+$/TCR$\alpha\beta^+$ T-cells and higher levels of CD161 and CD94 on TCR$\gamma\delta^+$ T-cells. Small expansions of one or more TCR-Vβ families accounting for 12 $\pm$ 7% of either the CD8$^+$/TCR$\alpha\beta^+$ or the CD4$^+$/TCR$\alpha\beta^+$ T-cell compartment were found in 12 of 14 patients studied, whereas the distribution of the TCR-Vγ and -Vδ repertoires tested in 2 of the individuals with expanded TCR$\gamma\delta^+$ T-cells was similar to that observed in control individuals. The results presented here provide evidence for an extensive T-cell activation during acute viral infection and establish the immunophenotype patterns associated with this condition.

Keywords: Infectious mononucleosis; T-cells; Flow cytometry; Immunophenotype; TCR-V repertoire

Introduction

The term acute infectious mononucleosis (AIM) is usually used to designate a syndrome that associates with acute viral infection [1]. Besides Epstein–Barr virus (EBV), other herpes viruses may also be involved [2–5] and primary infection with human retroviruses may occasionally exhibit a mononucleosis-like presentation [6,7]. In most occasions, AIM develops and evolves as a benign self-limited disease; however, some patients develop serious complications, occasionally with a fatal outcome. Fever, lymphadenitis, pharyngitis and jaundice are frequent clinical findings. In contrast, arthritis, gastroenteritis, pneumonitis, mediastinitis,

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encephalitis, orchitis, haemophagocytic syndrome, splenic infarct and rupture are much less frequent complications of the disease [8–18]. Laboratory findings include lymphocytosis with circulating atypical lymphocytes, liver cytolysis and colestasis. Severe cytopenias may be noted in some patients [19–21].

Previous studies suggest that B-cells are the primary target of EBV infection and that infected B-cells could act as a virus reservoir, although T-cells may be infected as well [22–24]. Additionally, evidence exists that patients with AIM show an increased proliferation of reactive activated $CD8^+$ T-lymphocytes [25–27], some of which are specific for viral antigens [28]. Increased levels of T-cell derived cytokines and other soluble factors may have a specific role in both the immune response and the clinical manifestations associated with AIM [29–31].

The relationship between EBV infection and the development of clonal lymphoproliferative disorders (LPD) has been extensively investigated in immunosuppressed patients, especially in transplant recipients [32,33]. Accordingly, it is now generally accepted that after viral infection these individuals may develop clonal expansions of lymphocytes that may end up in the so-called post-transplant LPD. Infection with EBV was also documented in both T- and NK-cell lymphomas as well as in other haematological and non-haematological malignancies [34,35] and there is evidence for a direct relationship between chronic EBV infection and lymphomagenesis [36,37]. Previous studies based on both immunophenotypic analysis of the T-cell receptor (TCR) Vβ repertoire and assessment of molecular clonality suggest the possibility of occurrence of both oligoclonal and monoclonal T-cell expansions during AIM in otherwise normal individuals, although data available in the literature are controverse [38–44].

A number of studies have focused on the immunophenotypic changes occurring during T-cell activation [45–56]. These reports show that both early and late T-cell activation stages can be identified though the analysis of specific phenotypic changes involving up- and down-regulation of different cell surface proteins. Accordingly, some antigens, such as CD69, are transiently up-regulated just after T-cell activation [47] whereas other markers have been recognized to appear at relatively early stages and persist thereafter; these include up-regulation of HLA-DR, CD38, CD2 and CD11a and a decreased expression of the CD28 T-cell costimulatory molecule [45–57]. Finally, expression of other antigens, such as CD57, typically occur in a later phase of the T-cell response [58–60]. In spite of these well-established changes occurring after T-cell activation, a comprehensive study describing the specific immunophenotypic features of T- lymphocytes during acute viral infection is still lacking in the literature; in addition, the frequency at which TCR-Vβ restricted T-cell expansions occur in AIM still remains to be established.

A detailed characterization of the T-cell phenotypes observed in AIM will provide the basis for the understanding of the biological changes (up- or down-regulation of cell receptors, adhesion molecules and other functional membrane proteins) occurring "in vivo" in conditions of "extreme" cellular activation. From the clinical point of view, it also has an immediate application in cases in which differential diagnosis with lymphoma is pertinent, especially for patients presenting with lymphadenopathies and/or organomegalies as well as for those individuals in whom serological screening for common viral diseases is not conclusive.

In the present paper we explore in detail the immunophenotypic characteristics of peripheral blood (PB) T-cells, including the analysis of their TCR-Vβ repertoire, in a group of 28 adult patients with well-documented AIM.

Material and methods

Patients

A total of 28 patients (16 males and 12 females with a mean age of 30 years; range 11 to 58 years) diagnosed as suffering from AIM were included in this study. None of these patients had previous evidence for an underlying immunodeficiency and none of them received previous immunosuppressive drugs. The possibility of ingestion of drugs that have been associated with an AIM-like syndrome was also excluded. Two patients had been previously splenectomised, one of them because of autoimmune haemolytic anaemia and the other due to a traumatic splenic rupture. Fever, malaise, lymphadenopathy, splenomegaly, hepatomegaly and pharingitis were found in 88%, 84%, 54%, 24%, 28% and 28% of the cases, respectively. Purpura and other muco-cutaneous haemorrhage occurred in 2 cases, both presenting with severe thrombocytopenia. Other less frequent clinical findings included ictericiae in 3 patients, skin rash in 2 cases, gastroenteritis, otitis, vertiginous syndrome, disturbed consciousness with unknown origin and autoimmune haemolytic anaemia, pneumonitis, uveitis and encephalitis observed in 1 patient each. All but 1 patient had an acute self-limited disease, with clinical resolution in 9 ± 8 weeks (range: 2 to 23 weeks). Twenty-five patients recovered spontaneously without need for specific therapy while in 2 patients treatment for severe thrombocytopenia and haemorrhage, consisting of high dose intravenous immunoglobulins and steroids, was needed and given. The remaining patient, who had been previously splenectomised for autoimmune haemolytic anaemia, evolved into a chronic disease with ophthalmic, pulmonary and neurological complications. This patient was treated with steroids, intravenous immunoglobulins and acyclovir without success and dyed with active CMV infection 1 year later.

Six normal healthy individuals (blood donors) were used as controls (3 males and 3 females with a mean age of 45 years, ranging from 37 to 51 years).

Table 1
Specificities, clones and sources of the monoclonal antibodies used in the present study

Specificity	Clone	Conjugate	Source[a]	Specificity	Clone	Conjugate	Source[a]	Specificity	Clone	Conjugate	Source[a]
CD2	SFCI3Pt2H9	FITC; PE	BC	CD62L	SK11	FITC	BD	TCR-Vβ8.1/.2	C2156C5.2	FITC	IOT
CD3	UCHT1; SK7	FITC; PerCP	IOT; BD	CD94	HP-3D9	FITC	PH	TCR-Vβ11.1	C21	FITC	IOT
CD4	SK3	FITC, PerCP or APC	BD	CD122	MIK-b	FITC	CLB	TCR-Vβ12.2	VER2.32.1	FITC	IOT
CD5	L17F12	PE	BD	CD158a	HP-3E4	FITC	BD	TCR-Vβ13.1	JU74.3	FITC	IOT
CD7	CLB-3A1/1,7F3; 3A1E-12H7	FITC; PE	CLB; BC	CD161	DX12	PE	BD	TCR-Vβ13.6	IMMV222	FITC	IOT
CD8	SK1	PE; APC	BD	Bcl2/p26	124	FITC	DK	TCR-Vβ14.1	CAS1.1.3	FITC	IOT
CD11a	CLB-LFA-1/2, TB133	FITC	CLB	HLA-DR	L243	PE	BD	TCR-Vβ16.1	TAMAYA1.2	FITC	IOT
CD11b	D12	PE	BD	NKB1	DX9	PE	BD	TCR-Vβ17.1	E17.5F3.15.13	FITC	IOT
CD11c	S-HCL-3	PE	BD	TCR-β	T10B9,1A-31	FITC	PH	TCR-Vβ18.1	BA62.6	PE	IOT
CD16	3G8	FITC; PE	BC; IOT	TCR-γ/δ	11F2; B1	PE; APC	BD; PH	TCR-Vβ20.1	ELL1.4	FITC	IOT
CD19	89B	FITC	BC	TCR-Vβ1	BL37.2	FITC	IOT	TCR-Vβ21.3	IG125	FITC	IOT
CD25	2A3	PE	BD	TCR-Vβ2.1	MPB2D5	FITC	IOT; BioD	TCR-Vβ22.1	IMMV546	FITC	IOT
CD28	L293	PE	BD	TCR-Vβ3.1	CH92; 8F10	FITC	IOT; END	TCR-Vβ23.1	AF23	PE	IOT
CD38	LD38; HB7	FITC; PE	CYT; BD	TCR-Vβ5.1	IMMV157	FITC	IOT	TCR-Vδ1	TCR2730	FITC	END
CD45	2D1	PerCP	BD	TCR-Vβ5.2	36213	FITC	IOT	TCR-Vδ2	P91464	FITC	BIO
CD45RA	L48	FITC	BD	TCR-Vβ5.3	3D11	PE	IOT	TCR-Vδ3	P91564	FITC	BIO
CD45RO	UCHL-1	PE	BD	TCR-Vβ6.1	CRI304.3	FITC	IOT	TCR-Vγ4	TCR2722	FITC	END
CD56	NCAM16.2; N901/NKH1	PE	BD; IOT	TCR-Vβ6.7	T145	FITC	END	TCR-Vγ9	TCR2720	FITC	END
CD57	HNK-1	FITC	BD	TCR-Vβ7.1	ZOE	FITC	IOT				

[a] BC, Beckman Coulter, Miami, FL, USA; IOT, Immunotech, Marseille, France; BD, Becton–Dickinson, San José, CA, USA; CLB, Amsterdam, The Netherlands; DK, Dako A/S, Glostrup, Denmark; CYT, Cytognos, Salamanca, Spain; PH, PharMingen, San Diego, CA, USA; BioD, Biodesign International, Kennebunk, ME, USA; END, Endogen, Woburn, MA, USA.

Samples

In all cases EDTA-K3 anti-coagulated PB samples were obtained at diagnosis for immunophenotypic analysis of circulating T- and NK-cells as well as other laboratory studies which are listed below.

Flow cytometry immunophenotypic studies

Immunophenotypic analysis of surface antigen expression on PB lymphocytes was performed in all cases using direct immunofluorescence techniques and a stain-and-then-lyse sample preparation protocol. The monoclonal antibodies (MoAb) used are shown in Table 1. Erythrocyte lysis and cell fixation were performed using either FACS lysing solution (Becton–Dickinson BioSciences, BD, San José, CA, USA)—for lymphocyte immunophenotyping—or Q-Prep reagents (Beckman Coulter, Miami, FL, USA)—for the analysis of the TCR-Vβ, TCR-Vγ and TCR-Vδ repertoires.

In all cases the following three-colour combinations of MoAb conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridin chlorophyll protein (PerCP) were initially used in order to quantify and characterize the major PB lymphocyte sub-populations (FITC/PE/PerCP): CD19/CD2/CD45, CD3/CD16+CD56/CD45, TCR$\alpha\beta$/TCR$\gamma\delta$/CD3, CD4/CD8/CD3 and CD8/HLA-Dr/CD3.

$CD8^+$/TCR$\alpha\beta^+$ and $CD4^+$/TCR$\alpha\beta^+$ T-cells were further characterized in 15 and 9 cases, respectively, using three- or four-colour stainings that combined allophycocyanin (APC) conjugated anti-CD8 and/or PerCP-conjugated anti-CD4 with the following pairs of MoAb (FITC/PE): CD2/CD7, CD7/CD5, CD62L/CD28, CD16/CD56, CD57/CD11c, CD38/CD11b, CD45RA/CD45RO, CD122/CD25, CD11a/HLA-DR, CD94/CD8, CD16/NKB1 and CD158a/CD161.

In addition, the TCR-Vβ repertoire was evaluated in 14 of these patients by flow cytometry using triple-stainings with anti-CD4-PerCP and anti-CD8-FITC (or PE) combined with a panel of MoAb specific for 23 different TCR-Vβ families (FITC or PE) (Table 1). This panel of MoAb identified a mean of 60 ± 4% of $CD4^+$/TCR$\alpha\beta^+$ and 46 ± 6% of $CD8^+$/TCR$\alpha\beta^+$ T-cells in the PB of age-matched normal healthy individuals.

Expression of p26/bcl2 was specifically explored on $CD8^+$/TCR$\alpha\beta^+$ T-cells using the Fix & Perm reagent kit (An Der Grub Bioresearch GmbH, Vienna, Austria) and strictly following the recommendations of the manufacturer.

In three of the patients who showed increased numbers of TCR$\gamma\delta^+$ T-cells, these cells were specifically studied by

Table 2
Blood cell counts observed at diagnosis in patients with AIM ($n = 28$)

	Patients with AIM			Controls, normal range
	Mean ± standard deviation (range)	% Cases with abnormal values		
		Decreased	Increased	
WBC ($\times 10^9$/L)	12.4 ± 5.9 (5.1–27.6)	0	50	4.0–10.0
Lymphocytes ($\times 10^9$/L)	8.2 ± 4.9 (2.1–21.7)	0	89	1.5–3.5
Neutrophils ($\times 10^9$/L)	3.1 ± 1.5 (0.6–6.9)	18	0	2.0–7.5
Haemoglobin (g/dl)	12.8 ± 2.2 (7.9–16.8)	32	0	12.0–17.0
Platelets ($\times 10^9$/L)	205 ± 100 (10–417)	32	4	150–400
T-cells ($\times 10^6$/L)	6963 ± 4030 (1384–16964)	0	96	1100–1700
T-cells (%)	85 ± 7% (67–94%)	0	89	67–76
NK-cells ($\times 10^6$/L)	636 ± 724 (94–3519)	21	57	200–400
NK-cells (%)	8 ± 5% (2–20%)	75	0	10–20
B-cells ($\times 10^6$/L)	260 ± 211 (50–955)	50	18	200–400
B-cells (%)	4 ± 3% (1–12%)	93	0	10–20
TCR$\alpha\beta^+$/CD4$^+$ T-cells ($\times 10^6$/L)	1290 ± 599 (570–2815)	11	61	700–1100
TCR$\alpha\beta^+$/CD4$^+$ T-cells (%)	18 ± 7% (9–37%)	100	0	38–46
TCR$\alpha\beta^+$/CD8$^+$ T-cells ($\times 10^6$/L)	5354 ± 3433 (620–13471)	0	96	500–900
TCR$\alpha\beta^+$/CD8$^+$ T-cells (%)	63 ± 12% (30–81%)	0	96	31–40
TCR$\gamma\delta^+$ T-cells ($\times 10^6$/L)	431 ± 795 (32–3594)	11	46	50–200
TCR$\gamma\delta^+$ T-cells (%)	6 ± 8% (1–32%)	61	11	3–10

using combinations of MoAb similar to those described above for TCR$\alpha\beta^+$ T-cells except that anti-TCR$\gamma\delta$-APC was used instead of anti-CD4 and anti-CD8. In addition, in 2 of these patients both the TCR-Vδ and TCR-Vγ repertoires were assessed using a panel of MoAb specific for the TCR-Vδ and TCR-Vγ chains (Table 1).

In all cases data acquisition was carried out using either a two-laser FACScalibur flow cytometer (Becton–Dickinson)—for lymphocyte immunophenotyping—or an EPICS-XL-MCL flow cytometer (Beckman/Coulter)—for analysis of p26/bcl2 and the TCR-Vβ repertoire; data analysis was performed using the Paint-A-Gate (Becton–Dickinson) and the XL2 (Beckman/ Coulter) software programs, respectively. For each marker, antigen expression was evaluated as percentage of positive cells, its mean fluorescence intensity (MFI) and coefficient of variation (CV).

Other laboratory tests

Sera from all patients were tested for the presence of antibodies against the following viruses and parasites: anti-EBV viral capside antigen IgG and IgM, anti-cytomegalovirus (CMV) immunoglobulins, anti-herpes simplex virus IgG and IgM, anti-herpes zoster virus IgG and IgM, anti-rubella virus IgG and IgM, anti-hepatitis A, B and C (HCV) viruses, anti-human immunodeficiency viruses (HIV) type 1 and 2, anti-human T cell leukaemia/lymphoma viruses type I and II and anti-*Toxoplasma gondii* IgG and IgM.

In 22/28 cases the infectious agent was identified by serological studies and corresponded to EBV in 11 cases, CMV in 9 cases (from whom one was associated to *T. gondii* and another to HCV infection), HCV in 2 cases (one of which associated to CMV infection) and recent HIV in 1 case. In the remaining 6 patients the serological screening gave negative results although both the clinical and other laboratory features at diagnosis and the clinical outcome were consistent with the diagnosis of AIM. One of these cases was persistently positive for the CMV antigen without serological evidence for acute CMV infection or reactivation of CMV infection.

Other laboratory parameters evaluated in all cases here studied included a full haematological cell count, a morphological evaluation of blood smears and a routine biochemical survey including liver enzymes, serum creatinine and lactic dehydrogenase (data not shown). Further biochemical, radiological, immunological, microbiological and histological studies were performed whenever necessary to confirm the diagnosis.

Statistical analysis

For all variables under study, median, mean, standard deviation, minimum and maximum were calculated. To establish the statistical significance of the differences observed between AIM patients and normal individuals, the Mann–Whitney *U* and the Student *t* tests were used.

Results

Blood cell counts

The mean PB cell counts are summarized in Table 2. As may be seen, most of the patients (25 of 28) presented with lymphocytosis ($>3.5 \times 10^9$/L) that was higher than 10×10^9/L in 9 individuals. Neutropenia was observed in 5

Table 3
Modulation of antigen expression on peripheral blood $CD8^+/TCR\alpha\beta^+$, $CD4^+/TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T-cell subsets in AIM patients

	$CD2^+$	$CD3^+$	$CD5^+$	$CD7^+$	$CD11a^+$	$CD28^+$	$CD38^+$	$CD45RA^+$	$CD45RO^+$	HLA-DR^+
$CD8^+/TCR\alpha\beta^{+a}$										
AIM	312 ± 103	141 ± 35	838 ± 302	745 ± 353	596 ± 91	120 ± 42	137 ± 68	26 ± 29	524 ± 241	1334 ± 634
(n = 15)	**282**	**141**	**817**	**632**	**608**	**114**	**130**	**19**	**423**	**1338**
Controls	259 ± 49	197 ± 32	1033 ± 153	2041 ± 712	368 ± 84	242 ± 82	79 ± 19	188 ± 52	574 ± 337	227 ± 42
(n = 6)	**266**	**196**	**1034**	**2115**	**358**	**231**	**74**	**166**	**511**	**246**
$CD4^+/TCR\alpha\beta^{+b}$										
AIM	293 ± 80	297 ± 88	1670 ± 350	1107 ± 421	385 ± 155	276 ± 119	126 ± 56	124 ± 45	771 ± 301	953 ± 431
(n = 10)	**262**	**261**	**1657**	**1180**	**350**	**243**	**136**	**116**	**673**	**932**
Controls	229 ± 32	433 ± 39	1735 ± 156	2163 ± 837	223 ± 54	413 ± 206	34 ± 10	155 ± 138	788 ± 593	410 ± 144
(n = 6)	**219**	**434**	**1740**	**2302**	**203**	**421**	**32**	**94**	**793**	**389**
$TCR\gamma\delta^{+c}$										
AIM	283 ± 42	588 ± 77	599 ± 360	886 ± 336	503 ± 97	153 ± 54	109 ± 62	76 ± 42	479 ± 104	791 ± 656
(n = 3)	**275**	**547**	**447**	**736**	**500**	**150**	**101**	**52**	**428**	**761**
Controls	250 ± 33	503 ± 69	658 ± 235	1678 ± 823	348 ± 111	417 ± 119	42 ± 16	230 ± 140	392 ± 235	93 ± 23
(n = 5)	**265**	**475**	**538**	**1404**	**388**	**421**	**42**	**206**	**388**	**96**

Note. Results are presented as the mean ± one standard deviation and median (bold) of the mean fluorescence intensity (MFI) of positive cells, expressed as arbitrary relative linear units scaled from 0 to 10,000. No differences were observed for the MFI of CD4 on $CD4^+/TCR\alpha\beta^+$ T-cells from patients with AIM and controls (3821 ± 792 versus 3606 ± 541 and 95 ± 39 versus 97 ± 39, respectively).

[a] AIM versus controls: CD2: $P > 0.05$; CD3: $P = 0.014$; CD5: $P = 0.02$; CD7: $P = 0.001$; CD11a: $P = 0.002$; CD28: $P = 0.003$; CD38: $P = 0.043$; CD45RA: $P = 0.001$; CD45RO: $P > 0.05$; HLA-DR: $P = 0.001$.

[b] AIM versus controls: CD2: $P > 0.05$; CD3: $P = 0.028$; CD5: $P > 0.05$; CD7: $P = 0.025$; CD11a: $P = 0.003$; CD28: $P > 0.05$; CD38: $P = 0.005$; CD45RA: $P > 0.05$; CD45RO: $P > 0.05$; HLA-DR: $P = 0.007$.

[c] AIM versus controls: CD2: $P > 0.05$; CD3: $P > 0.05$; CD5: $P > 0.05$; CD7: $P > 0.05$; CD11a: $P = 0.053$; CD28: $P = 0.025$; CD38: $P = 0.053$; CD45RA: $P = 0.053$; CD45RO: $P > 0.05$; HLA-DR: $P = 0.025$.

individuals, being severe ($<1.0 \times 10^9$/L) in 2 of these cases. Platelet counts $<150 \times 10^9$/L were found in 9 cases, of which 2—both of which were CMV^+—had severe thrombocytopenia ($<10 \times 10^9$/L) whereas haemoglobin levels <12 g/dl were noticed in 9 individuals.

As also shown in Table 2 increased absolute numbers of T- ($>1700 \times 10^6$/L), NK- ($>400 \times 10^6$/L) and B-($>400 \times 10^6$/L) cells were a relatively common finding in AIM: 96%, 57% and 18% of the patients, respectively. An abnormally low CD4/CD8 ratio was observed in 27/28 patients (mean of 0.32 ± 0.23, ranging from 0.11 to 1.23) being mainly due to the existence of increased absolute numbers ($>900 \times 10^6$/L) of $CD8^+/TCR\alpha\beta^+$ T-cells. $CD4^+/TCR\alpha\beta^+$ T-cell counts were either increased ($>1100 \times 10^6$/L; $n = 17$), normal ($n = 8$) or decreased ($<700 \times 10^6$/L; $n = 3$, one case being HIV^+). Increased numbers of $TCR\gamma\delta^+$ T-cells ($>200 \times 10^6$/L) were observed in 13 cases. In 3 of these cases—2 of which were EBV^+—there was a marked $TCR\gamma\delta^+$ T-cell lymphocytosis, ranging from 916 to 3594×10^6/L.

Immunophenotypic characteristics of PB lymphocytes

Peripheral blood T-cells from patients with AIM were phenotypically different from normal PB T-cells, concerning both the percentage of cells expressing each of the antigens analysed, their levels of expression (low versus high) and the pattern (homogeneous versus heterogeneous) of antigen expression. All patients had evidence of extensive T-cell activation affecting mainly the $CD8^+/TCR\alpha\beta^+$ T-cell compartment. These phenotypic changes were accompanied by a markedly decreased expression of p26/bcl2 on $CD8^+/TCR\alpha\beta^+$ T-cells towards negativity (mean of 89 ± 9% $bcl2^-$ cells; range 67 to 98%). $CD4^+/TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T-cells from AIM patients also showed evidence of cell activation and phenotypic changes were similar to those observed on $CD8^+/TCR\alpha\beta^+$ cells, but less pronounced. Bellow we describe in more detail the specific phenotypic features observed for these T-cell subsets.

$TCR\alpha\beta^+$ T-cells

Data concerning the immunophenotypic features of $CD8^+$ and $CD4^+/TCR\alpha\beta^+$ T-cells from patients with AIM and normal individuals are presented in Tables 2 and 3 and exemplified in Fig. 1. No statistically significant differences were observed in the percentage of $CD8^+$ and $CD4^+/TCR\alpha\beta^+$ T-cells that stained positively for CD2, CD5 and CD7, with more than 95% of cells expressing these markers in both patients and control individuals. In spite of this, most pan-T-cell markers showed activation-dependent modulation, consisting of either up- or down-regulation of their levels on the cell surface (Table 3). Accordingly, in AIM patients expression of CD7 was decreased on both $CD8^+$ ($P = 0.001$) and $CD4^+/TCR\alpha\beta^+$ T-cells ($P = 0.025$). Regarding CD2, a tendency towards an increased expression was observed on both $TCR\alpha\beta^+$ T-cell subsets, although differences did not reach statistical significance ($P > 0.05$). As a consequence, in patients with AIM $CD2^{+high}/CD7^{+low}$ T-cells accounted for a significantly higher proportions of both

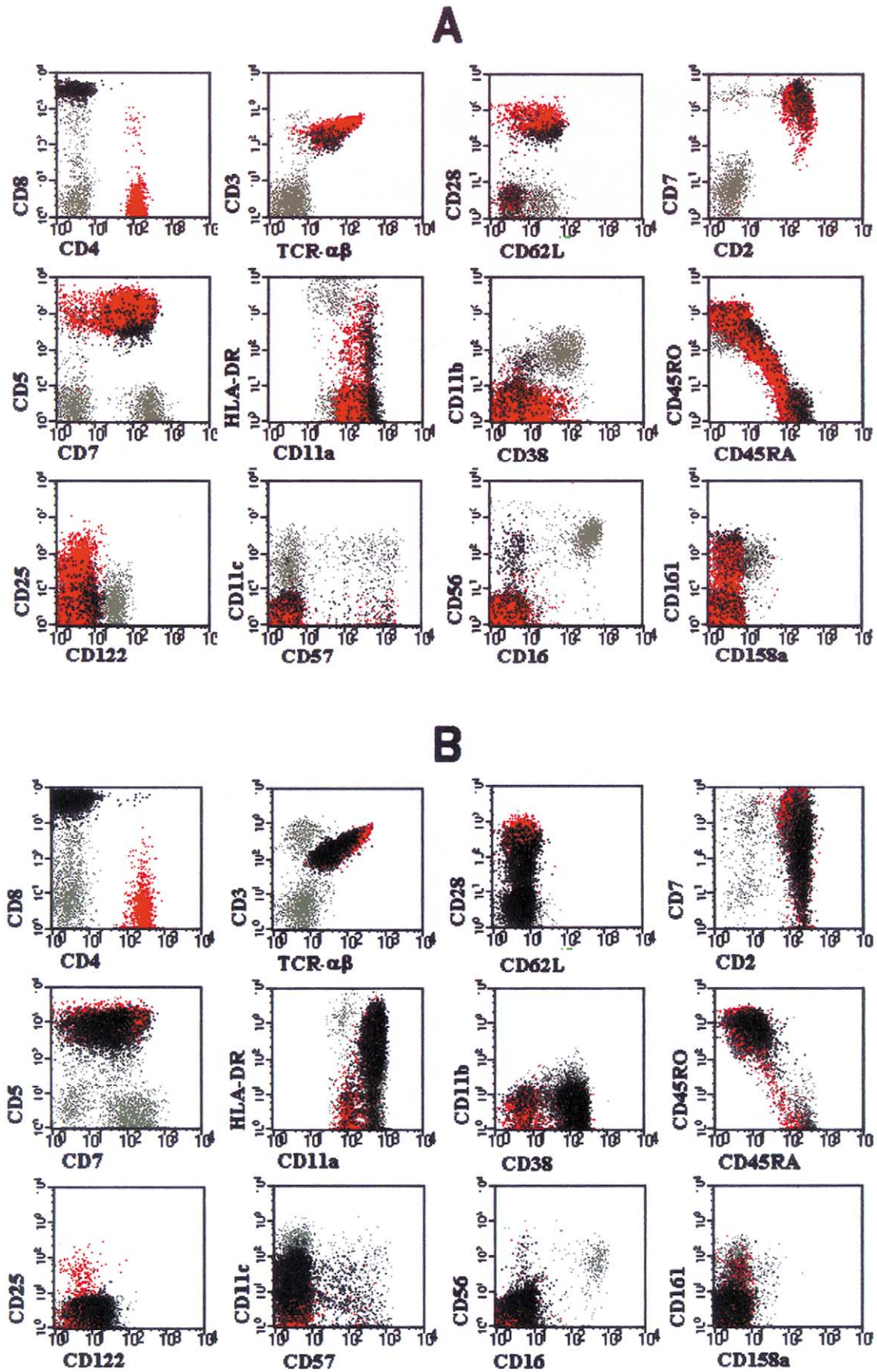

Fig. 1. Representative dot plots illustrating the main phenotypic features of $CD4^+$ (red dots) and $CD8^+$ (black dots) $TCR\alpha\beta^+$ PB lymphocytes in normal individuals (A) and in patients with AIM (B).

$CD8^+$ (93 ± 6% versus 59 ± 14%; $P = 0.001$) and $CD4^+$ (69 ± 13% versus 50 ± 19%; $P = 0.045$) $TCR\alpha\beta^+$ T-cells. Additionally, patients with AIM also showed decreased CD3 expression on both $CD8^+$ ($P = 0.014$) and $CD4^+$/$TCR\alpha\beta^+$ T-cells ($P = 0.028$), together with down-modulation of CD28, this latter phenomenon being statistically significant only for $CD8^+/TCR\alpha\beta^+$ T-cells ($P = 0.003$). In addition, the overall expression of CD28 on $CD8^+$/$TCR\alpha\beta^+$ T-cells from AIM patients was more heterogeneous (mean CV of 186.7 ± 70.2) than in controls (mean CV of 102.0 ± 13.0) ($P = 0.001$). Similar results were obtained for $CD4^+/TCR\alpha\beta^+$ T-cells, although differences were less pronounced (mean CV of 90.5 ± 44.7 and 52.1 ± 5.4, respectively; $P = 0.015$). There were also differences between $CD8^+$ and $CD4^+/TCR\alpha\beta^+$ T-cell subsets concerning the expression of CD5 consistent with reduction on the intensity of expression of this molecule on $CD8^+$ ($P = 0.02$) but not on $CD4^+/TCR\alpha\beta^+$ T-cells ($P > 0.05$). No significant differences were observed between AIM patients and controls as regards the expression of CD4 and CD8 antigens on $TCR\alpha\beta^+$ T-cells.

Regarding the expression of adhesion molecules, changes observed consisted mainly on the expression of higher levels of CD11a on both $CD8^+$ ($P = 0.002$) and $CD4^+/TCR\alpha\beta^+$ T-cells ($P = 0.003$) from AIM patients compared to controls (Table 3). In normal PB, expression of CD11a on T-cells showed a bimodal distribution, with two distinct $CD11a^+$ T-cell populations—$CD11a^{+low}$ and $CD11a^{+high}$—with $CD11a^{+high}$ T-cells being more frequent among the $CD8^+$ than the $CD4^+/TCR\alpha\beta^+$ PB T-cells. AIM patients showed increased percentages of $CD11a^{+high}$ T-cells within both the $CD8^+$ (94 ± 7% versus 55 ± 8%; $P = 0.001$) and the $CD4^+$ (56 ± 17% versus 33 ± 14%; $P = 0.013$) $TCR\alpha\beta^+$ T-cell populations. With respect to the expression of other adhesion molecules, $CD8^+/TCR\alpha\beta^+$ T-cells but not $CD4^+/TCR\alpha\beta^+$ T-cells from AIM patients showed a dim and heterogeneous expression of CD11c in the vast majority of cases (80%), resulting in an overall increase ($P = 0.002$) in the percentage of $CD8^+/CD11c^+$ cells among these patients (Table 4); in cases that stained positively for CD11c, this marker was expressed in a mean fraction of 58 ± 12% of $CD8^+/TCR\alpha\beta^+$ T-cells. In contrast, reactivity for CD11b was highly variable, this marker being dimly expressed on $CD8^+$ and on $CD4^+/TCR\alpha\beta^+$ in 33% and 22% of the cases, respectively. This variability of expression of CD11b explains why there were no statistically significant differences concerning the mean fraction of $CD11b^+$ cells between patients and controls when $CD11b^+$ and $CD11b^-$ cases were considered all together (Table 4). Up-regulation of adhesion molecules was accompanied by down-regulation of the CD62L homing receptor, resulting in a marked decrease on the percentage of $CD8^+/CD62L^+$ ($P = 0.002$) and of $CD4^+/CD62L^+$ $TCR\alpha\beta^+$ T-cells ($P = 0.02$) (Table 4).

Also probably reflecting the existence of an extensive T-cell activation, there was an over-expression of CD38 in both $TCR\alpha\beta^+$ T-cell populations from AIM patients, with a marked increase on both the percentage of $CD38^+$ cells ($P = 0.001$ and $P = 0.007$ for $CD4^+$ and $CD8^+$ T-cells, respectively) and the intensity of expression of CD38 ($P = 0.043$ and $P = 0.005$, respectively) (Tables 3 and 4). A similar behaviour to that of CD38 was found for the HLA-DR antigen, concerning both the fraction of HLA-DR^+ cells ($P = 0.001$ for both $CD8^+$ and $CD4^+/TCR\alpha\beta^+$ T-cells) and its intensity of expression ($P = 0.001$ and $P = 0.007$, respectively) (see also Tables 3 and 4). Up-regulation of CD11a, CD38 and HLA-DR was associated with both an increase in the percentage of $CD8^+/CD45RO^+$ cells ($P = 0.001$) and a decrease in the percentage of $CD8^+$/$CD45RA^+$ cells ($P = 0.005$) (Table 4). In 75% of the cases, $CD8^+$ T-cells expressed $CD45RO^{high}$ in the absence of a clear reactivity for CD45RA, whereas in the remaining 25% $CD8^+$ T-cells expressed lower levels of CD45RO ($P = 0.001$) and stained dimly for CD45RA. For that reason, expression of CD45RA was markedly decreased ($P = 0.001$), whereas the overall intensity of expression of CD45RO did not differ significantly from that observed in controls (Table 3). Modulation of CD45RA/CD45RO expression on $CD4^+$ T-cells was much more subtle than that observed on $CD8^+$ T-cells and consisted mainly on a decrease on the percentage of $CD4^+/CD45RO^+/CD45RA^+$ T-cells in patients with AIM (9 ± 3% versus 18 ± 9%) together with a correspondent increase on the proportion of $CD4^+/CD45RO^+/CD45RA^-$ T-cells ($P = 0.045$), no major differences being detected regarding either the overall percentage of total $CD4^+/CD45RA^+$ and $CD4^+/CD45RO^+$ $TCR\alpha\beta^+$ T-cells, or the overall intensity of CD45RA and CD45RO expression (Tables 3 and 4). Regarding the CD25 antigen no statistically significant differences were observed neither on the $CD8^+$ nor on the $CD4^+$ $TCR\alpha\beta^+$ T-cell compartments (data not shown).

In most AIM cases only a few proportion of $CD8^+$ and $CD4^+/TCR\alpha\beta^+$ T-cells were $CD56^+$ and/or $CD57^+$ (Table 4). It should be noted that the percentage of $CD8^+/CD57^+$ and/or $CD4^+/CD57^+$ T-cells exceeded 20% in only 3 cases—who also had the highest percentage of $CD8^+$/$CD56^+$ and $CD4^+/CD56^+$ T-cells—all of which exhibited increased proportions of large granular lymphocytes in the PB smear. Two of these cases corresponded to patients who had been previously splenectomised, the highest values being observed in the patient who became chronically infected with CMV. Other NK-cell associated markers and killer receptors (CD16, CD94, CD158a, CD161, NKBI) were either negative or expressed in relatively small proportions of both $CD8^+$ and $CD4^+/TCR\alpha\beta^+$ T-cells at values similar to or even lower than those observed in controls (see also Table 4).

$TCR\gamma\delta^+$ T-cells

The main immunophenotypic features of $TCR\gamma\delta^+$ T-cells from patients with AIM and normal individuals are

Table 4
Immunophenotypic characteristics of peripheral blood $CD8^+/TCR\alpha\beta^+$, $CD4^+/TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T-cells in AIM patients

	$CD2^{+high}$ $CD7^{+low}$	$CD11a^{+high}$	$CD11b^+$	$CD11c^+$	$CD16^+$	$CD28^+$	$CD38^+$	$CD45RA^+$	$CD45RO^+$	$CD45RA^+$ $CD45RO^+$	$CD56^+$	$CD57^+$	$CD62L^+$	$CD94^+$	$CD158a^+$	$CD161^+$	$HLADR^+$	$NKB1^+$
$CD8^+/TCR\alpha\beta^{+a}$																		
AIM	93 ± 6	94 ± 7	16 ± 24	47 ± 28	1 ± 1	49 ± 24	92 ± 7	26 ± 25	92 ± 5	19 ± 24	5 ± 5	12 ± 16	10 ± 14	0 ± 0	1 ± 1	10 ± 5	91 ± 10	1 ± 1
(n = 15)	**94**	**97**	**4**	**54**	**1**	**42**	**94**	**12**	**92**	**6**	**3**	**7**	**5**	**0**	**1**	**10**	**95**	**1**
Controls	59 ± 14	55 ± 8	16 ± 6	6 ± 5	6 ± 8	68 ± 7	4 ± 2	68 ± 16	53 ± 9	25 ± 3	18 ± 11	33 ± 5	51 ± 10	15 ± 9	5 ± 8	13 ± 7	17 ± 8	3 ± 4
(n = 6)	**63**	**53**	**16**	**4**	**0**	**66**	**3**	**75**	**51**	**27**	**14**	**32**	**50**	**11**	**1**	**10**	**13**	**1**
$CD4^+/TCR\alpha\beta^{+b}$																		
AIM	69 ± 13	56 ± 17	9 ± 10	4 ± 5	0 ± 0	92 ± 13	51 ± 16	26 ± 13	83 ± 11	9 ± 3	3 ± 4	11 ± 12	10 ± 10	0*	0 ± 0	26 ± 9	52 ± 16	0 ± 0
(n = 10)	**70**	**53**	**6**	**3**	**0**	**97**	**47**	**23**	**87**	**9**	**1**	**4**	**8**		**0**	**25**	**55**	**0**
Controls	50 ± 19	33 ± 14	1 ± 1	0 ± 0	0 ± 0	98 ± 3	27 ± 9	41 ± 16	78 ± 13	18 ± 9	1 ± 1	3 ± 2	74 ± 12	0 ± 0	0 ± 0	26 ± 9	7 ± 2	0 ± 0
(n = 6)	**42**	**34**	**0**	**0**	**0**	**99**	**26**	**47**	**83**	**16**	**1**	**2**	**78**	**0**	**0**	**27**	**7**	**0**
$TCR\gamma\delta^c$																		
AIM	N.D	96 ± 3	22 ± 14	33 ± 15	1 ± 1	84 ± 10	92 ± 7	20 ± 12	92 ± 11	8 ± 5	18 ± 7	4 ± 2	44*	85 ± 1	1 ± 1	73 ± 13	89 ± 12	1 ± 1
(n = 3)		**98**	**28**	**32**	**0**	**85**	**93**	**24**	**98**	**10**	**19**	**4**		**85**	**1**	**78**	**94**	**1**
Controls	N.D.	53 ± 18	51 ± 16	35 ± 14	16 ± 15	49 ± 16	4 ± 4	77 ± 20	65 ± 15	42 ± 17	37 ± 11	44 ± 16	46 ± 13	37 ± 13	10 ± 12	56 ± 14	52 ± 12	5 ± 5
(n = 5)		**61**	**61**	**31**	**17**	**42**	**3**	**88**	**75**	**40**	**35**	**45**	**46**	**38**	**2**	**56**	**49**	**3**

Note. Results are expressed as the mean percentage of positive cells from the indicated T-cell subset ± one standard deviation and median (bold). *Only one case was tested. n.d., not determined.

[a] AIM versus controls: $CD2^{+high}/CD7^{+low}$: $P = 0.001$; $CD11a^{+high}$: $P = 0.001$; $CD11b^+$: $P > 0.05$; $CD11c^+$: $P = 0.002$; $CD16^+$: $P > 0.05$; ; $CD28^+$: $P > 0.05$; $CD38^+$: $P = 0.001$; $CD45RA^+$: $P = 0.005$; $CD45RO^+$: $P = 0.001$; $CD45RA^+/CD45RO^+$: $P > 0.05$; $CD56^+$: $P = 0.004$; $CD57^+$: $P = 0.001$; $CD62L^+$: $P = 0.002$; $CD94^+$: $P = 0.015$; $CD158a^+$: $P > 0.05$; $CD161^+$: $P > 0.05$; HLA-DR^+: $P = 0.001$; $NKB1^+$: $P > 0.05$. No significant differences were observed between AIM patients and controls as regards the overall percentage of $CD2^+$ (99 ± 0 versus 99 ± 1), $CD5^+$ (99 ± 1 versus 94 ± 5), and $CD7^+$ (96 ± 5 versus 98 ± 2) cells.

[b] AIM versus controls: $CD2^{+high}/CD7^{+low}$: $P = 0.045$; $CD11a^{+high}$: $P = 0.013$; $CD11b^+$: $P > 0.05$; $CD11c^+$: $P = 0.019$; $CD16^+$: $P > 0.05$; $CD28^+$: $P > 0.05$; $CD38^+$: $P = 0.007$; $CD45RA^+$: $P > 0.05$; $CD45RO^+$: $P > 0.05$; $CD45RA^+/CD45RO^+$: $P = 0.045$; $CD56^+$: $P > 0.05$; $CD57^+$: $P > 0.05$; $CD62L^+$: $P = 0.02$; $CD158a^+$: $P > 0.05$; $CD161^+$: $P > 0.05$; HLA-DR^+: $P = 0.001$; $NKB1^+$: $P > 0.05$. No significant differences were observed between AIM patients and controls as regards the overall percentage of $CD2^+$ (99 ± 0 versus 99 ± 0), $CD5^+$ (99 ± 0 versus 99 ± 0), and $CD7^+$ (86 ± 14 versus 94 ± 2) cells.

[c] AIM versus controls: $CD11a^{+high}$: $P = 0.025$; $CD11b^+$: $P > 0.05$; $CD11c^+$: $P > 0.05$; $CD16^+$: $P > 0.05$; $CD28^+$: $P = 0.025$; $CD38^+$: $P = 0.025$; $CD45RA^+$: $P = 0.025$; $CD45RO^+$: $P = 0.025$; $CD45RA^+/CD45RO^+$: $P = 0.025$; $CD56^+$: $P = 0.025$; $CD57^+$: $P = 0.025$; $CD94^+$: $P = 0.040$; $CD158a^+$: $P > 0.05$; $CD161^+$: $P > 0.05$; HLA-DR^+: $P = 0.025$; $NKB1^+$: $P > 0.05$. No significant differences were observed between AIM patients and controls as regards the overall percentage of $CD2^+$ (99 ± 0 versus 99 ± 1), $CD5^+$ (96 ± 2 versus 86 ± 7), and $CD7^+$ (99 ± 1 versus 99 ± 1) cells.

shown in Tables 3 and 4. Normal PB TCR$\gamma\delta^+$ T-cells were rather heterogeneous and a relatively large fraction of them exhibited a cytotoxic-like immunophenotype, as defined by the expression of NK-associated markers and killer receptors (CD16, CD56, CD57, CD94, CD158a, CD161, NKB1) (Table 4). In contrast, in those patients with AIM who showed increased numbers of TCR$\gamma\delta^+$ T-cells the phenotype of these cells was quite homogeneous and, except for the high levels of expression of CD94 and CD161, they failed to express other NK-associated markers (Table 4). Overall, in these 3 AIM patients, more than 90% of TCR$\gamma\delta^+$ T-cells display an activation-associated phenotype: coexpression of HLA-DR$^+$ and CD38$^+$, low levels of CD5, CD7 and CD28 and high levels of CD2 and CD11a (Table 3). Moreover, as noted for the CD8$^+$/TCR$\alpha\beta^+$ T-cells, a variable fraction of TCR$\gamma\delta^+$ T-cells stained dimly for CD11b and CD11c (Table 4). Differences between patients and controls were found to be statistically significant only for a higher percentage of HLA-DR$^+$, CD38$^+$, CD11a^{+high}, CD45RO$^+$ and CD94$^+$ cells ($P = 0.04$ for CD94 and $P = 0.025$ for the remaining markers) together with a lower proportion of CD28$^-$; CD56$^+$, CD45RA$^+$, CD45RA$^+$/CD45RO$^+$ and CD57$^+$/TCR$\gamma\delta^+$ T-cells in AIM patients ($P = 0.025$ for all markers). Differences concerning the intensity of expression of each of these markers were found to be significant only for a few antigens, consisting of a lower reactivity for CD28 ($P = 0.025$) and CD45RA ($P = 0.05$) and higher expression of HLA-DR ($P = 0.025$), CD11a ($P = 0.05$) and CD38 ($P = 0.05$) on TCR$\gamma\delta^+$ cells from AIM patients.

TCR-Vβ, -Vγ and -Vδ repertoire of expanded PB T-cells

The study of the TCR-Vβ T-cell repertoire revealed the existence of small expansions of one or more TCR-Vβ families in 12 of 14 cases analysed (Table 5). From these cases, 5 showed expansions among CD8$^+$/TCR$\alpha\beta^+$, 2 among CD4$^+$/TCR$\alpha\beta^+$ and 5 in both T-cell subsets; overall, a total of 23 TCR-Vβ expansions were detected, 13 corresponding to CD8$^+$ and 10 to CD4$^+$/TCR$\alpha\beta^+$ T-lymphocytes. In the majority of cases, the TCR-Vβ expansions detected were small, being slightly above the cutoff values (mean + 2 standard deviation) obtained in normal individuals and in any case represented more than 30% of either the CD8$^+$ or the CD4$^+$/TCR$\alpha\beta^+$ T-cell compartments (mean of 12 ± 7%; range: 3 to 30%). The largest TCR-Vβ expansions occurring in CD8$^+$/TCR$\alpha\beta^+$ T-cells (Vβ2.1: 22% and Vβ17.1: 27%) were found in the two splenectomised patients who also showed the highest percentage of CD8$^+$/CD57$^+$ T-cells (65 and 26%, respectively) in the PB. As shown in Table 5, TCR-Vβ expansions were not restricted to particular TCR-Vβ families either within CD8$^+$ or among CD4$^+$/TCR$\alpha\beta^+$ T-cells.

In those patients presenting with increased numbers of TCR$\gamma\delta^+$ T-cells, the majority of TCR$\gamma\delta^+$ T-lymphocytes co-expressed the TCR-Vδ2 and the TCR-Vγ9 families, which represented 97% and 97% of TCR$\gamma\delta^+$ T-cells in case 1, 98% and 97% in case 2 and 80% and 80% in case 3, respectively; such distribution mimics what is observed in the PB of normal adult individuals (mean of 75 ± 21% and of 79 ± 16%, for TCR-Vδ2 and TCR-Vγ9 families, respectively).

Table 5
TCR-Vβ expansions within peripheral blood CD8$^+$/TCR$\alpha\beta^+$ and CD4$^+$/TCR$\alpha\beta^+$ T-cell subsets in AIM patients

Case number	% CD8$^+$/TCR$\alpha\beta^+$ T-cells[a]	% CD4$^+$/TCR$\alpha\beta^+$T-cells[a]
1	—	TCR-Vβ12.2: 12% (2%)
2	TCR-Vβ2.1: 18% (8%)	—
3	TCR-Vβ2.1: 22% (8%) TCR-Vβ20.1: 5% (3%)	TCR-Vβ2.1: 15% (11%) TCR-Vβ17.1:10% (7%)
4	TCR-Vβ3.1: 14% (11%)	TCR-Vβ23.1: 3% (1%)
5	—	TCR-Vβ8.1 + 8.2: 7% (5%); TCR-Vβ22.1: 7% (5%)
6	—	—
7	TCR-Vβ12.2 17% (2%)	—
8	TCR-Vβ20.1: 6% (3%)	—
9	—	—
10	TCR-Vβ3.1: 13% (11%)	TCR-Vβ8.1 + 8.2: 13% (5%)
11	TCR-Vβ8.1 + 8.2: 9% (7%) TCR-Vβ13.6: 7% (3%)	TCR-Vβ8.1 + 8.2: 6% (5%) TCR-Vβ22.1: 7% (5%)
12	TCR-Vβ17.1: 18% (10%) TCR-Vβ18.1: 5% (1%)	—
13	TCR-Vβ1: 11% (6%)	N.D.
14	Vβ17.1: 27% (10%)	TCR-Vβ13.6: 30% (2%)

Note. N.D., not determined.
[a] Results are expressed as the percentage of CD8$^+$ or CD4$^+$/TCR$\alpha\beta^+$ T-cells that expressed the expanded TCR-Vβ families (the specific normal upper limit is shown in parentheses).

Discussion

From the clinical point of view acute infectious mononucleosis is a relatively heterogeneous syndrome due to an underlying acute viral infection in accordance to what has been found in the present series. From the immunological point of view, acute viral infection has been reported to induce a redistribution of PB lymphocytes characterized by an increased of CD8$^+$ T-cells [25–27]. Interestingly, in the present study we have shown the existence of marked phenotypic changes consistent with an extensive activation of all the PB T-cell subsets, especially within the CD8$^+$/TCR$\alpha\beta^+$ T-cell compartment. CD8$^+$/TCR$\alpha\beta^+$ T-cells showed an intriguing stable immunophenotypic pattern—CD2^{+high}, CD7^{+dim}, CD11a^{+high}, CD28$^{-/+dim}$, CD38^{+high}, CD45RO^{+high}, CD45RA$^{-/+dim}$, HLA-DR^{+high}—which has been associated with a state of recent T-cell activation [45–57]. It should be noted that in agreement with previous reports [61] there was a marked decrease in the expression of p26/bcl2 on CD8$^+$ T-cells, indicating that activated

$CD8^+$ T-cells are prone to apoptosis. $TCR\gamma\delta^+$ and $CD4^+$/$TCR\alpha\beta^+$ T-cells also showed immunophenotypic evidence of being activated, although to a lesser extent. In fact, recently activated T-cells—defined as $CD2^{+high}$, $CD7^{+dim}$, $CD11a^{+high}$, $CD38^+$ and $HLA\text{-}DR^+$—accounted for about 95% of the total PB $CD8^+/TCR\alpha\beta^+$ T-cells in this series of AIM patients, whereas they only represented around half of circulating $CD4^+/TCR\alpha\beta^+$ T-lymphocytes. In that concerning the magnitude of $TCR\gamma\delta^+$ T-cell activation, although our results indicate that more than 90% of $TCR\gamma\delta^+$ T-cells displayed an activation-related immunophenotype in all three cases in whom the phenotype of $TCR\gamma\delta^+$ T-cells was completely characterized, results were probably biased by the fact that those patients were selected because of having the highest $TCR\gamma\delta^+$ T-cell counts. The fact that increased numbers of $TCR\gamma\delta^+$ T-cells were observed in only 46% of cases—compared to 61% and 96% for $CD4^+$ and $CD8^+/TCR\alpha\beta^+$ T-cells, respectively—would suggest that T-cell response in AIM would mainly involve $TCR\alpha\beta^+$ T-cells and is consistent with the existence of a predominant MHC-restricted T-helper type 1 (Th1) mediated cytotoxic response. Besides this extensive T-cell activation, AIM patients also showed evidence for NK-cell activation suggested by both the increased numbers of total PB NK cells and the fraction of activated ($HLA\text{-}DR^+$) NK-cells (data not shown).

Except for differences observed in a few antigens, the phenotypic features of activated $CD4^+/TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ were similar to that described for $CD8^+/TCR\alpha\beta^+$ T-cells, but less pronounced. Specific differences consisted of the following: (i) down-regulation of CD5 was not observed and decreased expression of CD28 was much less evident on $CD4^+/TCR\alpha\beta^+$ than on the $CD8^+/TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T-cells; (ii) there was no evidence for down-regulation of CD3 on activated $TCR\gamma\delta^+$ T-cells, levels of CD3 being higher than those observed in normal $TCR\gamma\delta^+$ T-cells; (iii) in contrast to what was observed for $TCR\alpha\beta^+$ T-cells, that were either negative of expressed low levels of CD94 and CD161, activated $TCR\gamma\delta^+$ T-cells displayed high reactivity for these lectin-like killer receptors; (iv) activated $CD4^+/TCR\alpha\beta^+$ T-cells did not express CD11c, whereas this adhesion molecule was dimly expressed on a variable fraction of $CD8^+/TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T-cells in the majority of cases.

The majority of the alterations described here have already been recognized as occurring after T-cell activation using in vitro or in vivo models, including up-regulation of CD2, CD11a, CD11b and CD11c adhesion molecules, CD38, CD45RO and HLA-DR together with down-regulation of CD3, CD7, CD28 and CD45RA [45–57]. Reactivity for CD25, which has also been described to increase after T-cell activation both in vitro and in vivo, did not prove to be a good indicator of T-cell activation in this model.

The immunophenotypic pattern described above identifies an early/intermediate phase of T-cell activation. Late-activation antigens, such as CD57, were only present in a small proportion of $CD8^+/TCR\alpha\beta^+$ T-cells, the percentage of $CD8^+/CD57^+$ exceeding 20% in only three cases. Interestingly, two of these patients had been previously splenectomised, supporting the hypothesis that the increased proportion of $CD8^+/CD57^+$ T-cells could be more likely related to splenectomy than to the acute viral infection itself [62]. An alternative possibility would be that expression of CD57 could represent a marker directly related to disease outcome. In this sense, it should be noted that the only patient that became chronically infected with CMV was the one showing the highest proportion of $CD8^+/CD57^+$ cells. $CD8^+/CD57^+$ T-cells were previously characterized as a particular subtype of large granular lymphocytes with an activated/memory T-cell phenotype that exhibit immunoregulatory suppressive functions and that are preferentially increased in conditions characterized by chronic antigen stimulation [58–60]. Several studies also provided evidence for the existence of dynamic changes in the phenotype of PB T-cells after viral infection; accordingly, it has been shown that the majority of $CD8^+/TCR\alpha\beta^+$ T-cells observed during the first weeks after primary CMV infection are $CD57^-$ and that $CD8^+/CD57^-$ T-cells are progressively replaced by $CD8^+/CD57^+$ T-cells in patients that become chronically infected [59].

In order to gain further insights into the immune mechanisms involved in AIM, we have also analysed both the TCR-Vβ and the TCR-Vγ/Vδ repertoires in representative subgroups of patients with AIM. Interestingly, whereas $TCR\gamma\delta^+$ T-cells showed a TCR-Vγ/Vδ distribution similar to that of normal $TCR\gamma\delta^+$ T-cells, small expansions of specific TCR-Vβ families were found in both the $CD8^+$ and $CD4^+/TCR\alpha\beta^+$ T-cell compartments in the majority of cases analysed, in agreement with previous observations [41,43,44]. However, in contrast to some of these studies [43] we did not find evidence for a selective expansion of any of the TCR-Vβ families. Even more, the type of TCR-Vβ family did not show any correlation with the specific virus identified as responsible for AIM (data not shown). The magnitude of the TCR-Vβ expansion never exceeded 40% of the $CD8^+$ or the $CD4^+$ T-cells, which favours the hypothesis of a polyclonal/oligoclonal, rather than monoclonal, T-cell proliferation [63] in accordance with previous studies [42]. Interestingly, it should be noted that the highest expansions of specific TCR-Vβ families occurred in those patients that showed the greatest levels of expression of CD57. This observation would support previous reports in which it has been demonstrated that $CD8^+/CD57^+$ T-cells have a restricted TCR-Vβ repertoire [64]. Further re-evaluation of this group of patients with AIM at different time periods after the acute episode could probably shed some light on whether $CD57^+$ TCR-Vβ restricted T-cell expansions occurring in AIM are directly related to the persistent stimulation of T-cells by either viral antigens or superantigens.

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