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Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis

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Epidemiological studies consistently demonstrate that patients with multiple sclerosis are almost universally infected with Epstein–Barr virus (EBV) and that the risk of developing the disease increases with the level of EBV-specific antibody titers. The EBV-encoded nuclear antigen-1 (EBNA1) maintains the viral episome in replicating infected human B cells, and EBNA1-specific $CD4^+$ T cells have been identified as a crucial part of the EBV-specific immune control in healthy individuals. We studied 20 untreated EBV seropositive patients with multiple sclerosis and 20 healthy EBV carriers matched demographically and for the expression of multiple sclerosis-associated HLA-DR alleles for their immunological control of EBV latency at the level of EBNA1-specific T cells. Using 51 overlapping peptides covering the C-terminal of EBNA1 domain of EBNA1 (amino acids 400–641), peptide-specific $CD4^+$ memory T cells in patients with multiple sclerosis were found to be strikingly elevated in frequency, showed increased proliferative capacity and an enhanced interferon-γ production. In contrast to EBNA1, T-cell responses to three other latent and three other lytic immunodominant EBV antigens and human cytomegalovirus (HCMV) epitopes did not differ between patients and controls, indicating a distinct role for EBNA1-specific T-cell responses in multiple sclerosis. $CD4^+$ T cells from healthy virus carriers preferentially recognized multiple epitopes within the centre part of the C-terminal, whereas the stimulatory epitopes in multiple sclerosis patients covered the entire sequence of this domain of EBNA1. Quantification of EBV viral loads in peripheral blood mononuclear cells (PBMC) by real-time polymerase chain reaction (PCR) showed higher levels of EBV copy numbers in some patients with multiple sclerosis, although the overall difference in viral loads was not statistically significant compared with healthy virus carriers. We suggest that the accumulation of highly antigen-sensitive EBNA1-specific Th1 cells in multiple sclerosis is capable of sustaining autoimmunity by cross-recognition of autoantigens or by TCR-independent bystander mechanisms.

Keywords: multiple sclerosis; Epstein–Barr virus; T cell; autoimmunity

Abbreviations: CMV = cytomegalovirus; EBV = Epstein–Barr virus; EBNA1 = Epstein–Barr virus-encoded nuclear antigen-1; HD = healthy donors; IFN-γ = interferon-γ; IL = interleukin; PBMC = peripheral blood mononuclear cells; SI = stimulation indices; TCL = T-cell lines

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Introduction

Multiple sclerosis is considered a chronic T-cell-mediated inflammatory disease of the CNS. It develops in young adults with a complex genetic predisposition and is thought to require an inciting environmental insult such as a viral infection to trigger the disease (Hafler, 2004; Hohlfeld and Wekerle, 2004; Sospedra and Martin, 2005). Studies on migrants indicated that the risk of developing multiple sclerosis is established by an early environmental exposure occurring most likely in the first two decades of life (Soldan and Jacobson, 2001). However, despite many claims that a transmissible agent was specifically found in the CNS compartment of multiple sclerosis patients and despite numerous reports on a disease-specific higher sero-prevalence to various viral and bacterial candidates, a specific infectious agent has so far not been linked convincingly to multiple sclerosis. The most consistent data for a potential role in the disease exist for Epstein–Barr virus (EBV) and human herpesvirus 6 (HHV6) owing to the detection of viral DNA in brain specimens derived from multiple sclerosis lesions (in the case of HHV6) and by convincing sero-epidemiological studies. Both are ubiquitous viruses that act at the population level and produce latent, recurrent infections.

Approximately 90% of the human adult population is seropositive for EBV, which can be considered a classic example for effective immunological control of persistent viral infections in humans. Its lifelong latency is maintained through long-lived infected memory B cells in the absence of EBV protein expression. However, latent EBV nuclear antigen-1 (EBNA1) is expressed in all EBV-positive proliferating cells in healthy EBV carriers (Thorley-Lawson, 2001; Hochberg *et al.*, 2004) and all EBV-associated malignancies (Thorley-Lawson and Gross, 2004). During cell division, EBNA1 initiates viral replication by binding to the EBV circular DNA or episome with its C-terminal domain and cross-links the episome to mitotic chromosomes as a protein anchor, thereby accomplishing the transmission of the episomes into progeny cells (Munz, 2004). In healthy EBV carriers, the growth-transforming capacity and potential pathogenicity of EBV for associated tumors such as Hodgkin's disease, Burkitt's lymphoma and nasopharyngeal carcinoma need to be tightly and efficiently controlled by innate and adaptive immune responses. T cells specific for EBNA1 are considered to be a crucial component of EBV-specific immune control. $CD4^+$ T cells of healthy EBV carriers consistently respond to EBNA1 (Munz *et al.*, 2000), are Th1 in function (Bickham *et al.*, 2001), recognize autologous EBV-transformed B-cell lines (B-LCL) (Voo *et al.*, 2002, 2005; Long *et al.*, 2005) and have the capacity to kill EBNA1-expressing targets via CD95/CD95L (Paludan *et al.*, 2002) as well as the ability to inhibit the outgrowth of B-LCL *in vitro* (Nikiforow *et al.*, 2003) and of EBNA1 positive Burkitt's lymphoma cells *in vivo* (Fu *et al.*, 2004).

Several large prospective sero-epidemiological studies demonstrated that multiple sclerosis patients are universally, that is, close to 100%, seropositive for EBV (Ascherio *et al.*, 2001). The analysis of serum samples collected before the onset of disease showed that the risk of developing multiple sclerosis within a mean period of 4 years increased with the level of EBV-antibody titers, and the strongest association was found for EBNA1-specific IgG (Levin *et al.*, 2005). The strong and highly significant association between EBNA1-specific titers and the risk of multiple sclerosis could be confirmed in another independent study (Sundstrom *et al.*, 2004). EBNA1-specific IgG antibodies were reported to occur more frequently in the CSF of patients with multiple sclerosis (Bray *et al.*, 1992). More recently, using a cDNA library derived from foetal brain tissue and epitope mapping techniques to analyse the specificity of oligoclonal IgG antibodies in the patients' CSF compared with other neuroinflammatory diseases, the two most frequent multiple sclerosis-specific and high-affinity epitopes identified were both derived from EBV, that is, EBNA1 and another less characterized EBV protein (BRRF2) (Cepok *et al.*, 2005). Given the association of EBV infections with the incidence of multiple sclerosis and the correlation between elevated IgG titers to EBNA1 and increased risk for disease, we were interested in characterizing the EBNA1-specific T-cell responses in patients with multiple sclerosis and healthy virus carriers.

Patients and methods

Patients and healthy donors

Twenty untreated multiple sclerosis patients [19 with clinically definite, 17 with relapsing-remitting, 2 with secondary progressive multiple sclerosis and 1 with clinically isolated demyelinating syndrome fulfilling MRI criteria for multiple sclerosis (McDonald *et al.*, 2001)] and 20 age-, sex- and major histocompatibility complex (MHC) class II-matched healthy donors (HD) recruited from the National Institutes of Health, Department of Transfusion Medicine database were included in this study (Table 1). All individuals were infected by EBV as assessed by positive IgG antibody titers against EBV-encoded viral capsid antigen (VCA). The study was approved by the National Institute of Neurological Disorders and Stroke (NINDS) Institutional Review Board, and all subjects provided informed consent.

Antigens

Fifty-one peptides covering the C-terminal domain of EBV nuclear antigen-1 (amino acids 400–641) were purchased from the Proteomics Resource Center of the Rockefeller University (Table 2). Five peptides (six for pool #10) were combined in each of 10 different pools. Influenza hemagglutinin (Flu-HA) amino acids 306–318 served as a control peptide.

Interleukin-7- modified primary proliferation

Peripheral blood mononuclear cells (PBMC) were isolated from leukapheresis by density gradient centrifugation and seeded with 1×10^5 cells/well in 96-well U-bottom microtiter plates in T-cell medium (IMDM, Gibco, Grand Islands, NY, USA) containing 2 mM L-glutamine, 50 μg/ml gentamicin and 100 U/ml penicillin/streptomycin (all Whittacker Bioproducts,

Table 1 Multiple sclerosis patients and HD controls

		Multiple sclerosis (n = 20)	HD (n = 20)
Demographic data	Age (median; range)	32 (24–57)	34 (28–61)
	Gender (male; female)	1 : 1.86	1 : 1.5
HLA-DR haplotype	HLA-DR2/DRx	10 (50%)	11 (55%)
	HLA-DR2/DR4	7 (35%)	7 (35%)
	HLA-DRx/DR4	3 (15%)	2 (10%)
Clinical characteristics	Disease duration in years from first clinical symptom (median; range)	2.5 (1–21)	N/A
	EDSS*	1.5 (0–6.5)	N/A

*Expanded disability status score.

Gaithersburg, MD, USA), 5% pooled human AB-serum and enriched with 10 ng/ml interleukin-7 (IL-7) (PeproTech, Rocky Hill, NJ, USA). Twenty wells (2×10^6 cells) were tested with each of the 10 peptide pools, with the control Flu-HA antigen as a positive control and no antigen as negative control, using 240 wells/donor. All peptides were used at a final concentration of 1 μM. After 7 days of incubation at 37°C and in 5% CO_2, each culture was re-suspended and half of the total volume (100 μl) was transferred into new plates, which were pulsed with [^{3}H] thymidine (Amersham, Arlington Heights, IL, USA) for 8 h at 1 μCi/well. T-cell medium enriched with IL-2 (20 IU/ml; National Institutes of Health/National Cancer Institute, Frederick, MD, USA) was added to the remaining non-pulsed wells to propagate the cultures. The incorporated radioactivity (counts per minute, c.p.m.) in 'daughter' plates was measured by scintillation counting (1450 Microbeta; Wallac/PerkinElmer Life Sciences, Boston, MA, USA). Individual wells were considered positive if their stimulation index (SI = c.p.m. of wells with antigen/mean c.p.m. of 20 negative control wells) was >2 and their c.p.m. was at least 3 SD above the average of negative control wells. Estimated precursor frequencies of EBNA-1-specific cells were determined by the number of positive wells for each group divided by the number of seeded PBMC.

Re-stimulation of positive cultures and generation of EBNA1-specific T-cell lines

Positive cultures were identified on the corresponding 'mother' plates and re-stimulated on day 10 in 48-well plates with 1.5×10^6 autologous irradiated (3000 rad) antigen-pulsed (1 μM per peptide) PBMC. IL-2-enriched medium was added immediately and again 3 days later. Cultures were again re-stimulated after 7 days. After the second and before the third re-stimulation (day 24), the cells were tested for CD4, CD8 and CD45RA versus RO isoform expression and for cytokine production. Long-term cultures were then re-stimulated every 12–14 days in 24-well plates with 2.5×10^6 autologous irradiated antigen-pulsed PBMC. IL-2 (20 IU/ml) was added immediately after re-stimulation and on day 3, 7 and 10.

Flow cytometry-based phenotypic analysis

Previous studies have shown that gradual loss of CD45RA expression in naïve CD4$^+$ cells and lack of its re-expression in memory cells during the first 3–4 stimulation cycles allow identification of the naïve versus memory origin of antigen-specific *in vitro* cultured cells (Bielekova *et al.*, 1999; Muraro *et al.*, 2000). After the second re-stimulation (day 24 *ex vivo*), cells were washed with staining buffer [Dulbecco's phosphate-buffered saline (PBS) with 1% heat-inactivated fetal calf serum (FCS) and 0.1% sodium azide] and incubated with antibodies [CD45RO-FITC, CD45RA-PE, CD4-Cy5, CD8-APC and the isotype-matched mouse IgG controls, all purchased from BD PharMingen (San Diego, CA, USA)] at saturating concentrations for 30 min on ice, then washed twice with staining buffer and analysed (FACSCalibur, Beckton-Dickinson) using Cell-Quest software. A minimum of 5000 lymphocytes was acquired per sample. CD45 isoform expression was analysed on double-gated populations of CD4$^+$ and CD8$^+$ lymphocytes, respectively. Memory CD4$^+$ T-cell lines (TCL) were defined by a proportion > 90% of CD45RA-CD45RO$^+$ in the CD4$^+$ gate.

Antigen avidity

Following their phenotypic characterization by fluorescence-activated cell sorting (FACS), memory CD4$^+$ TCL were re-stimulated on day 31 at 2×10^4 T cells/well, and irradiated (3000 rad) autologous PBMC (1×10^5/well) and antigen (0.01, 0.1, 1 and 10 μM) were added in duplicate for 36 h. For the last 8 h, cells were pulsed with [^{3}H]thymidine, and proliferation was measured as above. The EC_{50}, that is, the half-maximal stimulatory concentration, was derived from individual antigen-avidity curves.

Cytokine production

The secretion of prototypic TH1 [interferon-γ (IFN-γ)] and TH2 (IL-4) cytokines was determined in supernatants of EBNA-1-specific TCL stimulated 36 h after stimulation with 1 μM of the corresponding antigen. Analyses were performed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' recommendations (DuoSet ELISA, R&D systems, Minneapolis, MN, USA). Samples were measured in duplicates and in 1 : 2 dilutions for both cytokines.

Intracellular cytokine staining assay

PBMC (2×10^6 cells in 0.5 ml RPMI 1640 complete medium containing 5% pooled human AB-serum) were stimulated with peptide mixtures for 6 h in the presence of 1 μg/ml of co-stimulatory monoclonal antibodies to CD28 and CD49d (BD Biosciences, Immunocytometry systems, San Jose, CA, USA) and Brefeldin A at a concentration of 10 μg/ml (Sigma, St Louis, MO, USA). EBNA1 peptides were added at a final concentration of 3.5 μM per peptide per reaction. After examining peptide concentrations between 1 and 5 μM, 3.5 μM was determined to provide the most consistent responses in EBV seropositive individuals with the lowest background in EBV seronegative donors. Negative controls included co-stimulatory antibodies and Brefeldin A at the same concentration without peptides. Positive controls included, aside from Streptococcus Enterotoxin B (SEB; 1.5 μg/ml), known peptide mixtures of immunodominant T-cell epitopes from EBV and HCMV proteins at a final concentration of 1 μM per peptide. These peptides constitute the EBV- and HCMV-derived portion of the control peptide pool, which has previously been used as positive control in immunomonitoring studies of patients infected with HIV and contains CD8$^+$ T-cell epitopes recognized by most individuals (Currier *et al.*, 2002).

Table 2 EBNA1 peptides

Peptide pool #	Description	Sequence	Length (amino acids)
1	EBNA1 (400–414)	PGRRPFFHPVGEADY	15
	EBNA1 (405–418)	FFHPVGEADYFEYH	14
	EBNA1 (409–422)	VGEADYFEYHQEGG	14
	EBNA1 (413–429)	DYFEYHQEGGPDGEPDV	17
	EBNA1 (420–434)	EGGPDGEPDVPPGAI	15
2	EBNA1 (425–439)	GEPDVPPGAIEQGPA	15
	EBNA1 (430–451)	PPGAIEQGPADDPGEGPSTGPR	22
	EBNA1 (435–451)	EQGPADDPGEGPSTGPR	17
	EBNA1 (442–458)	PGEGPSTGPRGQGDGGR	17
	EBNA1 (449–461)	GPRGQGDGGRRKK	13
3	EBNA1 (452–465)	GQGDGGRRKKGGWF	14
	EBNA1 (456–469)	GGRRKKGGWFGKHR	14
	EBNA1 (460–474)	KKGGWFGKHRGQGGS	15
	EBNA1 (465–478)	FGKHRGQGGSNPKF	14
	EBNA1 (469–482)	RGQGGSNPKFENIA	14
4	EBNA1 (473–487)	GSNPKFENIAEGLRA	15
	EBNA1 (478–491)	FENIAEGLRALLAR	14
	EBNA1 (482–496)	AEGLRALLARSHVER	15
	EBNA1 (487–503)	ALLARSHVERTTDEGTW	17
	EBNA1 (494–508)	VERTTDEGTWVAGVF	15
5	EBNA1 (499–510)	DEGTWVAGVFVY	12
	EBNA1 (501–514)	GTWVAGVFVYGGSK	14
	EBNA1 (505–518)	AGVFVYGGSKTSLY	14
	EBNA1 (509–522)	VYGGSKTSLYNLRR	14
	EBNA1 (513–527)	SKTSLYNLRRGTALA	15
6	EBNA1 (519–532)	NLRRGTALAIPQCR	14
	EBNA1 (523–536)	GTALAIPQCRLTPL	14
	EBNA1 (527–541)	AIPQCRLTPLSRLPF	15
	EBNA1 (532–544)	RLTPLSRLPFGMA	13
	EBNA1 (535–548)	PLSRLPFGMAPGPG	14
7	EBNA1 (539–554)	LPFGMAPGPGPQPGPL	16
	EBNA1 (545–559)	PGPGPQPGPLRESIV	15
	EBNA1 (549–563)	PQPGPLRESIVCYFM	15
	EBNA1 (554–566)	LRESIVCYFMVFL	13
	EBNA1 (557–571)	SIVCYFMVFLQTHIF	15
8	EBNA1 (562–576)	FMVFLQTHIFAEVLK	15
	EBNA1 (566–580)	LGTHIFAEVLKDAIK	15
	EBNA1 (571–584)	FAEVLKDAIKDLVM	14
	EBNA1 (575–588)	LKDAIKDLVMTKPA	14
	EBNA1 (579–593)	IKDLVMTKPAPTCNI	15
9	EBNA1 (584–597)	MTKPAPTCNIRVTV	14
	EBNA1 (588–600)	APTCNIRVTVCSF	13
	EBNA1 (591–604)	CNIRVTVCSFDDGV	14
	EBNA1 (595–609)	VTVCSFDDGVDLPPW	15
	EBNA1 (600–614)	FDDGVDLPPWFPPMV	15
10	EBNA1 (605–619)	DLPPWFPPMVEGAAA	
	EBNA1 (610–625)	FPPMVVEGAAAEGDDG	15
	EBNA1 (616–629)	EGAAAEGDDGDDGDE	16
	EBNA1 (620–634)	EGDDGDDGDEGGDGD	15
	EBNA1 (625–639)	DDGDEGGDGDEGEEG	15
	EBNA1 (630–641)	GGDGDEGEEGQE	12
Control antigen	Flu-HA (306–318)	PKYVKQNTLKLAT	13

After 6 h of incubation at 37°C in 5% CO_2, each sample received 4 μl of 0.5 M ethylenediamine tetraacetic acid (EDTA) and 4.5 ml FACS lysing buffer (BD Immunocytometry Systems) before storage at −80°C overnight. After thawing, cells were re-suspended in 0.5 ml of permeabilization solution [0.1% bovine serum albumin (BSA), 0.1% saponin, in PBS], and left at room temperature for 10 min. After an additional centrifugation, the permeabilization solution was decanted and the cells were stained with directly labelled fluorochrome antibodies ($CD8^+$-FITC, IFN-γ-PE, CD4-PerCP, CD3-APC, all from BD PharMingen, San Diego, CA, USA) for 15 min at room temperature. After two washes, cells were re-suspendend in 200 μl of FACS buffer solution (0.1% BSA and 0.1% sodium azide in PBS). At least 50 000 events were analysed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) by gating on $CD3^+$

lymphocytes. In order to reduce the sample number, the 10 peptide pools that contained the entire C-terminal domain of EBNA1 were combined to a total of 5 pools. PBMC derived from 16 patients with multiple sclerosis and 12 healthy virus carriers, from whom cells were available, were stimulated with the respective peptides and analysed for their frequencies of $CD3^+$ gated $CD4^+$ and $CD8^+$ antigen-specific IFN-γ-producing T cells using FlowJo software (Tree Star, Ashland, OR, USA). According to criteria used in ELISPOT analyses, a positive response required a frequency at least 2-fold above background (no antigen) and at least 10 IFN-γ^+ events. The frequencies of antigen-specific T cells were determined by subtracting the background frequency from the frequency of antigen-stimulated positive samples.

Viral loads

EBV DNA was quantified from PBMCs by quantitative real-time polymerase chain reaction (PCR) using a TaqMan PCR kit and a Model 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). DNA was extracted from PBMCs using the QIAamp DNA Blood Mini Kit (Qiagen) following the manufacturer's protocol. A region from the BamHI W fragment of EBV was amplified using primers GGACCACTGCCCCTGGTA and TTTGTGTGGACTCCTGGG and detected with fluorogenic probe FAM-TCCTGCAGCTATTTCTGGTCGCATCA-TAMRA. The human bcl-2 gene was amplified using primers CCTGCCCTCCTTCCGC and TGCATTTCAGGAAGACCCTGA and detected with fluorogenic probe FAM-CTTTCTCATGGCTGTCC-TAMRA. The EBV BamHI W fragment copy number per cell was calculated using the formula $N = 2 \times W/B$, where N is the EBV BamHI W copy number/cell, W is the EBV BamHI W copy number and B is the bcl-2 copy number. Two samples derived from HD showed exceedingly high EBV copy numbers ($>5 \times 10^6/10^6$ PBMC) presumably due to contamination and were omitted from the analysis. All samples were tested in triplicates.

Statistical analysis

Statistics were performed using commercial software (PRISM 4, GraphPad Software, San Diego, CA, USA). Comparisons between multiple sclerosis patients and HD were based on the non-parametric Mann–Whitney U rank sum test. For correlation analyses between clinical disease parameters and T-cell responses, we used the non-parametric Spearman correlation.

Results

Latent EBV nuclear antigen-1-specific T cells are increased in frequency and enhanced in their proliferative capacity in patients with multiple sclerosis

Twenty untreated multiple sclerosis patients exhibiting an IgG response to EBV and 20 healthy EBV carriers matched for age and gender were studied for their immunological control of EBV latency at the level of EBNA1-specific T cells. In order to minimize influences of MHC binding on the T-cell repertoire and response patterns, patients and healthy volunteers were further matched for their HLA-DR haplotype. All individuals included in this study carried multiple sclerosis-associated HLA-DR2 or HLA-DR4 alleles (Table 1). An influenza hemagglutinin (Flu-HA) peptide (amino acids 306–318) chosen for its reported promiscuous MHC class II binding and its immunodominance in humans served as control recall antigen (Gelder *et al.*, 1996). We used 10 overlapping sets of 5 peptides covering the C-terminal domain of EBNA1 (amino acids 400–641) (Table 2), since most of the previously reported EBNA1-specific $CD4^+$ and $CD8^+$ T-cell responses have been elicited by peptides located in this region (Blake *et al.*, 2000; Leen *et al.*, 2001; Paludan *et al.*, 2002; Lee *et al.*, 2004; Tellam *et al.*, 2004; Voo *et al.*, 2004; Long *et al.*, 2005). To assess the frequency and reactivity of EBNA1-reponsive T cells and to generate EBNA1-specific TCL, we performed a primary proliferation assay modified by the addition of IL-7 (Bielekova *et al.*, 1999, 2000; Lunemann *et al.*, 2004).

Under these conditions, T-cell responses to the recall Flu-HA (amino acids 306–318) antigen were comparable between multiple sclerosis patients and healthy controls. In sharp contrast, we found substantially different responses to EBNA1 between the two groups (Fig. 1). Pooling the data for all EBNA1 peptides, multiple sclerosis patients had significantly higher numbers of positive cultures (median: 27.5 for multiple sclerosis versus 12.5 for HD; $P = 0.0006$) with estimated precursor frequencies of 1.7×10^{-6} versus 0.59×10^{-6} as well as higher median stimulation indices (5.91 versus 3.74; $P = 0.0027$). Aside from one individual (multiple sclerosis #9), patients with above-average high numbers of positive cultures (>60) were not identical with the three patients showing above-average SI of cultures (>8). Within the patient group, there was no correlation between the frequency nor the reactivity of EBNA1-specific T-cell responses to clinical disease parameters such as the extent of clinical disability (EDSS), and the duration of disease.

The enhanced responsiveness to EBNA1 in multiple sclerosis patients was associated with a higher avidity of reactive T cells. Since our positive cultures predominantly consisted of memory $CD4^+$ T cells as described below and since memory $CD4^+$ T cells have been identified as a crucial component of EBV-specific immune responses, EBNA1-specific TCL were selected for their $CD4^+$ memory phenotype and re-stimulated with the peptide pool that elicited the primary proliferative response in dose titration experiments (0.01, 0.1, 1 and 10 μM). The functional avidity of the TCL was defined by their EC_{50} value (dose of an antigen that leads to 50% of the maximal proliferative response) as calculated from individual dose titration curves. As shown in Fig. 2, TCL from patients with multiple sclerosis showed a moderately, but significantly higher, avidity to EBNA1 with EC_{50} values being consistently lower than 1 μM.

The enhanced T-cell response is associated with a broadened EBNA1-epitope recognition in multiple sclerosis

We next tried to identify immunodominant epitopes within the EBNA1 protein by analyzing the frequency of

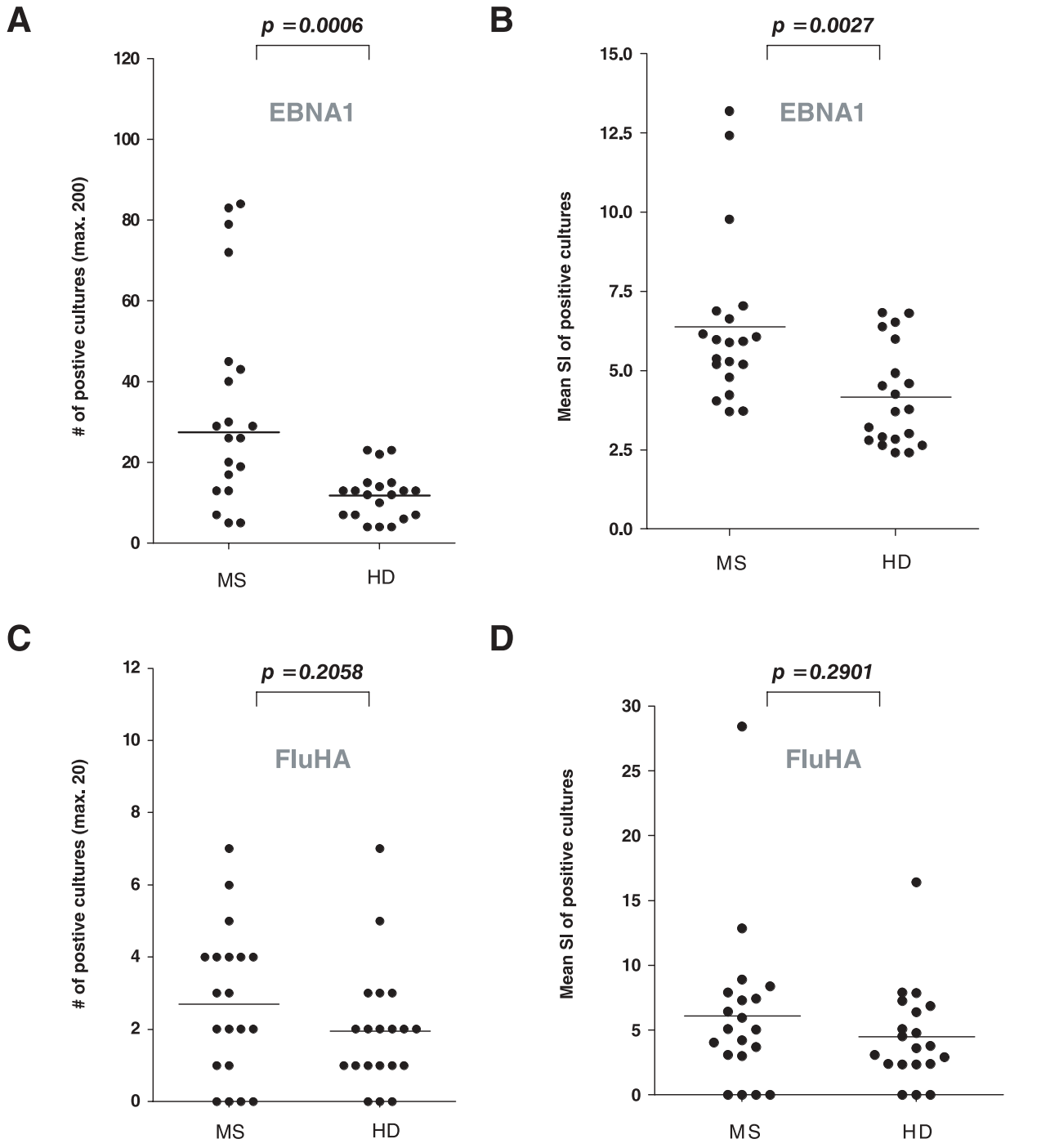

Fig. 1 Increased frequency and enhanced proliferative capacity of EBNA1-specific T cells in patients with multiple sclerosis. Depicted are primary proliferation data from peripheral blood cells derived from multiple sclerosis patients ($n = 20$) and HD matched for their multiple sclerosis-associated HLA-DR alleles ($n = 20$). Bars represent median values. Both groups differed from each other in (**A**) the number of growing cultures stimulated with EBNA1 peptides covering the region (amino acids 400–641), against which T-cell reactivity is focused in healthy virus carriers, and (**B**) in their mean SI. In contrast to EBNA1, no differences were observed in the (**C**) frequency and (**D**) magnitude of Flu-HA-specific T-cell responses.

EBNA1-responsive T cells separately for each pool. In healthy HLA-DR2/DR4 positive EBV carriers, the vast majority of positive cultures were obtained with peptide pool #4 (amino acids 473–508). Interestingly, no clear immunodominance was detected in patients with multiple sclerosis. The stimulatory potential of peptide pool #4 was comparable with what was observed in healthy controls with estimated frequencies of 2.65×10^{-6} in multiple sclerosis versus 2.55×10^{-6} in HD. However, patients with multiple sclerosis showed T-cell recognition of a much broader panel of EBNA1 epitopes

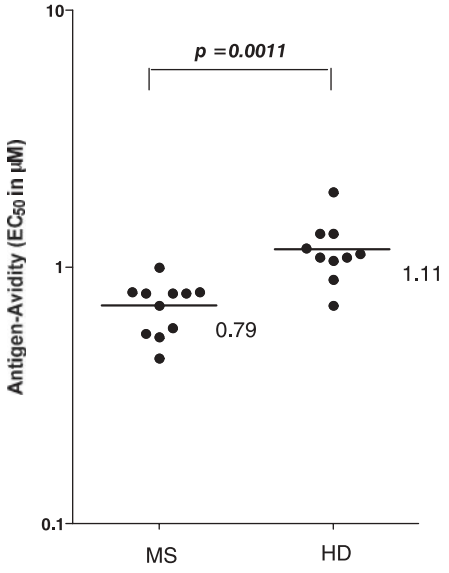

Fig. 2 Enhanced responsiveness to EBNA1 in multiple sclerosis patients is reflected by a higher functional avidity of reactive T cells. The figure demonstrates the results of dose titration experiments with 21 TCL selected for their $CD4^+$ memory phenotype. The functional antigen avidity was defined by their EC_{50} value (dose of the antigen that leads to 50% of the maximal proliferative response) as calculated from individual titration curves. Bars and numbers represent mean EC_{50} values.

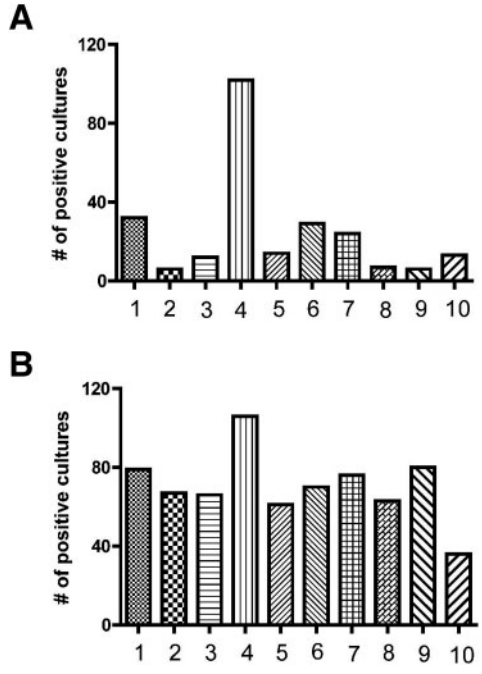

Fig. 3 Diversified EBNA1-epitope recognition in multiple sclerosis. Displayed are the numbers of all EBNA1-specific T-cell cultures derived from (**A**) healthy individuals and (**B**) patients with multiple sclerosis in the order of stimulatory peptide pools (1–10). In healthy HLA-DR2/DR4 positive EBV carriers, the majority of positive EBNA1-specific T-cell cultures were obtained by peptide pool #4 (EBNA1 amino acids 473–508). In contrast, multiple sclerosis patients showed T-cell recognition to a much broader panel of EBNA1 epitopes covering the protein's entire C-terminal domain.

covering the protein's whole C-terminal domain with almost equally distributed frequencies (Fig. 3). Thus, a diversified epitope recognition pattern rather than a dominant response to a single region of the protein appears to be responsible for or contribute to the enhanced EBNA1-directed T-cellular reactivity in multiple sclerosis.

TCL from healthy individuals specific for pool #4 were further tested with five single peptides at 1 μM concentration in order to define immunodominant EBNA1 epitopes in the context of HLA-DR2 and HLA-DR4. As depicted in Table 3, the TCL tested did not uniformly respond to a single peptide but exhibited a strong response to multiple epitopes within one region, most of them either with EBNA1 (amino acids 473–487) or EBNA1 (amino acids 494–508). Thus, multiple epitopes within the area of EBNA1 (amino acids 473–508) appear to be responsible for the immunodominance in healthy HLA-DR2/DR4 positive EBV carriers.

Differences between multiple sclerosis patients and healthy EBV carriers can be attributed to EBNA1-specific memory $CD4^+$ T cells

In order to characterize the phenotype of EBNA1-responsive T cells and to identify the population mediating the differences between patients and controls, we analysed 132 TCL (98 derived from multiple sclerosis patients, 34 from HD) after the second re-stimulation (day 24 *ex vivo*) for CD4 and CD8 as well as for CD45 isoform expression. Previous work by our group on the time kinetics of the naïve to memory phenotype transition after TCR stimulation using purified CD45RA/RO subsets demonstrated that the naïve versus memory phenotype before the third *in vitro* re-stimulation is still representative for their *ex vivo* origin and can easily be assessed by surface CD45RA/RO staining (Bielekova *et al.*, 1999; Muraro *et al.*, 2000).

As depicted in Fig. 4, EBNA1-specific TCL (n = 119) derived from both multiple sclerosis patients and healthy individuals predominantly consisted of $CD4^+$ lymphocytes (median: 72.2% for $CD4^+$ versus 11.5% for $CD8^+$ of gated lymphocytes; $P < 0.0001$). In particular, 59 out of 119 EBNA1-TCL (49.6%) contained >90% $CD4^+$ and <10% $CD8^+$ cells, while only 4 of 119 (3.4%) contained predominantly (>80%) $CD8^+$ cells. Fifty-six out of 119 (47.0%) cell lines could not be clearly attributed to either lineage, but comprised more $CD4^+$ than $CD8^+$ cells (median: 61.3% for $CD4^+$, and 16.9% for $CD8^+$). $CD4^+$ lymphocytes were also significantly more abundant in Flu-HA-specific TCL (n = 13), although to a much lesser extent than with the EBNA1-TCL (median: 58.7% $CD4^+$ versus 39.0% for $CD8^+$ gated lymphocytes; $P = 0.0014$).

In the $CD4^+$ cell subset, almost all virus-specific TCL displayed a memory phenotype (median: 92.1% $CD45RA^-RO^+$

Table 3 Fine-specificity of 10 selected TCL responsive to EBNA1 pool #4 (473-508)

TCL	EBNA1 (473–487) GSNPKFENIAEGLRA	EBNA1 (478–491) FENIAEGLRALLAR	EBNA1 (482–496) AEGLRALLARSHVER	EBNA1 (487–503) ALLARSHVERTTDEGTW	EBNA1 (494–508) VERTTDEGTWVAGVF
HD4-3	**396.7**	17.6	5.1	7.9	2.9
HD4-1	1.2	2.4	10.3	12.8	**78.4**
HD6-9	2.2	1.1	1.3	15.0	**214.6**
HD6-11	1.1	1.2	1.1	2.3	**21.0**
HD6-13	1.03	1.08	0.98	2.89	**30.3**
HD6-15	**135.3**	3.6	5.2	4.9	8.5
HD9-6	2.2	2.1	**140.1**	10.8	37.0
HD9-4	**213.5**	19.7	4.2	2.2	1.4
HD11-2	**89.2**	8.9	4.8	4.1	5.4
HD12-8	**36.2**	13.1	4.2	2.0	1.8

Depicted are SI of single peptide-stimulated T cells seeded in duplicate wells. Bold numbers represent the highest SI achieved by one of five peptides for each TCL.

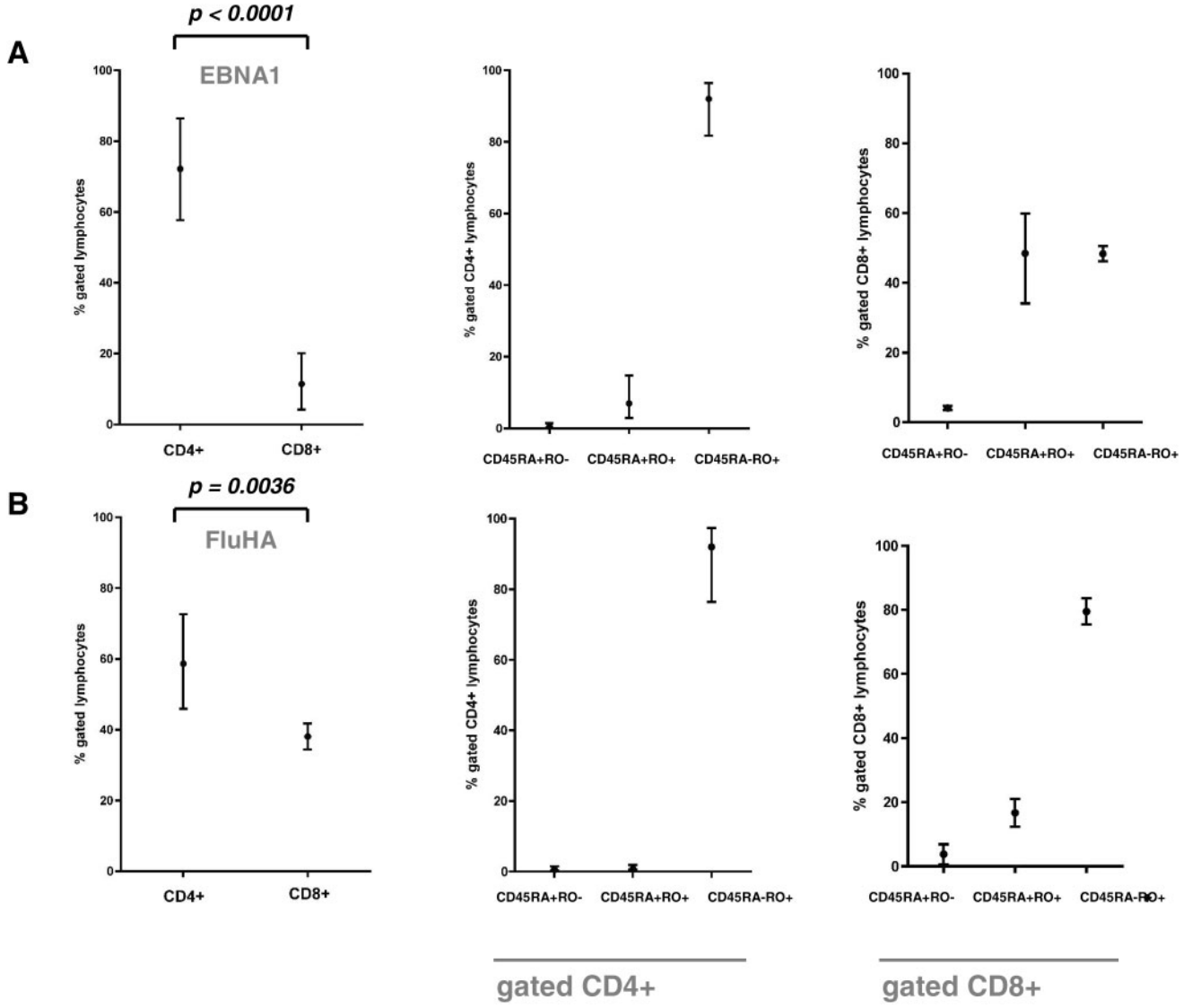

Fig. 4 EBNA1-specific T cells are predominantly $CD4^+$ and originate from the memory pool. T-cell lines specific (**A**) for EBNA1 ($n = 119$) and (**B**) for Flu-HA ($n = 13$) were analysed by flow cytometry after their second re-stimulation on day 24 *ex vivo* for the surface expression of CD4 and CD8 as well as for CD45 isoforms to detect whether they originated from the naïve versus memory T-cell pool. Memory $CD4^+$ lymphocytes predominated in the EBNA1- and Flu-HA-specific T-cell repertoire in every individual tested. Symbols and bars represent median and interquartile range values.

of gated $CD4^+$). EBNA1- and Flu-HA-specific lines did not differ from each other in the frequency of $CD4^+$ lymphocytes originating from the memory pool (median, 92.0% $CD45RA^-RO^+$ for $CD4^+$ Flu-HA-specific TCL). Single $CD45RO^+$ lymphocytes equally predominated in the $CD8^+$ compartment in Flu-HA-specific lines (median: 78.4% of gated $CD8^+$). In EBNA1-TCL, however, we observed a significantly lower number of $CD45RA^-RO^+$ (median, 46.8%) but an increase of double-positive $CD45RA^+RO^+$ (median, 48.5%). However, since CD45RA is reported to be re-expressed upon activation of effector memory $CD8^+$ cells (Hamann *et al.*, 1997), we cannot draw definite conclusions on the naïve versus memory origin of EBNA1-specific $CD8^+$ lymphocytes.

Comparing multiple sclerosis patients and healthy individuals, we did not detect any significant differences between the two groups for any cell subset. Memory $CD4^+$ T cells were clearly predominant in the EBNA1-specific T-cell repertoire of every individual tested and appeared thus to be the cell population sustaining the enhanced frequency and reactivity observed in patients with multiple sclerosis.

EBNA1-specific T cells are Th1 in function and show enhanced IFN-γ production in multiple sclerosis

Previous studies demonstrated that cytotoxic effector functions of EBNA1-specific $CD4^+$ T cells are restricted to Th1-type lymphocytes and can be read out by their ability to produce IFN-γ (Bickham *et al.*, 2001). Therefore, we analysed 119 EBNA1-TCL (91 derived from multiple sclerosis patients, 28 from HD) and in parallel 15 Flu-HA-specific TCL (7 from patients with multiple sclerosis, and 8 derived from HD) 72 h after their third re-stimulation (day 24 *ex vivo*) for IFN-γ and IL-4 production by ELISA.

IL-4 could not be detected in any of these TCL. In contrast, most of the EBNA1-specific lines (77% of the TCL derived from patients versus 82% of TCL from HD) produced detectable levels of IFN-γ (median, 133.0 pg/ml ± for all IFN-γ-producing TCL). As depicted in Fig. 5, multiple sclerosis-derived EBNA-1-specific T cells showed a significantly enhanced production of IFN-γ following stimulation with their cognate EBNA1-antigen compared with healthy EBV carriers (median: 279.0 pg/ml for multiple sclerosis versus 37.1 pg/ml for HD; $P < 0.0001$). This difference was preserved in the supernatants of TCL incubated with the maximal stimulatory antigen concentration in dose titration experiments, indicating an intrinsically greater capacity rather than an antigen concentration-dependent mechanism to be responsible for the increased IFN-γ production. In Flu-HA-specific TCL, IFN-γ could be detected in the supernatants of all T-cell cultures with median levels of 616.7 pg/ml and with no significant differences between both groups (median: 691.8 pg/ml for multiple sclerosis versus 507.9 for HD; $P = 0.1893$).

Enhanced $CD4^+$ Th1 responses to EBNA1 but not to other latent and lytic EBV or to cytomegalovirus (CMV) antigens in multiple sclerosis patients

Since the proliferation assay used in this study favoured $CD4^+$ T-cell responses, we additionally analysed $CD8^+$ T-cell reactivity by a short-term *ex vivo* intracellular cytokine staining assay. Furthermore, we used pooled latent and lytic EBV antigens (derived from EBNA3A, EBNA3B, EBNA3C, BZLF1, BRLF1 and BMLF1) and CMV pp65 epitopes in order to compare the stimulatory potential of EBNA1 to other viral antigens (Table 4). Consistent with the

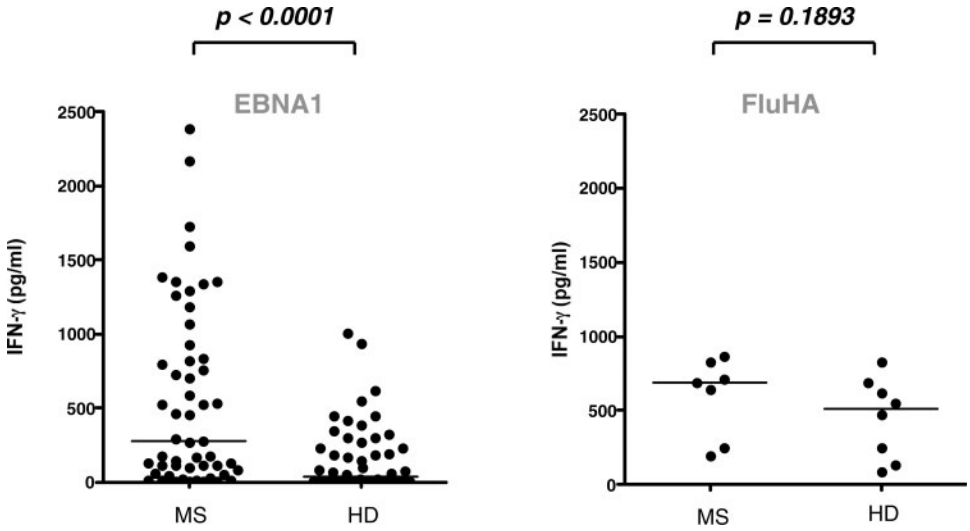

Fig. 5 EBNA1-specific T cells are Th1 in function and show enhanced IFN-γ production in multiple sclerosis. Depicted are the amounts of IFN-γ measured in the supernatants of EBNA1 ($n = 119$) and Flu-HA ($n = 15$) specific T-cell lines derived from patients and HD. IL-4 could not be detected in any of these lines. Bars represent median values.

Table 4 Additional HLA class I-restricted control antigens used in the flow cytometry-based assay

Antigen	Sequence	HLA restriction	Virus
EBNA3A (158–166)	QAKWRLQTL	HLA-B8	EBV
EBNA3A (325–333)	FLRGRAYGL	HLA-B8	EBV
EBNA3A (379–387)	RPPIFIRRL	HLA-B7	EBV
EBNA3A (458–466)	YPLHEQHGM	HLA-B35	EBV
EBNA3A (603–611)	RLRAEAQVK	HLA-A3	EBV
EBNA3B (416–424)	IVTDFSVIK	HLA-A11	EBV
EBNA3C (258–266)	RRIYDLIEL	HLA-B27	EBV
EBNA3C (281–290)	EENLLDFVRF	HLA-B44	EBV
BZLF1 (190–197)	RAKFKQLL	HLA-B8	EBV
BRLF1 (28–37)	DYCNVLNKEF	HLA-A24	EBV
BRLF1 (134–143)	ATIGTAMYK	HLA-A11	EBV
BRLF1 (148–156)	RVRAYTYSK	HLA-A3	EBV
BMLF1 (259–267)	GLCTLVAML	HLA-A2	EBV
pp65 (417–426)	TPRVTGGGAM	HLA-B7	HCMV
pp65 (495–503)	NLVPMVATV	HLA-A2	HCMV
pp65 (512–521)	EFFWDANDIY	HLA-B44	HCMV

proliferation assays, multiple sclerosis patients showed significantly higher frequencies of EBNA1-specific and IFN-γ-producing $CD4^+$ T cells (Fig. 6). Positive responses ranged from 0.044% to 0.275% (mean: 0.149%) in 13 out of 16 multiple sclerosis patients (81%), and from 0.049 % to 0.117 % (mean: 0.073%) in 8 out of 12 healthy volunteers (67%).

Also in line with our proliferation-based T-cell assay, healthy volunteers predominantly recognized the centre part of the C-terminal domain (amino acids 452–548) of EBNA1 (20 out of 28 positive samples; 71%), whereas $CD4^+$ T cells derived from patients showed a much broader recognition pattern with stimulatory epitopes equally distributed over the entire C-terminal domain (amino acids 400–641).

$CD8^+$ T-cell responses to EBNA1 tended also to be higher in the patient group (mean: 0.135% versus 0.080% in HD), but these differences did not reach an overall statistical significance. Furthermore, frequencies of $CD4^+$ and $CD8^+$

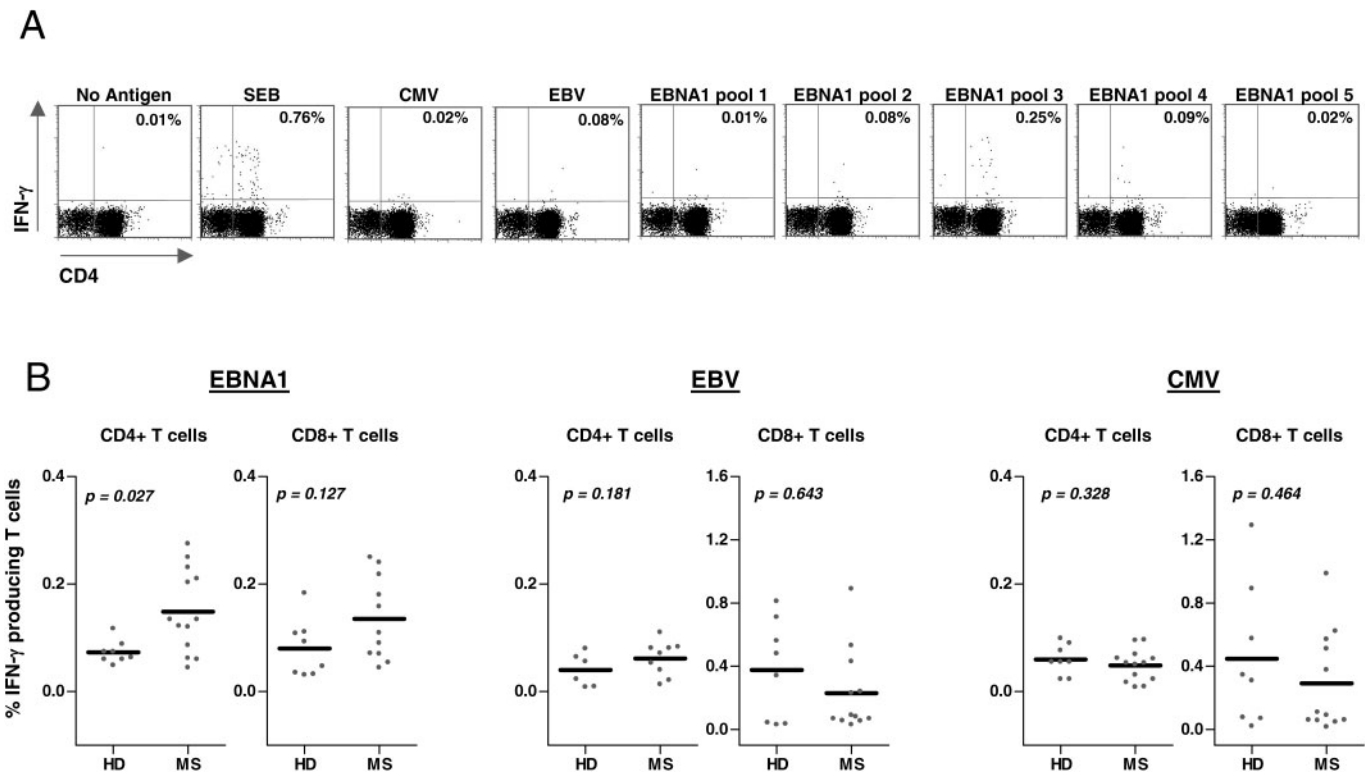

Fig. 6 Confirmation of enhanced $CD4^+$ Th1 responses to EBNA1 but not to other EBV-derived latent and lytic antigens in patients with multiple sclerosis by a short-term flow cytometry-based intracellular cytokine staining assay. (**A**) Depicted is an analysis of $CD3^+$ gated $CD4^+$ and $CD8^+$ T cells following a 6 h stimulation of PBMC with SEB, EBNA1 peptide pools (10 pools were combined to 5 pools in these experiments), other pooled T-cell epitopes of latent and lytic EBV antigens (derived from EBNA3A, EBNA3B, EBNA3C, BZLF1, BRLF1 and BMLF1) and CMV pp65 epitopes. This patient shows a positive response of IFN-γ-producing $CD4^+$ T cell to EBNA pool 3 (499–548). (**B**) Frequency of EBNA1-, other EBV latent and lytic antigen- and CMV-specifc $CD4^+$ and $CD8^+$ T cells in 16 patients with multiple sclerosis and 12 HD. Consistent with our findings achieved with the proliferation assay, patients showed significantly higher frequencies of EBNA1-specific and IFN-γ-producing $CD4^+$ T cells. Dots represent the cumulative frequency of positive responses to all EBNA1 peptide pools per patient. $CD8^+$ T-cell responses to EBNA1 tended also to be higher in the patient's group, but these differences did not reach statistical significance. In contrast to EBNA1, T-cell responses to other immunodominant EBV antigens and pp65 CMV epitopes did not differ between both groups, indicating a distinct role for EBNA1-specific T-cell responses in multiple sclerosis.

T cells specific for the other EBV antigens and the CMV epitopes did not differ significantly between the two groups (mean frequencies for EBV-specific $CD4^+$ T cells: 0.061% versus 0.040%, and for $CD8^+$ lymphocytes, 0.230% versus 0.376%; mean frequencies of CMV-specific $CD4^+$ T cells of 0.048% versus 0.060% and 0.292% versus 0.447% for CMV-specific $CD8^+$ cells in patients versus controls, respectively). The frequency of EBV and CMV-specific T-cell responses in our cohorts is consistent with previously reported results achieved in healthy virus carriers (Tan *et al.*, 1999).

Thus, in contrast to EBNA1, T-cell responses to three other latent and three other lytic immunodominant EBV antigens and pp65 CMV epitopes did not differ between both groups, indicating a distinct role for EBNA1-specific T-cell responses in multiple sclerosis.

EBV viral loads are not increased in patients with multiple sclerosis

Finally, we investigated whether the enhanced EBNA1-specific T-cell response in multiple sclerosis was associated with an increase in EBV viral loads. To this end, we quantified EBV DNA copy numbers in PBMC by real-time PCR in a cross-sectional analysis using bcl-2 DNA to determine copy number per genome. Although some patients showed elevated EBV copy numbers, the overall difference between the two cohorts was not statistically significant (mean ± SEM: 163.6 ± 82.5 for multiple sclerosis versus 65.6 ± 17.1 for HD per 10^6 PBMC, respectively; $P = 0.8247$) (Fig. 7). Thus, although the mechanisms of EBV immunosurveillance differ between patients and healthy virus carriers, we did not find any evidence for a deficient control of EBV infection in patients with multiple sclerosis.

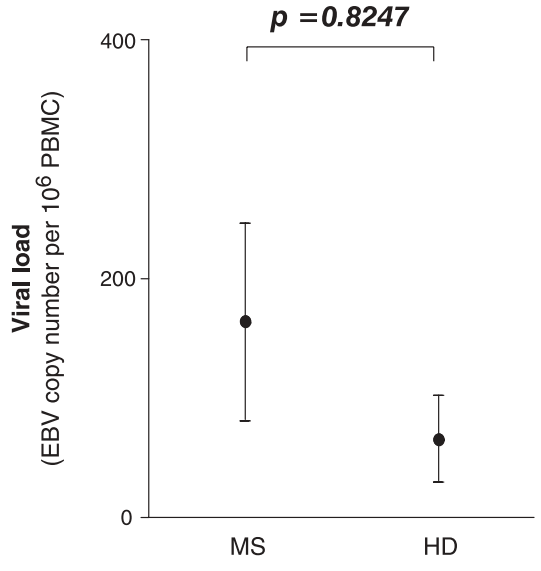

Fig. 7 EBV viral loads from patients with multiple sclerosis and healthy controls. EBV DNA was quantified from PBMC by quantitative real-time PCR in relation to bcl-2 expression. All samples were tested in triplicates. Symbol and bars represent mean and SEM values.

Discussion

Large prospective epidemiological studies consistently demonstrate that patients with multiple sclerosis are universally, that is, close to 100%, infected with EBV (Ascherio *et al.*, 2001) and that the risk of developing multiple sclerosis in healthy individuals increases with the level of EBV-specific and, in particular, EBNA1-specific antibody titers (Sundstrom *et al.*, 2004; Levin *et al.*, 2005). EBNA1-specific $CD4^+$ T cells are a crucial component of the EBV-specific immune control in healthy virus carriers (Munz, 2004). In this study, we found a qualitatively and quantitatively distinct EBNA1-specific T-cell repertoire in patients with multiple sclerosis, who exhibited a substantially increased frequency, reactivity, functional avidity and enhanced IFN-γ production of EBNA1-targeting $CD4^+$ T cells despite normal viral loads.

Strengthening the *in vivo* relevance of our findings, EBNA1-specific $CD4^+$ T cells almost exclusively originated from the memory pool. As expected for subjects who were also exposed to the virus, TCL derived from HD contained also >90% EBNA1-specific $CD4^+$ $CD45RA^-RO^+$ T cells. The increased number of positive cultures derived from multiple sclerosis patients, however, demonstrates that this population accounts for the differential response pattern in patients versus controls. In our primary proliferation assay, the frequency of EBNA1-specific $CD8^+$ T cells was significantly lower than the frequency of EBNA1-specific $CD4^+$ cells in both patients and controls. The difference in frequencies of Flu-HA-specific $CD4^+$ and $CD8^+$ cells was minor when compared with EBNA1-specific lymphocytes. EBNA1 was previously thought to be immunologically invisible for $CD8^+$ T cells since its glycine–alanine (GA) repeat domain was reported to prevent antigen processing by the proteasome and thereby recognition by cytotoxic $CD8^+$ cells (Blake *et al.*, 1997). However, recent studies that employ sensitive IFN-γ detection assays instead of cytotoxicity as readouts demonstrate that B-LCL recognition by autologous EBNA1-specific $CD8^+$ cells does occur and suggest that the $CD8^+$ T-cell epitopes of EBNA1 can be separated from the GA domain by certain serine proteases, making primarily the defective ribosomal products (DRiPs) of EBNA1 accessible to proteasomal degradation and MHC class I presentation (Voo *et al.*, 2004). Therefore, we additionally analysed $CD4^+$ and $CD8^+$ T-cell reactivity by a short-term *ex vivo* intracellular cytokine staining assay. Data from these assays confirmed enhanced $CD4^+$ Th1 responses to EBNA1 shortly after activation of polarized effector cells. Furthermore, we could indeed detect low-frequency $CD8^+$ T-cell responses to EBNA1, which tended to be higher in the patients' group, but these differences did not reach an overall statistical significance. By comparison, there was no increase in the responses of $CD4^+$ and $CD8^+$ T cells specific for other latent and lytic EBV and HCMV antigens, which altogether indicates a distinct role for EBNA1-specific $CD4^+$ T-cell responses in multiple sclerosis.

Using an overlapping set of peptides covering the entire C-terminal domain of EBNA1, we identified EBNA1 (amino acids 473–508) as the preferentially recognized region containing multiple immunodominant epitopes in healthy EBV carriers in the context of HLA-DR2/DR4. In recent studies, using an enzyme linked immunospot assay (ELISPOT) assay for IFN-γ release, CD8-depleted PBMCs from healthy EBV seropositive individuals not matched for HLA alleles stimulated with overlapping peptide pools covering the whole EBNA1-sequence also detected frequent recognition of the EBNA1 (amino acids 475–489) and EBNA1 (amino acids 485–499) peptides (Leen *et al.*, 2001; Long *et al.*, 2005). In patients with multiple sclerosis, we found that the EBNA1-specific T-cell response was not focused on a distinct immunodominant region, but covered virtually the entire C-terminal domain of EBNA1 (amino acids 400–641) with comparable frequencies of responding T cells, although the frequency of T cells specific for EBNA1 (amino acids 473–508) was preserved.

Dendritic cells (DCs) are thought to prime EBNA1-specific $CD4^+$ T-cell immunity by presentation of dead virus-transformed B cells (Munz *et al.*, 2000; Subklewe *et al.*, 2001; Bickham *et al.*, 2003) and could have mediated the intramolecular determinant spreading in patients with multiple sclerosis. In the neurotropic Theiler's virus-induced animal model of multiple sclerosis, epitope spreading from viral to myelin antigens (Miller *et al.*, 1997) and molecular mimicry mechanisms between foreign and self epitopes (Olson *et al.*, 2001) are important steps in the initiation of autoimmune demyelinating disease. At the B-cell level, EBNA1-specific IgG antibodies were very recently demonstrated to cross-react with autoantigenic epitopes derived from the ribonucleoproteins 60 kDa Ro and 25 kDa Sm in early stages of systemic lupus erythematosus (Sundar *et al.*, 2004; McClain *et al.*, 2005) and susceptible animals immunized with both target epitopes Ro (amino acids 169–180) and EBNA1 (amino acids 58–72) equally acquired clinical symptoms of lupus (McClain *et al.*, 2005). In multiple sclerosis, a similar scenario was suggested since EBNA1-specific antibodies in the CSF occur more frequently in multiple sclerosis patients compared with controls and two pentapeptide identities between EBNA1 and myelin basic protein (MBP) were identified in the same study (Bray *et al.*, 1992).

None of the EBNA1-specific TCL produced detectable amounts of IL-4, whereas stimulation with EBNA1-antigens elicited strong IFN-γ responses in the vast majority of T cells. Cytotoxicity of EBNA1-specific $CD4^+$ T cells is restricted to IFN-γ producers (Bickham *et al.*, 2001). TCL derived from patients with multiple sclerosis produced substantially higher amounts of IFN-γ. Moreover, patients with multiple sclerosis did not show increased EBV viral loads. Although we cannot exclude that a particular subgroup of patients might carry higher EBV copy numbers, our findings do not argue for an insufficient immune control of EBV in multiple sclerosis as it has been suggested for other autoimmune diseases. Patients with systemic lupus erythematosus (SLE) are reported to have an up to 40-fold increased EBV copy numbers (Kang *et al.*, 2004) and patients with rheumatoid arthritis an ~10-fold (Balandraud *et al.*, 2003) increase of EBV viral loads in PBMC. On the contrary, we suggest that (i) a loss of functional suppression at the T-cell level and/or (ii) an abnormal regulation of EBV infection in the B-cell compartment potentially contributed to the distinct characteristics of EBNA1-specifc T cells in multiple sclerosis. $CD4^+CD25hi$ regulatory T cells (Treg) are reported to be decreased in frequency and impaired in function in patients with multiple sclerosis (Viglietta *et al.*, 2004). *In vitro* peptide stimulation with EBNA1 (amino acids 561–573) and EBNA1 (amino acids 607–619) has lately been shown to elicit both $CD4^+$ T helper as well as Treg cell responses (Voo *et al.*, 2005). The latter expressed CD25, the glucocorticoid-induced tumor necrosis factor receptor-related protein, Forkhead Box P3, and were capable of suppressing EBNA1-effector T cells exhibiting the same antigen-specificity. Tregs could thus predominantly neutralize effector memory cells specific for epitopes different from EBNA1 (amino acids 473–508) in healthy individuals, thereby preventing potentially cross-reactive and harmful effector T-cell responses, and potentially fail to do so in multiple sclerosis. Alternatively, a more effective EBNA1-specific T-cell response might be required and secondary to an increased antigenic challenge and abnormal regulation of EBV infection at the B-cell level. Increased frequencies of EBV-infected blood cells quantified by a limiting dilution PCR, and aberrant expression of viral lytic and latency genes were reported to occur in patients with SLE (Gross *et al.*, 2005). Both scenarios—loss of functional suppression and abnormal EBV regulation—eventually lead to a circle of constant antigenic challenge and immune activation. Repeated stimulation with increased concentrations of high-affinity TCR ligands was shown to mediate memory Th1 phenotype differentiation in naïve T cells (Constant *et al.*, 1995; Brogdon *et al.*, 2002). Since memory T cells have less stringent co-stimulatory requirements (Holzer *et al.*, 2003), we suggest that the accumulation of highly antigen-sensitive EBNA1-specific memory Th1 cells in multiple sclerosis possibly sustains autoimmunity by cross-recognition of CNS autoantigens or by TCR-independent bystander activation mechanisms. In line with this hypothesis are the strong and highly significant association between EBNA1-IgG titers and the risk of developing multiple sclerosis (Sundstrom *et al.*, 2004; Levin *et al.*, 2005) as well as the correlation between disease activity and EBV reactivation/viremia in patients followed over a period of 1 year (Wandinger *et al.*, 2000).

The results of our study demonstrate that the EBNA1-mediated immunological control of latent EBV infection is deregulated at the T-cell level in patients with multiple sclerosis. EBNA1 is currently being investigated as a promising key antigen for T-cell-mediated immunotherapeutic approaches in EBV-associated malignancies (Munz, 2004). We suggest that strategies aiming at modulating the enhanced $CD4^+$ T-cell response associated with EBV persistence might

also have therapeutic merit in the prevention and treatment of multiple sclerosis and other autoimmune diseases.

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