Expansion of Peripheral CD8+ CD28– T Cells in Response to Epstein-Barr Virus in Patients with Rheumatoid Arthritis

TATJANA KLATT, QIN OUYANG, THOMAS FLAD, INA KOETTER, HANS-JÖRG BÜHRING, HUBERT KALBACHER, GRAHAM PAWELEC, and CLAUDIA A. MÜLLER

ABSTRACT. ***Objective.*** To investigate control of Epstein-Barr virus (EBV) infection in rheumatoid arthritis (RA) by comparing the frequency phenotypes and function of peripheral CD8+ EBV-peptide antigen-specific T cells in patients with RA and healthy longterm carriers of EBV.

Methods. The frequency of interferon-γ (IFN-γ)-producing HLA-A2 or HLA-B8-restricted EBV-reactive CD8+ T cells in peripheral blood mononuclear cells (PBMC) from 49 RA patients and 26 healthy EBV carriers was evaluated in Elispot assays with 12 lytic/latent peptide epitopes. Direct staining with HLA-peptide tetramers containing 3 of these peptides was performed for comparison. The phenotype and function of these T cells was determined by FACS and cytotoxicity testing.

Results. IFN-γ production patterns in Elispot assays revealed that EBV-specific CD8+ T cells were directed predominantly against the lytic epitopes A2/GLC and B8/RAK and to a minor extent to all the other lytic and latent epitopes tested, with no significant differences of the frequencies in patients and controls. However, although similar frequencies of CD8+ T cells stained with A2/GLC or B8/RAK tetramers in both groups, the fraction of A2/GLC or B8/RAK-reactive T cells producing IFN-γ in response to specific peptide antigen was significantly lower in RA patients than controls. The A2/GLC or B8/RAK tetramer-positive T cells were also substantially enriched in CD28–CD27– T cells of a late-differentiated phenotype in RA patients but not in controls.

Conclusion. RA patients show clonal expansion of dysfunctional, terminally differentiated CD8+ EBV-specific T cells in their T cell responses to immunodominant lytic peptide EBV epitopes, which could be a sign of specific impairment of virus-host interactions in RA. (J Rheumatol 2005; 32:239–51)

Key Indexing Terms:
EPSTEIN-BARR VIRUS-SPECIFIC T CELLS
RHEUMATOID ARTHRITIS
CD8+ MEMORY T CELLS
T CELL DYSFUNCTION

In rheumatoid arthritis (RA), microbial agents are suspected to function as environmental triggers of autoreactivity[1]. Among several viruses, Epstein-Barr virus (EBV) has been suggested to be involved in the pathogenesis of RA on the basis of clinical and experimental observations[2-4]. In particular, altered humoral and cellular immune responses towards different viral antigens, such as the Epstein-Barr nuclear antigen-1 (EBNA-1) and gp110, have been reported in patients with RA and shown to include cross-reactivities with self-antigens[5-12].

In healthy individuals, EBV usually establishes an asymptomatic life-long persistent infection, in which B cells become latently infected and are thought to represent the virus reservoir *in vivo*, whereas viral lytic replication is focused on permissive epithelial cells of the oropharynx[13,14]. Compared with latently infected healthy controls, RA patients have been reported to exhibit a higher degree of productive EBV infection in epithelial cells of the oropharyngeal mucosa[15,16], as well as a higher EBV load and frequency of infected B cells in the peripheral blood[17,18]. Several studies have also shown that EBV-DNA can be readily detected in inflamed synovial tissues of a large proportion of patients with RA[19-21] concomitant with synovial accumulations of EBV-specific T cells[22,23]. The importance of T cells in controlling EBV infection has been recognized in patients after transplantation developing EBV associated lymphoproliferative diseases due to immunosuppression of

From the Section for Transplantation-Immunology and Immunohematology; Department II of Internal Medicine, University Clinics, Tuebingen; and the Institute of Physiological Chemistry, University of Tuebingen, Tuebingen, Germany.

Supported in part by a grant from the Federal Ministry of Education and Research (Fö. 01KS9602) and the Interdisciplinary Center of Clinical Research (IZKF), project II C5, Med. Faculty, Univ. Tuebingen, Germany; and by grant PA 361/7-1 from the German Research Foundation (DFG).

T. Klatt, MD; Q. Ouyang, MD; T. Flad, PhD; G. Pawelec, PhD; C.A. Müller, MD, Section for Transplantation–Immunology and Immunohematology; I. Koetter, MD; H.J. Bühring, PhD, Department II of Internal Medicine, University Clinics, Tuebingen; H. Kalbacher, PhD, Center of Medical and Natural Sciences, Institute of Physiological Chemistry, University of Tuebingen.

Dr. Klatt and Dr. Ouyang contributed equally to this work.

Address reprint requests to Prof. C.A. Müller, Section for Transplantation–Immunology and Immunohematology, ZMF, Waldhoernlestrasse 22, 72072 Tuebingen, Germany.
E-mail: claudia.mueller@uni-tuebingen.de

Submitted March 26, 2004; revision accepted September 29, 2004.

T cells[24,25]. In healthy longterm carriers of EBV, CD8+ T cell responses against the immediate early and early lytic cycle EBV proteins are readily detectable in peripheral blood, and are usually more abundant than those specific for latent proteins[26-30]. In RA patients, EBV-specific CD8+ T lymphocytes also appeared to be predominantly directed against epitopes from lytic cycle proteins and to accumulate in synovial fluids[31,32]. Previous analysis had claimed that the function of EBV-cytotoxic T cells could be disturbed in patients with RA[33,34]. In recent studies of T cell abnormalities in RA, decreased expression of signal transduction molecules for lymphocyte activation was found as a sign of generalized dysregulation of T cells, particularly in synovial lymphocytic infiltrates[35,36]. Thus, one of the reasons for the observed higher rate of productive EBV infection in patients could be defective T cell control of EBV replication.

In this study, peripheral CD8+ T cell memory responses against 12 different lytic and latent EBV-peptide epitopes were analyzed in 49 patients with RA in comparison to 26 healthy longterm EBV carriers by Elispot and direct staining with HLA-peptide tetrameric complexes. The results indicate that patients with RA enrich terminally differentiated CD8+ T cells with signs of dysfunction in their dominant T cell immune reactivity against lytic epitopes of EBV.

MATERIALS AND METHODS

Patient and control sample. Heparinized and EDTA peripheral blood samples were collected from 49 patients who met the RA criteria of the American College of Rheumatology (ACR)[37], as well as from 26 healthy volunteer controls (Table 1). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll 400 (Amersham, Freiburg, Germany) and cryopreserved in 10% dimethylsulfoxide, 20% fetal calf serum (FCS; Sigma Aldrich, Taufkirchen, Germany), and 70% RPMI-1640 medium (Invitrogen Life Technologies, Karlsruhe, Germany). Thirty-four of the 49 patients previously had received methotrexate (10–25 mg) and/or corticosteroids (5–15 mg) as immunosuppressive therapy. The study was approved by the Ethics Committee of the Medical Faculty, University of Tübingen, and all patients and controls gave informed consent.

Cell lines. The HLA-A*0201 homozygous EBV-transformed B-lymphoblastoid cell lines JY and Boleth, as well as the HLA-A2 negative cell line Cox (derived from the 10th International Histocompatibility Workshop, IHW 9287, IHW 9031, and IHW 9022) and the TAP-deficient, HLA-A2-positive cell line T2 (CRL-1992; American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin (Roche Diagnostics, Mannheim, Germany), and 10% heat inactivated FCS (Sigma Aldrich).

Diagnosis of EBV infection. Prior EBV infection was diagnosed by the detection of IgG antibodies to EBNA and of IgM antibodies to viral capsid antigen (VCA) in sera from RA patients and control participants by ELISA. All patients were EBNA IgG-positive, and 5 (Patients RA3, RA4, RA30, RA31, RA36) also were VCA IgM-positive. Controls and patients were considered EBV seronegative if the antibody titers were < 1:10 (below the level of detection). In addition, 200 ng of DNA isolated from 5×10^6 PBMC were amplified and hybridized for presence of DNA sequences of the EBNA-LP region as described[20].

Human leukocyte antigen (HLA) typing. Molecular HLA typing was conducted using Inno-LiPa HLA-A, -B, DRB1 typing kits (Innogenetics, Zwijndrecht, Belgium). For subtyping of HLA-A2 alleles, direct sequencing was performed as described[38]. HLA-A*02 or B*08 homozygous individuals are indicated in the tables.

Table 1. Clinical characteristics of the RA patients and healthy controls.

	RA Patients, n = 49	Controls, n = 26
Age, mean ± SD yrs	55 ± 13.8	43 ± 13.2
Men, n	12	15
Women, n	37	11
Serological EBV status		
EBNA IgG positive	49	26
VCA IgM positive	6	0
EBV-DNA PCR		
+ to +++ positive (no. of individuals analyzed)	10 (23)	5 (16)
HLA genotype		
HLA-A*0201	23 (homozygous 2)	20 (homozygous 1)
HLA-A*0206	1	0
HLA-B*08	19 (homozygous 3)	6
DRB1*04	15	10
DRB1*01	10	5
RA grading		
I	4	—
II	26	—
III	8	—
IV	3	—
Undefined	8	—
Immunosuppression		
Methotrexate (10–25 mg) + corticosteroids (5–15 mg)	15	—
Corticosteroids (5–15 mg)	10	—
Methotrexate (10–25 mg)	9	—

Synthesis and purification of peptides. All EBV and control peptides were synthesized by the solid-phase method using standard fluorenylmethoxycarbonyl (FMOC) chemistry on a MilliGen 9050 Pep Synthesizer (Millipore, Bedford, MA, USA), purified to greater than 95% homogeneity by reverse-phase high performance liquid chromatography (HPLC) and validated by electrospray ionization (ESI) mass spectrometry. In addition to published EBV-peptide epitopes, new potential T cell epitopes of EBV proteins were predicted by established peptide binding motifs for HLA-A2 and –B8 using the online program *SYFPEITHI* (available at: http://syfpeithi.bmi-heidelberg.com). Only peptide epitopes with a theoretical binding score greater than 24 were selected[39] (Table 2). As a control the HLA-A*0201 binding peptide KTWGQYWQV derived from gp100 was used.

Elispot assay for interferon-γ (IFN-γ)-secreting cells. IFN-γ-producing cells in response to stimulation with EBV-peptide epitopes were assessed on PBMC and T cell clones as described[40]. We used 96-well MAHA S4510 plates (Millipore) precoated with 10 µg/ml of the anti-IFN-γ monoclonal antibody 1-DIK (Hoelzel, Koeln, Germany) overnight at 4°C and then blocked with complete medium (RPMI-1640 containing 10% FCS) for 2 h at room temperature. PBMC were added in triplicate wells (1×10^5 cells/well) and incubated for 24 h at 37°C in 5% CO_2 in the presence or absence (negative control) of 10 µM synthetic EBV-peptide or with 1% phytohemagglutinin (Invitrogen) as positive control. The following day, the cells were discarded and the second anti-IFN-γ monoclonal antibody, biotin-labeled 7-B6-1 (Hoelzel), was added at 1 µg/ml for 3 h, followed by the addition of avidin-bound biotinylated horseradish peroxidase H (Vectastain Elite Kit; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Individual IFN-γ-producing cells were detected as

Table 2. Synthetic peptide epitopes from EBV lytic and latent cycle proteins used in this study.

HLA Restriction	Peptide	Sequence	AA Position	EBV Protein	Binding Score*	Reference
A*0201	A2/GLC	GLCTLVAML	280–288	BMLF1	28	28
	A2/QLL	QLLQHYREV	195–203	BZLF1	24	UP
	A2/VMA	VMATLLPPV	520–528	EBNA-4	27	UP
	A2/LLD	LLDFVRFMGY	284–293	EBNA-6	12	43
	A2/LLW	LLWTLVVLL	329–337	LMP2	30	44
	A2/CLG	CLGGLLTMN	426–434	LMP2	17	45
B*08	B8/RAK	RAKFKQLL	190–197	BZLF1	34	28
	B8/FLR	FLRGRAYGL	325–333	EBNA-3	31	46
	B8/GPK	GPKVKRPPI	242–250	EBNA-3	34	UP
	B8/AYR	AYRRRWRRL	234–242	LMP2	24	UP
	B8/AAR	AARQRLQDI	147–154	EBNA-6	24	UP
A*02/B*08	A2/B8 SVR	SVRDRLARL	464–472	EBNA-3	23/26	47

* See Materials and Methods and reference 39. UP: unpublished.

dark purple spots after 8 min incubation with 3-amino-9-ethyl-carbazol (AEC) dissolved in dimethylformamide (Sigma Aldrich) and diluted in sodium acetate buffer (pH 5), as well as with the addition of H_2O_2. The colored spots were counted by an automated Elispot reader (Aelvis GmbH, Hannover, Germany) and expressed as number of spots/10^6 PBMC after subtraction of the negative control values. Responses were defined as undetectable if the numbers of spots did not exceed the mean of the background values plus 3 SD.

HLA-A2 and –B8 tetrameric complexes. Soluble HLA-A*0201/peptide tetramers were synthesized as described[41,42]. HLA-B8/RAK tetrameric complexes were purchased from ProImmune (Oxford, UK).

Cell staining and sorting by flow cytometry. Frozen PBMC were thawed, washed in PBS with 2% FCS and 0.1% sodium azide (staining buffer), and then incubated with phycoerythrin (PE)-conjugated HLA-A*0201 tetrameric complexes (200 ng tetramers per 1 × 10^6 cells) or with 2 µl PE-conjugated HLA-B*0801 tetrameric complexes together with saturating amounts of a PerCP-conjugated anti-CD8 mAb (clone SK1, Becton Dickinson Biosciences, Heidelberg, Germany). Background labeling of tetramers was validated by staining of PBMC obtained from HLA-A*0201–/EBV+ and HLA-A*0201+/EBV– or HLA-B*0801–/EBV+ individuals as negative controls. In several experiments triple-staining was performed by the additional use of one of the following fluorescein-isothiocyanate (FITC) conjugated mAb: anti-CD4 (BD Biosciences), anti-CD45RO (Dako, Carpenteria, CA, USA), anti-CD45RA (Immunotech, Marseille, France), anti-CD28 (Immunotech), anti-HLA-DR (Dako), or anti-CD69, -CD27 or -CD57 (BD Biosciences). Prior to analysis on a FACScan flow cytometer samples were fixed in 2% formaldehyde. Samples that were used for cell sorting were not fixed. Tetramer-positive cells were sorted on a FACSVantage flow cytometer using CellQuest software (BD Biosciences). After sorting, cells were expanded and cloned as described below.

Intracellular cytokine assay. Staining for intracellular IFN-γ production was done as follows: 1 × 10^6/200 µl PBMC were stimulated with 10 µg/ml A2/GLC-peptide after 30 min preincubation with the respective tetramer at 37°C or were incubated with PMA/ionomycin (positive control) or not stimulated (medium alone as negative control) at 37°C for 4 h with brefeldin A (Sigma Aldrich). After washing, cells were stained with HLA-A2/GLC tetramers (PE) and PerCP-conjugated mAb CD8 (BD Biosciences) in PBS supplemented with 0.5% BSA on ice for 20 min. Cells were washed with PBS/BSA, fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences Pharmingen), and stained intracellularly with IFN-γ-FITC (BD Biosciences) for 30 min at 4°C. Data files containing 100,000 events within the lymphocyte gate were acquired using a FACScan flow cytometer. Frequencies of IFN-γ-positive cells within the CD8+-PerCP and HLA-A2/GLC-PE tetramer-positive fluorescence lymphocyte gate were determined using CellQuest software. Negative controls showed less than 0.01% of IFN-γ-positive cells.

Expansion and cloning of antigen-specific CD8+ T cells. The polyclonal CD8+ T cell population obtained by flow cytometry sorting of HLA-A2/GLC tetramer-stained CD8+ T cells was immediately plated in round-bottom 96–well culture plates in the presence of irradiated feeder cells (allogeneic pooled PBMC and the HLA-A*0201+ B-lymphoblastoid cell lines JY or Boleth) at a responder to stimulator ratio of 1:10,000 in X-vivo 15 medium (BioWhittaker, Taufkirchen, Germany) and of 50 U/ml of interleukin 2 (IL-2; R&D Systems, Wiesbaden, Germany). The T cells were restimulated weekly with the same stimulators as described above and fed with 50 U/ml of IL-2 two days later. After 3 stimulation cycles the expanded T cell lines were tested for cytolytic activity.

In addition, T cells were cloned by limiting dilution in 96-well round–bottom microtiter plates in the presence of irradiated feeders (PBMC and B-lymphoblastoid cell lines), in X-vivo 15 medium and 50 U/ml of IL-2.

Cytotoxicity assay. The colorimetric Cytotoxicity Detection Kit (LDH) (Roche) based on the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into the supernatant was used to evaluate cytotoxicity of EBV-peptide-specific T cells. Triplicates of 1 × 10^4 T2 cells before and after loading with 10 µg/ml EBV-peptide were incubated with increasing numbers of expanded sorted tetramer-positive T cells in 200 µl X-vivo 15 medium in 96-well microtiter plates. After 4 h, 100 µl cell-free supernatant of each well was collected and incubated with the reaction mixtures of the kit. The amounts of LDH in the supernatants was determined as direct correlates to the amount of a formazan dye formed during a time period of 25 min at 25°C and measured as absorbance (optical density, OD) of the samples (S) at 492 nm wavelength using an ELISA reader. Background absorbance values were determined in control wells with the assay medium only and subtracted from all other test values. Spontaneous LDH release was analyzed as absorbance of wells with target or effector cells only. As positive control values, maximum release of LDH was induced in triplicate wells of target cells by the addition of 100 µl/well of Triton X-100. Specific lysis was calculated by the following formula:

$$\text{Specific lysis} = \frac{(\text{sample}) - (\text{spontaneous release effector} + \text{spontaneous release target})}{(\text{maximum release target}) - (\text{spontaneous release target})} \times 100\%$$

Statistical analysis. All statistical procedures were performed with WinStat for Microsoft Excel (Fitsch Software, Staufe, Germany). The Mann-Whitney test was used to determine statistical significance of differences of all comparisons between RA patients and controls. P values less than 0.05 were considered significant.

RESULTS

Detection of EBV-specific CD8+ T lymphocytes. To determine the frequencies of EBV antigen-specific CD8+ T cells in peripheral blood of 49 RA patients and 26 healthy controls (Table 1), we first used the Elispot assay, which gave the numbers of IFN-γ-producing T cells after stimulation with 12 different EBV-peptides (Table 2). T cells reactive against HLA-A2 binding epitopes from the EBV latent and lytic proteins were evaluated in 23 EBV-seropositive HLA-A*02+ patients with RA and compared to those of 16 healthy controls (Tables 3 and 4). With each of the peptides no false-positive results were observed with EBV-immune donors, such as Control HC18, lacking the restricting HLA-A2 antigen (Figure 1) or with an EBV-seronegative HLA-A*02+ donor (data not shown). T cells reactive against the newly predicted lytic epitope A2/QLL or responding to any one of the 5 latent epitopes tested were rarely present in the RA patients and EBV-seropositive controls (Table 3). T cells responsive to the lytic A2/GLC-epitope were detected in all controls and RA patients except one (Patient RA16), who carried an HLA-A*0206 suballele (Table 4). These A2/GLC-specific T cells were present at similar levels in RA patients (mean 99/10^6 PBMC) and healthy EBV carriers (mean 104/10^6 PBMC).

In addition, 18 RA patients and 6 healthy EBV-immune controls were analyzed for T cells reacting against 6 lytic and latent EBV-peptide epitopes restricted by HLA-B8. As shown in Table 5, T cells reacting against the lytic epitope B8/RAK and the latent epitope B8/FLR were present in all patients except one (Patient RA33), and predominated over the frequencies of T cells directed against the 4 other latent B8-restricted EBV epitopes tested. The B8/RAK-reacting T cells were slightly but not significantly reduced in RA patients (mean 287/10^6 PBMC) compared to those in controls (mean 443/10^6 PBMC; p = 0.18). In 4 RA patients and 4 controls with an HLA-A2+ as well as -B8+ HLA-genotype, T cells responding to B8/RAK appeared to be 2 to 10 times more frequent than those reacting to HLA-A2-restricted lytic or latent peptide antigens.

Enumeration of EBV antigen-specific T cells using MHC-peptide tetrameric complexes. The frequencies of T cells reactive with the EBV lytic epitopes A2/GLC, B8/RAK, and the latent epitope A2/LLD were then analyzed using PE-labeled tetrameric HLA-peptide complexes. The tetrameric complexes did not stain PBMC obtained from EBV-seropositive HLA-A*02-negative or B*08-negative donors (Figure 1, Controls HC18 and HC14) or cells from EBV-seronegative HLA-A*02-positive individuals (data not shown). Figure 1 also shows repeated staining of PBMC from Patients RA1 and RA30, in whom 5.5% and 1.9% of the CD8+ T cells, respectively, reacted with the A2/GLC and B8/RAK tetramer in comparison to the staining of T cells from the EBV-immune healthy controls HC1 and HC9 (0.4% and 2% of the CD8+ T cells). Between the RA patients and EBV-immune controls there were no significant differences in the percentages of either A2/GLC tetramer (patients: mean 0.59%, controls: mean 0.21%; p = 0.08) or B8/RAK tetramer (patients: mean 1.07%, controls: mean 0.88%; p = 0.92) binding CD8+ T cells (Tables 4 and 5). However, when looking at the absolute numbers, the A2/GLC-binding T cells appeared to be elevated in PBMC of the RA patients (mean 442 cells/10^6 PBMC) in comparison to the controls (mean 195 cells/10^6 PBMC; p = 0.046), whereas B8/RAK labeled T cells showed only slightly

Table 3. Frequencies of IFN-γ-producing T cells reactive against HLA-A2 or -B8-restricted EBV peptides in PBMC of RA patients and EBV-immune controls measured by Elispot assay.

EBV Peptides	RA Patients		Controls	
HLA-A2 restricted	Reacting A2+ individuals, n = 23*	IFN-γ+ T cells/10^6 PBMC, Mean (range)	Reacting A2+ individuals, n = 16	IFN-γ+ T cells/10^6 PBMC, mean (range)
Latent antigens				
A2/SVR	5†	19.8 (10–37)	1	22
A2/VMA	6	28.9 (10–64)	1	22
A2/LLW	10	31.1 (14–45)	4	48.2 (37–53)
A2/CLG	9	37.6 (13–75)	3	41.6 (30–50)
Lytic antigen				
A2/QLL	6	39.6 (17–54)	1	102
HLA-B8 restricted	Reacting B8+ individuals, n = 9*	IFN-γ+ T cells/10^6 PBMC, Mean (range)	Reacting B8+ individuals, n = 6	IFN-γ+ T cells/10^6 PBMC, mean (range)
Latent antigens				
B8/AAR	2†	14.0 (8–20)	2	25.0 (10–40)
B8/GPK	1	16	0	0
B8/SVR	1	15	1	20
B8/AYR	3	30.0 (11–66)	1	30

* Tested individuals. † Number of reacting individuals.

Table 4. Frequencies of EBV-specific T cells reactive against A2/GLC lytic or A2/LLD latent peptides of EBV in peripheral blood of RA patients and controls: comparison of Elispot and HLA-tetramer staining.

	EBV Peptides			
	A2/GLC		A2/LLD	
	Elispot[a]	Tetramer[a,b]	Elispot[a]	Tetramer[a,b]
RA patients				
RA1[c]	790	2361 (5.6)	50	168 (0.55)
RA2	39	100 (0.08)	24	74 (0.01)
RA3[d]	31	63 (0.08)	UD	UD
RA4[d]	85	844 (0.7)	UD	UD
RA5	6	68 (0.09)	UD	12 (0.01)
RA6	21	275 (0.4)	UD	UD
RA7	50	110 (0.08)	UD	15 (0.01)
RA8	11	66 (0.1)	30	36 (0.03)
RA9	51	195 (0.2)	UD	UD
RA10	10	49 (0.03)	UD	UD
RA11	126	486 (0.7)	33	20 (0.03)
RA12	12	579 (0.4)	UD	UD
RA13	80	320 (0.2)	UD	UD
RA14	26	340 (0.7)	UD	57 (0.05)
RA15	146	NT	UD	UD
RA16	UD	NT	UD	NT
RA17	78	378 (0.1)	UD	NT
RA18	20	191 (0.2)	UD	NT
RA19[c]	133	400 (0.23)	UD	NT
RA20	130	328 (0.7)	UD	NT
RA21	63	223 (0.2)	UD	NT
RA22	135	1471 (1.1)	25	NT
RA23	253	NT	16	NT
Healthy controls				
HC1	215	320 (0.4)	10	22 (0.02)
HC2	30	31 (0.04)	20	19 (0.02)
HC3	60	65 (0.11)	UD	31 (0.11)
HC4	16	38 (0.03)	13	19 (0.01)
HC5	230	461 (0.9)	UD	13 (0.05)
HC6	10	20 (0.01)	UD	11 (0.01)
HC7	40	90 (0.04)	UD	28 (0.13)
HC8	47	50 (0.03)	UD	20 (0.02)
HC9	310	630 (0.5)	41	49 (0.04)
HC10	138	300 (0.46)	10	18 (0.02)
HC11[c]	230	306 (0.2)	132	135 (0.04)
HC13	UD	36 (0.04)	UD	NT
HC14	260	491 (0.5)	UD	NT
HC15	41	118 (0.1)	UD	NT
HC16	UD	15 (0.02)	UD	NT
HC17	50	151 (0.1)	UD	NT
HC18[e]	UD	UD	UD	UD

[a] Values are given per 10^6 PBMC. [b] Values in parentheses are given as percentage CD8+ T cells. [c] Homozygous individuals. [d] VCA IgM positive. [e] A2 negative control. UD: undetectable, NT: not tested.

increased frequencies in RA patients (mean 948/10^6 PBMC) and controls (mean 811/10^6 PBMC). In addition, A2/LLD-reactive T cells were detected in only 7 of 15 RA patients at similar levels (range 12 to 168/10^6 PBMC) as in all 11 EBV-seropositive controls tested (Table 4).

Reduced proportions of functional T cells among the EBV-specific CD8+ T cells in RA patients. To compare the functional capacity of EBV-specific CD8+ T cells, the proportion of IFN-γ-producing T cells among the A2/GLC or B8/RAK tetramer-positive T cells was calculated by dividing the individual frequencies of IFN-γ-producing peptide-specific T cells per 10^6 PBMC determined by Elispot by the total number of the tetramer-binding T cells in 20 HLA-A*02 and 13 HLA-B*08 RA patients as compared to 14 HLA-A*02 and 6 HLA-B*08 EBV-immune controls. As shown in Figure 2A, RA patients possessed significantly reduced proportions of CD8+ T cells (mean 22.9%, range 2–49%) producing IFN-γ in response to stimulation with the immunodominant

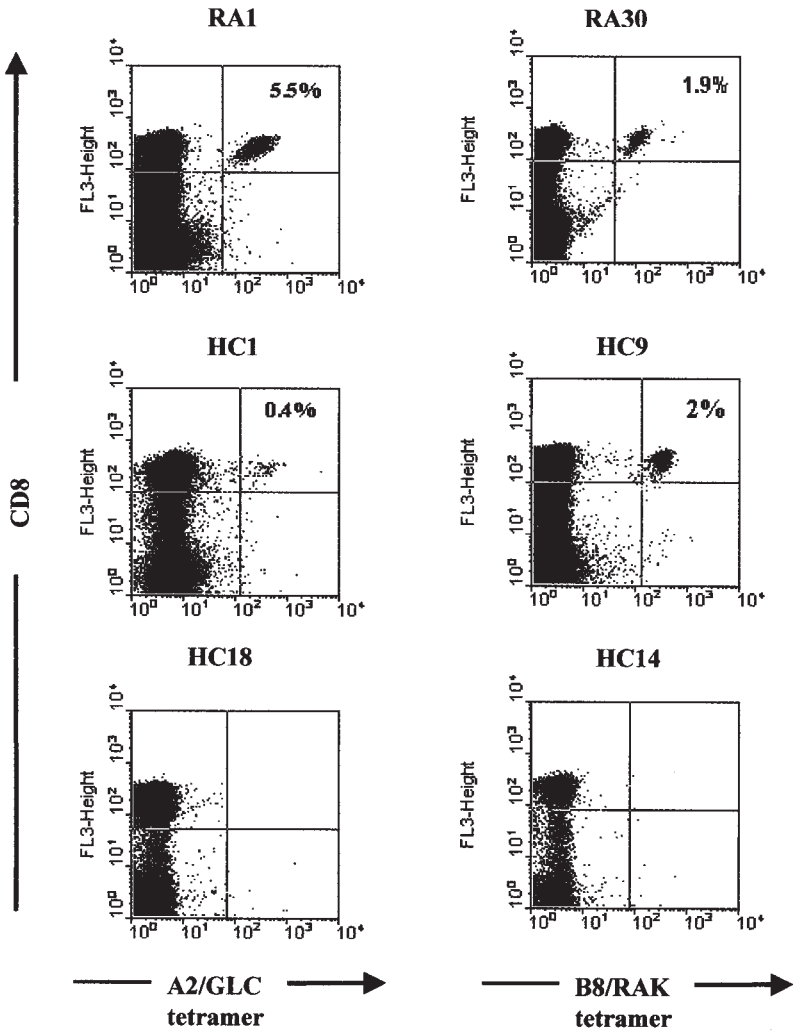

Figure 1. FACS analysis of T cells binding HLA-A2/GLC or HLA-B8/RAK tetrameric complexes in RA patients and controls. PBMC from 2 RA patients (RA1: HLA-A2+, homozygous; RA30: HLA-B8+, VCA IgM-positive) and 4 controls (HC1: HLA-A2+; HC9: HLA-B8+) were stained with PE-conjugated tetrameric complexes (y-axis) and PerCP-labeled anti-CD8 mAb (x-axis); 50,000 cells were included in each assay. Numbers in the upper right corners show percentages of tetramer-positive CD8+ T cells. The staining patterns of PBMC from two HLA-A2-negative or B8-negative, but EBV-seropositive healthy controls (HC18, HC14) show that the tested tetrameric complexes did not label CD8+ T cells in HLA-A2 or B8-negative controls.

A2/GLC-peptide compared to the controls (mean 59%, range 33–97%; p = 0.00001). In a similar analysis of the B8/RAK tetramer-specific T cells, we also observed significant differences in the proportions of T cells responding with IFN-γ production after stimulation with the B8/RAK-peptide in RA patients in comparison with controls (Figure 2B). Direct intracellular staining of the A2/GLC tetramer-binding CD8+ T cells after stimulation with the peptide also revealed reduced proportions of IFN-γ CD8+ T cells in patients with RA in comparison to controls, as illustrated in Figure 2C. Stimulation of CD8+ T cells with PMA as a positive control, however, showed that CD8+ T cells in general did not have a significantly reduced capacity to produce IFN-γ in RA patients (n = 8; range 12–60%, mean 26%) in comparison to controls (n = 8; range 13–46%, mean 32%; p = 0.14). Also, the correlation between the numbers of T cells detected by the Elispot assays and those obtained by direct staining with A2/GLC tetrameric complexes on PBMC in 20 controls (r = 0.93, p = 0.02) and to a slightly lesser extent in 23 HLA-A*02+ RA patients (r = 0.86, p < 0.001) was relatively high, suggesting no major interindividual differences in test sensitivity. A similar correlation of Elispot and FACS analysis was seen with the B8/RAK-peptide and tetramers in 6 HLA-B8+ patients (r = 0.74, p = 0.005). Both assays were found to be reproducible and similarly sensitive on frozen and fresh peripheral blood cells (data not shown).

Table 5. Frequencies of EBV-specific T cells reactive against B8/RAK lytic or B8/FLR latent peptides in PBMC of RA patients and controls: comparison of Elispot and HLA-tetramer staining.

	EBV Peptides		
	B8/RAK		B8/FLR
	Elispot[a]	Tetramer[a,b]	Elispot[a]
RA patients			
RA3[c]	561	NT	135
RA6	245	NT	123
RA13	1003	NT	140
RA23	566	NT	261
RA24	110	NT	180
RA25	25	577 (1.1)	38
RA26	121	1062 (1.0)	730
RA27[d]	83	182 (0.4)	126
RA28	16	170 (0.3)	20
RA29[d]	200	1098 (0.6)	130
RA30[c]	210	1945 (1.9)	20
RA31[c]	175	711 (0.5)	20
RA32	445	2742 (4.4)	90
RA33[d]	621	2058 (2.0)	UD
RA34	20	164 (0.3)	125
RA35	25	76 (0.2)	20
RA36[c]	620	1110 (0.8)	296
RA37	121	438 (0.5)	296
Healthy controls			
HC3	350	620 (0.8)	90
HC5	670	942 (1.0)	92
HC9	973	1866 (2.0)	540
HC10	130	240 (0.4)	55
HC12	125	208 (0.2)	10
HC15	415	990 (0.9)	UD

[a] Values are given per 10^6 PBMC. [b] Values in parentheses are given as percentage CD8+ T cells. [c] VCA IgM positive. [d] Homozygous individuals. NT: not tested, UD: undetectable.

To investigate whether the reduction of IFN-γ-producing CD8+ T cells was also associated with a decrease of lytic function, HLA-A2/GLC tetramer-positive CD8+ T cells were sorted from RA patients and controls by FACS and expanded as polyclonal T cell lines over 3 weeks by mitogen-driven stimulation, as described in Materials and Methods. As shown in Figure 3A, CD8+ tetramer-positive, but not CD8+ tetramer-negative expanded T cell lines from 2 RA patients (RA1 and RA9) specifically and significantly killed HLA-A2-positive T2 cells as target-cells (97% and 93% at an E:T ratio of 10:1) after pulsing with the peptide A2/GLC. T2 cells loaded with the A2-binding control peptide gp100 or without peptide were not lysed. Another CD8+ tetramer-positive T cell line (from Patient RA12) revealed a much weaker cytolytic activity (27% at an E:T ratio of 10:1) than tetramer-positive cell lines from 2 controls (54% and 51%) and from the 2 other RA patients tested. Additionally, allogeneic EBV-transformed lymphoblastoid cell lines carrying the relevant restricting HLA antigen A2 (JY, Boleth) were lysed after A2/GLC-peptide pulsing (Figure 3B). Maximal cytotoxic activity was induced with titrated A2/GLC-peptide at a concentration as low as 0.1 µg/ml (Figure 3C).

Phenotype of peripheral EBV-epitope-specific CD8+ cells in RA patients and controls. In parallel experiments, we analyzed the phenotype of EBV-epitope-specific CD8+ T cells. Figure 4 shows representative staining profiles of flow cytometric analyses from PBMC of Patient RA1 and Control HC1 after gating the CD8+ lymphocytes stained with PE-labeled HLA-A2/GLC tetramer, PerCP-labeled anti-CD8 mAb, and the FITC-labeled mAb against CD28. Summarized phenotypic results of the A2/GLC tetramer-positive CD8+ T cells surface phenotyping with a series of T cell differentiation markers on PBMC from additional patients and controls are given in Table 6. CD28 as a co-stimulatory molecule on T cells[43] was found to be significantly reduced on tetramer–specific CD8+ T cells in RA patients in comparison to the controls (A2/GLC tetramer-positive T cells: range 26–42%, mean 31%, in patients versus range 63–87%, mean 73%, in controls; $p < 0.001$). A similar reduction of CD28 was also seen on B8/RAK tetramer-positive CD8+ T cells (RA patients: range 15–69%, mean 43%; controls: range 54–78%, mean 64%; $p = 0.01$; Figure 4B). CD28 was not found to be decreased in general on CD8+ T cells of RA patients ($n = 11$; mean 70.9%) when compared to controls ($n = 10$; mean 69.3%; Figure 4C). In addition, CD57 appeared to be upregulated

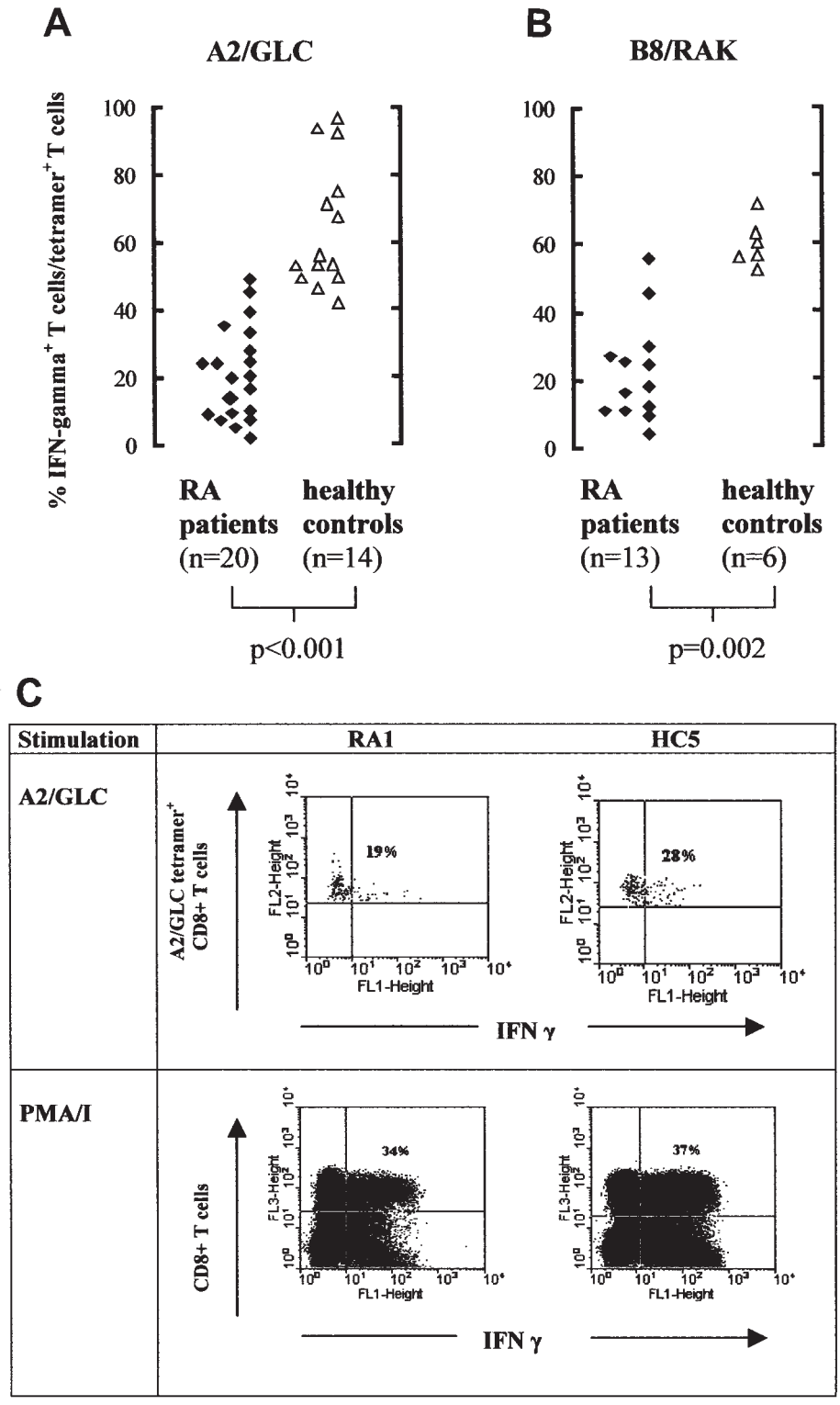

Figure 2. A and B: Comparison of the functionality of tetramer-positive EBV-specific CD8+ T cells in RA patients and controls. Percentages of IFN-γ-producing EBV-peptide-specific T cells in PBMC of RA patients and controls were determined as ratios of the numbers of EBV-peptide-specific T cells determined by Elispot and of the tetramer-binding T cells. Significant differences in the percentages of IFN-γ-producing A2/GLC (A) or B8/RAK (B) tetramer-binding T cells between RA patients and controls are shown. C: Intracellular staining of IFN-γ production of CD8+ T cells in Patient RA1 and Control HC5 after stimulation with A2/GLC-peptide or PMA/ionomycin (positive control): the gating for positive IFN-γ content was set by running a permeabilized isotype control alongside each sample. All events shown were first gated through CD8. Numbers in the upper panel represent percentages of A2/GLC tetramer-binding CD8+ T cells expressing IFN-γ. Lower panel: total IFN-γ-positive CD8+ T cells after PMA/ionomycin stimulation.

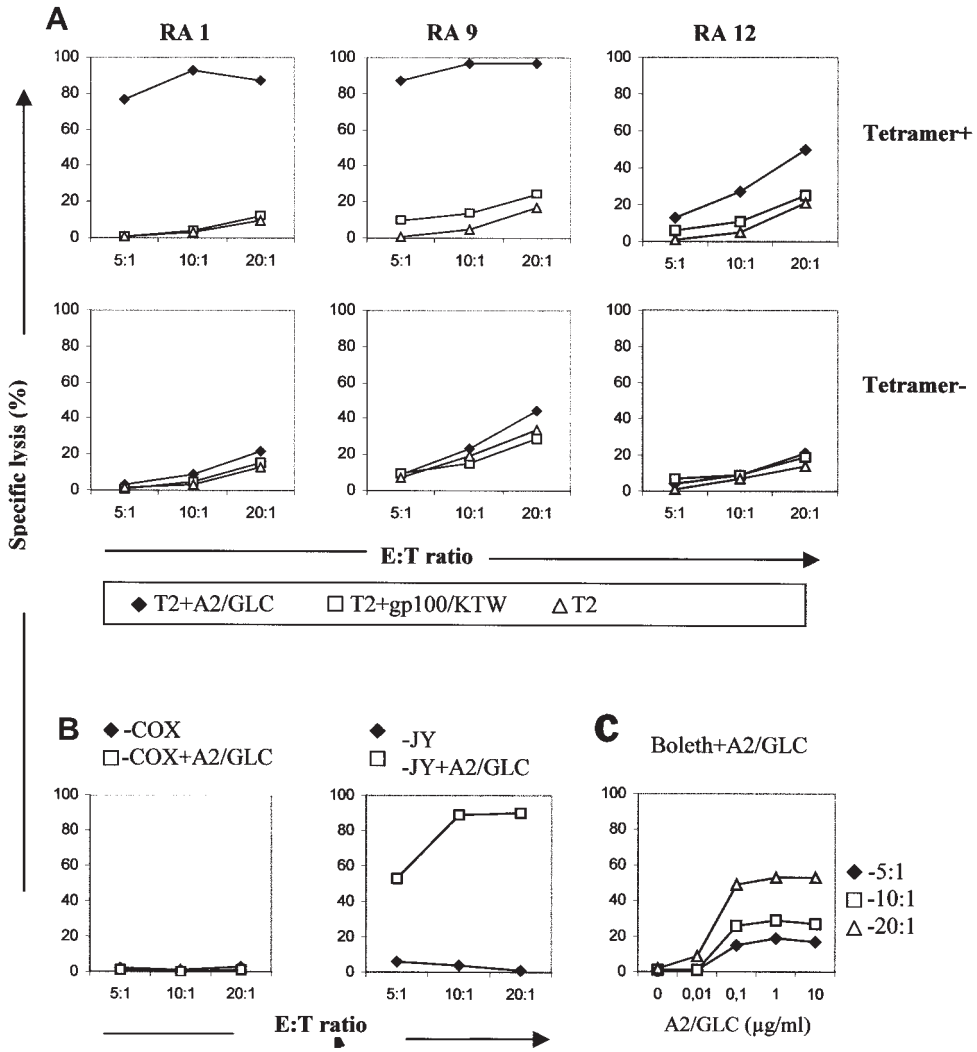

Figure 3. A: CD8+ T cells from 3 RA patients (RA1, RA9, RA12) were sterile-sorted into A2/GLC tetramer-positive and -negative populations. After expansion for 3 weeks in the presence of irradiated allogeneic PBMC and phytohemagglutinin, each cell fraction was tested for lytic activity against T2 cells in the absence (white triangles) or presence (black diamonds) of 10 µg/ml of peptide A2/GLC, or in the presence of the control peptide gp100/KTW (10 µg/ml; white squares). B: Expanded and sorted A2/GLC tetramer-positive T cells from Patient RA1 were tested for their lytic activity against the HLA-A2-negative EBV-transformed cell line COX in the absence (black diamonds) or presence (white squares) of peptide A2/GLC, and against the HLA-A2-positive EBV-transformed cell line JY in the absence (black diamonds) or presence (white squares) of peptide A2/GLC. C: Specific lysis of the HLA-A2-positive EBV-transformed cell line Boleth in the absence or presence of different concentrations of peptide A2/GLC by expanded and sorted A2/GLC tetramer-positive T cells from Patient RA1. Percentages of specific lysis recorded at E:T cell ratios of 5:1 (black diamonds), 10:1 (white squares), or 20:1 (white triangles) are shown.

on slightly higher proportions of tetramer-reactive T cells in RA patients (range 4–44%, mean 32%) than in controls (range 12–31%, mean 22%; p = 0.17). The A2/GLC tetramer-positive T cells in the controls were also almost entirely positive for CD27 (range 82–97%, mean 89%), but showed reduced expression of this marker in the RA patients (range 62–90%, mean 75%; p = 0.03). However, RA patients and controls showed no differences in the expression of CD27 on B8/RAK tetramer-positive T cells (RA patients: n = 5, mean 70%; controls: n = 4, mean 70%). Coexpression of CD45RO as a marker of previously activated antigen-specific T cells as well as of CD45RA (previously suggested to characterize naive as well as terminally differentiated effector T cell subsets) was similar on A2/GLC and B8/RAK tetramer-positive CD8 T cells in RA patients and controls (Table 6 and data not shown). As well, the extent of HLA-DR and CD69 expression was not strikingly different on A2/GLC or B8/RAK tetramer-positive CD8+ T cells in RA patients and controls. (Table 6 and data not shown.)

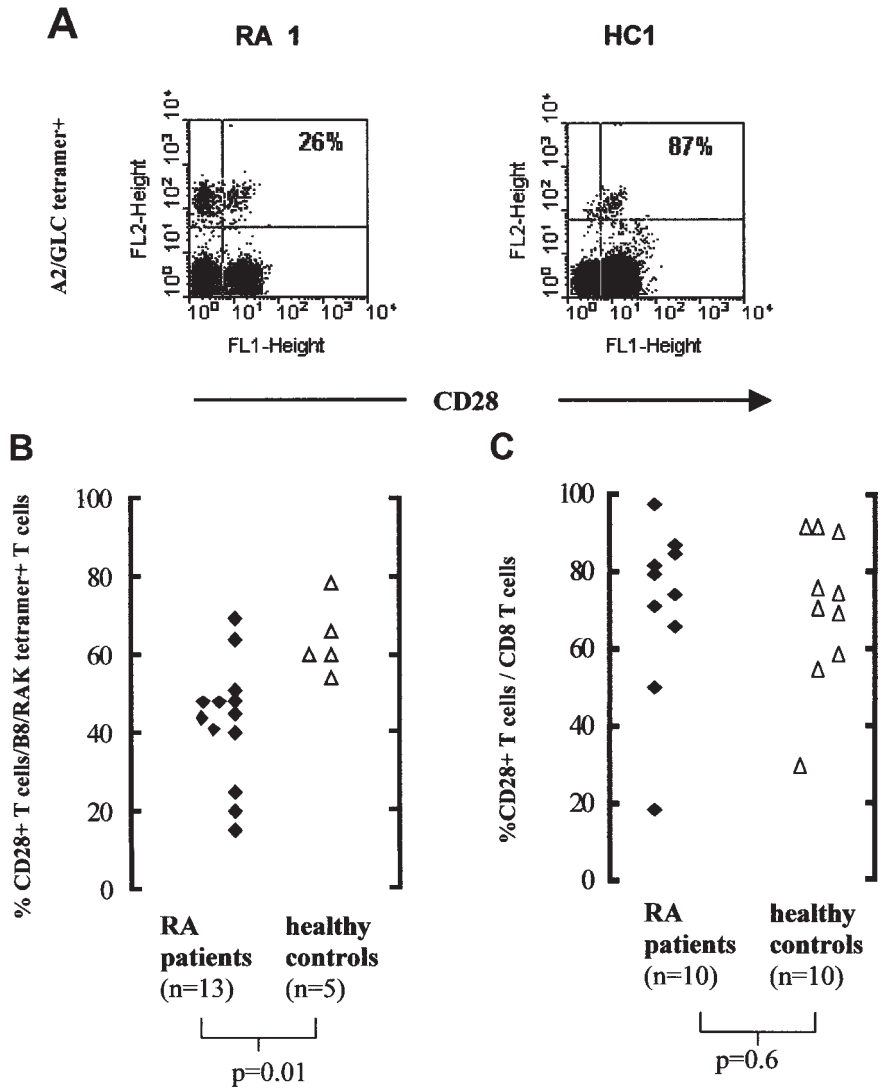

Figure 4. A: Phenotypic analysis of HLA-A2/GLC-specific CD8+ T cells: PBMC of Patient RA1 and Control HC1 were triple-stained with PE-HLA-A2/GLC tetrameric complexes, PerCP-conjugated anti-CD8, and a FITC-conjugated mAb specific for CD28; 50,000 cells were included in each analysis. The number of tetramer-reactive CD8+ T cells that stained with the relevant phenotypic marker is given in the upper right corner as the percentage of the total CD8+ population. B: Comparison of percentages of B8/RAK tetramer-positive CD8+ T cells expressing CD28 in PBMC of RA patients and controls after triple-staining and FACS analysis. C: Comparison of percentages of total CD8+ T cells expressing CD28 in PBMC of RA patients and controls after FACS analysis.

DISCUSSION

In addressing the role of EBV in RA, we investigated the frequency and function of peripheral memory CD8+ T cell responses against 12 lytic or latent EBV epitopes in RA patients in comparison to healthy controls. Seven of the studied epitopes had been shown to be immunodominant or subdominant in EBV-specific T cell immune responses of healthy individuals[28-30,50], whereas 5 further epitopes were selected from computerized prediction of binding to HLA-A2 or –B8. We observed that EBV-specific memory CD8+ T cells in peripheral blood of RA patients were predominantly directed against the lytic epitopes A2/GLC and B8/RAK, as in healthy EBV carriers, and to only a minor extent to the 9 latent EBV-peptide antigens tested. Although the frequencies of the A2/GLC or B8/RAK tetramer-binding CD8+ T cells were only slightly raised in the RA patients compared to controls, they contained significantly lower proportions of CD8+ T cells that were capable of producing IFN-γ upon antigen challenge. Further, these EBV-peptide antigen-specific CD8+ T cells showed a marked increase in CD28–, CD27– phenotypes, suggesting antigen-specific accumulation of terminally differentiated T cell populations as a sign of dysregulation of immunodominant T cell immune responses against EBV in the patients with RA.

Table 6. Phenotypic analysis of A2/GLC tetramer-specific CD8+ T cells.

	Percentage of A2/GLC Tetramer+ CD8+ T Cells						
	CD45RO	CD45RA	CD28	CD27	CD57	HLA-DR	CD69
RA patients							
RA1	70	31	26	90	38	49	5
RA7	88	49	41	81	44	12	15
RA9	93	14	24	64	4	15	7
RA11	96	18	26	84	31	50	18
RA12	94	36	29	62	37	66	8
RA14	73	19	42	71	38	84	12
Mean	86	28	31	75	32	46	11
Controls							
HC1	87	22	87	97	12	39	4
HC7	77	40	69	92	24	47	8
HC9	69	41	69	87	17	29	7
HC10	96	16	76	89	25	12	7
HC11	83	30	63	82	31	29	7
Mean	82	30	73	89	22	31	7

In general, the specificities of the EBV memory CD8+ T cell responses in peripheral blood of RA patients appeared to be similar to those reported in healthy individuals[30,50]. T cell responses against the two A2/GLC and B8/RAK lytic EBV protein epitopes were detectable at higher levels than responses against the 9 latent epitopes tested. The newly predicted lytic peptide A2/QLL, also derived from the immediate early protein BZLF1, stimulated only a small number of T cells in one of 11 healthy controls (9%) and in 6 of the 22 RA patients (27%) to respond with IFN-γ production. Interestingly, in almost all HLA-A*02+ and -B*08+ patients and controls the memory response against the lytic B8/RAK epitope exceeded the frequency of A2/GLC-reactive T cells at least 2-fold, suggesting relative immunodominance of the HLA-B8 over the HLA-A2-restricted EBV-specific longterm CD8+ T cell memory in these individuals, as has been reported for another persistent Herpes virus, cytomegalovirus[51]. Among the 9 latent epitopes we tested, T cell responses against the B8/FLR epitope were immunodominant in RA patients and controls, as reported[46,47], but were still less abundant than those reacting against the lytic epitopes A2/GLC and B8/RAK.

There was a good correlation between the estimates of T cell frequencies from the Elispot assays and the tetramer staining for the A2/GLC and the B8/RAK epitopes in healthy individuals and RA patients. However, total numbers of EBV-specific T cells obtained after stimulation with these peptide epitopes from the Elispot assays were on average about 7-fold lower in RA patients than those obtained from tetramer staining, but about only 1.7-fold lower than those obtained in controls. RA patients thus appeared to accumulate T cells with impaired IFN-γ production in the immunodominant A2/GLC or B8/RAK-peptide-specific T cell responses against EBV. This increase of IFN-γ-nonproducing EBV-specific T cells was seen in RA patients irrespective of immunosuppressive therapies. RA patients compared to controls also did not show a generalized reduced capacity of the whole CD8+ T cell population to produce IFN-γ after PMA stimulation. However, we could not exclude that previously reported defects in signal transduction pathways[35,36] contributed to the observed reduced fractions of CD8+ T cells with IFN-γ production upon lytic EBV antigen challenge in RA patients. A similar loss of functional lytic EBV antigen-specific CD8+ T cells had previously been observed in human immunodeficiency virus-1 infected patients and correlated with a concomitant higher EBV load and a decline of CD4+ T cells[52,53], as well as in the elderly[42]. Thus we were not able to correlate peripheral EBV-DNA load as determined by semiquantitative polymerase chain reaction with reduction of IFN-γ-producing EBV-specific T cells. Further, all our RA patients except 2 exhibited normal CD4/CD8 ratios in the peripheral lymphocyte populations (data not shown).

For further characterization of the peripheral EBV epitope-specific CD8+ T cells, we performed immunophenotyping for markers discriminating subsets that are thought to represent naive, memory, or effector T cells. The lytic A2/GLC and B8/RAK epitope-binding CD8+ T cell populations appeared to contain higher numbers of cells with downregulation of CD28 and concomitantly also of CD27 in the RA patients than in the EBV-immune controls. Such phenotypic differences between RA patients and controls were not seen on the whole CD8 populations, although expansions of unusual T cell populations with loss of CD28 have been described, particularly for the CD4+ T cell compartment, and associated with diminished proliferative capacity[48,54] and premature senescence of T cells in RA[55]. Extraarticular involvement of RA, reported to correlate with the expansion of CD28– CD4+ T cells, was rarely present in our patient group. In healthy individuals EBV-specific memory CD8+ T cells during chronic infection have been described to be enriched in early-differentiated CD28+,

CD27+ phenotype subsets[56]. Together with a decreased expression of CD27 on A2/GLC tetramer-positive T cells, however, we observed that RA patients, in contrast to controls, accumulate terminally differentiated CD28–CD27–CD8+ memory subsets in the response to specific lytic EBV epitopes. Several studies on the frequencies and sites of latently or productively EBV-infected cells in blood and tissues suggest[17-21] that in comparison to healthy individuals RA patients may exhibit a higher load of EBV, probably with a higher level of antigenic challenge, at least for some hosts. Thus, we hypothesize that in RA patients recurrent antigen restimulation leads to terminal differentiation of a larger proportion of the EBV lytic antigen-specific CD8+ T cells than in controls. At least some of these T cells seem to fail to respond to antigen in terms of IFN-γ production and maybe also of cytotoxic activity, as shown on isolated A2/GLC tetramer-binding T cell lines. Such T cells could also represent an anergic CD28–CD27– phenotype and belong to a suppressive T cell subset, as suggested[57], and/or for as yet unknown reasons may have failed to undergo normal apoptosis. In the elderly, such clonal expansions of non-responding, terminally differentiated virus-specific CD8+ T cells have been associated with an immune-risk phenotype as a relevant factor for an age-related increasing morbidity and mortality[58]. Such dysfunctional T cells were suggested to fill immunological space and, although dispensable, to influence the available T cell repertoire for other antigens[59]. It is still unclear whether similar changes in CD8+ T cell responses against EBV are a further sign of premature immunosenescence and are related to a primary defect of T cell differentiation and proliferation in RA[60]. To date we do not know whether EBV is only one of the viruses with broad infection and persistence in human populations that leads to such dysregulated T cell proliferations in RA. However, growing insufficiency in virus control leading to a higher risk for EBV-related lymphoma, as reported to occur in some RA patients[61], might also be a consequence of these abnormalities. Further analyses of the virus-host equilibrium need to be performed in RA to understand the complex relationship of EBV infection to the development and progression of this disease.

ACKNOWLEDGMENT

We thank Inge Steiert for HLA typing and Jon Tolson and Ashly Knights for their comments on the manuscript.

REFERENCES

1. Vaughan JH. Infection and rheumatic disease: a review. Bull Rheum Dis 1990;39:1-8.
2. Bonneville M, Scotet E, Peyrat MA, Saulquin X, Houssaint E. Epstein-Barr virus and rheumatoid arthritis. Rev Rheum Engl Ed 1998;65:365-8.
3. Vaughan JH. The Epstein-Barr virus in autoimmunity. Springer Semin Immunopathol 1995;17:203-30.
4. Lotz M, Roudier J. Epstein-Barr virus and rheumatoid arthritis. In: Smolen J, Kalden JR, Maini RN, editors. Rheumatoid arthritis. Berlin: Springer Verlag; 1992:257-80.
5. Alspaugh MA, Tan EM. Serum antibody in rheumatoid arthritis reactive with a cell-associated antigen: demonstration by precipitation and immunofluorescence. Arthritis Rheum 1976;19:711-9.
6. Billings PB, Hoch SO, White PJ, Carson DA, Vaughan JH. Antibodies to the Epstein-Barr virus nuclear antigen and the rheumatoid arthritis nuclear antigen identify the same polypeptide. Proc Natl Acad Sci USA 1983;80:7104-8.
7. Youinou P, Buisson M, Berthelot JM, et al. Anti-Epstein-Barr virus nuclear antigen 1, 2A and 2B antibodies in rheumatoid arthritis patients and their relatives. Autoimmunity 1992;13:225-31.
8. Kouri T, Petersen J, Rhodes G, et al. Antibodies to synthetic peptides from Epstein-Barr nuclear antigen-1 in sera of patients with early rheumatoid arthritis and in preillness sera. J Rheumatol 1990;17:1442-9.
9. Baboonian C, Venables PJ, Williams DG, Williams RO, Maini RN. Cross-reaction of antibodies to a glycine/alanine repeat sequence of Epstein-Barr virus nuclear antigen-1 with collagen, cytokeratin, and actin. Ann Rheum Dis 1991;50:772-5.
10. Roudier J, Rhodes G, Petersen J, Vaughan JH, Carson DA. The Epstein-Barr virus glycoprotein gp110, a molecular link between HLA-DR4, HLA-DR1, and rheumatoid arthritis. Scand J Immunol 1988;27:367-71.
11. Roudier J, Petersen J, Rhodes G, Luka J, Carson DA. Susceptibility to rheumatoid arthritis maps to a T-cell epitope shared by the HLA-Dw4 and DRß-1 chain and the Epstein-Barr virus glycoprotein gp110. Proc Natl Acad Sci USA 1989;86:5104-8.
12. La Cava A, Nelson JL, Ollier WE, et al. Genetic bias in immune responses to a cassette shared by different microorganisms in patients with rheumatoid arthritis. J Clin Invest 1997;100:658-63.
13. Rickinson AB, Kieff E. Epstein-Barr virus. In: Fields BN, Knipe DM, Howley PM, editors. Virology. 7th ed. Philadelphia: Lippincott-Raven; 1996:2397-446.
14. Sixbey JW, Nedrud JG, Raab-Traub N, Hanes RA, Pagano JS. Epstein-Barr virus replication in oropharyngeal epithelial cells. N Eng J Med 1984;310:1225-30.
15. Newkirk MM, Watanabe Duffy KN, Leclerc J, Lambert N, Shiroky JB. Detection of cytomegalovirus, Epstein-Barr virus and herpes virus-6 in patients with rheumatoid arthritis with or without Sjögren's syndrome. Br J Rheumatol 1994;33:317-22.
16. Yao QY, Rickinson AB, Gaston JS, Epstein MA. Disturbance of the Epstein-Barr virus-host balance in rheumatoid arthritis patients: a quantitative study. Clin Exp Immunol 1986;64:302-10.
17. Tosato G, Steinberg AD, Yarchoan R, et al. Abnormally elevated frequency of Epstein-Barr virus-infected B cells in the blood of patients with rheumatoid arthritis. J Clin Invest 1984;73:1789-95.
18. Balandraud N, Meynard JB, Auger I, et al. Epstein-Barr virus load in the peripheral blood of patients with rheumatoid arthritis. Arthritis Rheum 2003;48:1223-8.
19. Takeda T, Mizugaki Y, Matsubara L, Imai S, Koike T, Takada K. Lytic Epstein-Barr virus infection in the synovial tissue of patients with rheumatoid arthritis. Arthritis Rheum 2000;43:1218-25.
20. Saal JG, Krimmel M, Steidle M, et al. Synovial Epstein-Barr virus infection increases the risk of rheumatoid arthritis in individuals with the shared HLA-DR4 epitope. Arthritis Rheum 1999;42:1485-96.
21. Edinger JW, Bonneville M, Scotet E, Houssaint E, Schumacher HR, Posnett DN. EBV gene expression not altered in rheumatoid synovia despite the presence of EBV antigen-specific T-cell clones. J Immunol 1999;162:3694-701.
22. Scotet E, Peyrat MA, Saulquin X, et al. Frequent enrichment for CD8 T-cells reactive against common herpes viruses in chronic inflammatory lesions: towards a reassessment of the physiopathological significance of T-cell clonal expansions found in autoimmune inflammatory processes. Eur J Immunol

1999;29:973-85.
23. David-Ameline J, Lim A, Davodeau F, et al. Selection of T-cells reactive against autologous B lymphoblastoid cells during chronic rheumatoid arthritis. J Immunol 1996;157:4697-706.
24. Zutter MM, Martin PJ, Sale GE, et al. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. Blood 1988;72:520-9.
25. Papadopoulos EB, Ladanyi M, Emanuel D, et al. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. N Engl J Med 1994;330:1185-91.
26. Murray RJ, Kurilla MG, Brooks JM, et al. Identification of target antigens for the human cytotoxic T-cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. J Exp Med 1992;176:157-68.
27. Khanna R, Burrows SR, Kurilla MG, et al. Localization of Epstein-Barr virus cytotoxic T-cell epitopes using recombinant vaccinia: implications for vaccine development. J Exp Med 1992;176:169-76.
28. Steven NM, Annels NE, Kumar A, Leese AM, Kurilla MG, Rickinson AB. Immediate early and early lytic cycle proteins are frequent targets of the Epstein-Barr virus-induced cytotoxic T-cell response. J Exp Med 1997;185:1605-17.
29. Callan MF, Tan L, Annels N, et al. Direct visualization of antigen-specific CD8+ T-cells during the primary immune response to Epstein-Barr virus in vivo. J Exp Med 1998;187:1395-402.
30. Tan LC, Gudgeon N, Annels NE, et al. A re-evaluation of the frequency of CD8+ T-cells specific for EBV in healthy virus carriers. J Immunol 1999;162:1827-35.
31. Scotet E, David-Ameline J, Peyrat MA, et al. T-cell response to Epstein-Barr virus transactivators in chronic rheumatoid arthritis. J Exp Med 1996;184:1791-800.
32. Tan LC, Mowat AG, Fazou C, et al. Specificity of T-cells in synovial fluid: high frequencies of CD8(+) T-cells that are specific for certain viral epitopes. Arthritis Res 2000;2:154-64.
33. Gaston JS, Rickinson AB, Epstein MA. Epstein-Barr virus-specific cytotoxic T-cell responses in rheumatoid arthritis patients. Rheumatol Int 1982;2:155-9.
34. Fawcett MC, Walker DJ, Griffiths ID. Demonstration of impaired T-cell regulation of Epstein Barr virus stimulated B lymphocytes in rheumatoid arthritis with HLA identical, disease discordant sibling pairs. Ann Rheum Dis 1988;47:372-6.
35. Takei M, Ishiwata T, Mitamura K, et al. Decreased expression of signaling lymphocytic-activation molecule-associated protein (SAP) transcripts in T-cells from patients with rheumatoid arthritis. Int Immunol 2001;13:559-65.
36. Romagnoli P, Strahan D, Pelosi M, Cantagrel A, van Meerwijk JP. A potential role for protein tyrosine kinase p56(lck) in rheumatoid arthritis synovial fluid T lymphocyte hyporesponsiveness. Int Immunol 2001;13:305-12.
37. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
38. Kurz B, Steiert I, Heuchert G, Müller CA. New high resolution typing strategy for HLA-A locus alleles based on dye terminator sequencing of haplotypic group-specific PCR-amplicons of exon 2 and exon 3. Tissue Antigens 1999;53:81-96.
39. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol 1994;152:163-75.
40. Yang J, Lemas VM, Flinn IW, Krone C, Ambinder RF. Application of the ELISPOT assay to the characterization of CD8(+) responses to Epstein-Barr virus antigens. Blood 2000;95:241-8.
41. Altman JD, Moss PA, Goulder PJ, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science 1996;274:94-6.
42. Ouyang Q, Wagner W, Walter S, et al. Age-related increase in CD8+ T-cells carrying receptors for an immunodominant EBV epitope, but decrease in their antigen-specific responsiveness. Mech Ageing Dev 2003;124:477-85.
43. Kerr BM, Kienzle N, Burrows JM, et al. Identification of type B-specific and cross-reactive cytotoxic T-lymphocyte responses to Epstein-Barr virus. J Virol 1996;70:8858-64.
44. Lee SP, Thomas WA, Murray RJ, et al. HLA A2.1-restricted cytotoxic T-cells recognizing a range of Epstein-Barr virus isolates through a defined epitope in latent membrane protein LMP2. J Virol 1993;67:7428-35.
45. Lee SP, Tierney RJ, Thomas WA, Brooks JM, Rickinson AB. Conserved cytotoxic T lymphocyte (CTL) epitopes within Epstein-Barr virus (EBV) latent membrane protein 2, a potential target for CTL-based tumor therapy. J Immunol 1997;158:3325-34.
46. Burrows SR, Sculley TB, Misko IS, Schmidt C, Moss DJ. An Epstein-Barr virus-specific cytotoxic T-cell epitope in EBNA3. J Exp Med 1990;171:345-50.
47. Burrows SR, Gardner J, Khanna R, et al. Five new cytotoxic T-cell epitopes identified within Epstein-Barr virus nuclear antigen 3. J Gen Virol 1994;75:2489-93.
48. Azuma M, Philips JH, Lanier LL. CD28-lymphocytes T antigenic and functional properties. J Immunol 1993;150:1147-59.
49. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:659-60.
50. Rickinson AB, Callan MF, Annels NE. T-cell memory: lessons from Epstein-Barr virus infection in man. Philos Trans R Soc Lond B Biol Sci 2000;355:391-400.
51. Khan N, Shariff N, Cobbold M, et al. Cytomegalovirus seropositivity drives the CD8T cell repertoire toward greater clonality in healthy elderly individuals. J Immunol 2002;169:1984-92.
52. Catalina MD, Sullivan JL, Brody RM, Luzuriaga K. Phenotypic and functional heterogeneity of EBV epitope-specific CD8+ T-cells. J Immunol 2002;168:4184-91.
53. Hamann D, Baars PA, Rep MH, et al. Phenotypic and functional separation of memory and effector human CD8+ T-cells. J Exp Med 1997;186:1407-18.
54. Martens PB, Goronzy JJ, Schaid D, Weyand CW. Expansion of unusual CD4+ T cells in severe rheumatoid arthritis. Arthritis Rheum 1997;40:1106-14.
55. Weyand CM, Goronzy JJ. Premature immunosenescence in rheumatoid arthritis. J Rheumatol 2002;29:1141-6.
56. Appay V, Dunbar PR, Callan M, et al. Memory CD8+ T-cells vary in differentiation phenotype in different persistent virus infections. Nature Med 2002;8:379-85.
57. Filaci G, Suciu-Foca N. CD8(+) T suppressor cells are back to the game: are they players in autoimmunity? Autoimmun Rev 2002;1:279-83.
58. Pawelec G, Ouyang Q, Colonna-Romana G, Candore G, Lio D, Caruso C. Is human immunosenescence clinically relevant? Looking for "immunological risk phenotypes". Trends Immunol 2002;23:330-2.
59. Franceschi C, Bonafe M, Valensin S. Human immunosenescence: the prevailing of innate immunity, the failing of clonotypic immunity, and the filling of immunological space. Vaccine 2000;18:1717-20.
60. Ponchel F, Morgan AW, Bingham SJ, et al. Dysregulated lymphocyte proliferation and differentiation in patients with rheumatoid arthritis. Blood 2002;100:4550-6.
61. Baecklund E, Sundstrom C, Ekbom A, et al. Lymphoma subtypes in patients with rheumatoid arthritis: increased proportion of diffuse large B cell lymphoma. Arthritis Rheum 2003;48:1543-50.