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Frequency analysis of HLA-B7-restricted Epstein-Barr virus-specific cytotoxic T lymphocytes in patients with multiple sclerosis and healthy controls

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Abstract

The Epstein-Barr virus (EBV) has been implicated in the pathogenesis of multiple sclerosis (MS), however, the mechanisms by which EBV may be involved in MS are unknown. We here have investigated the frequency of EBV-specific cytotoxic T lymphocytes (CTL) in human leukocyte antigen (HLA)-B7$^+$ patients with MS and healthy controls using enzyme-linked immunospot assays and seven previously characterized HLA-B7-restricted immunogenic EBV peptides. Overall, there were no significant differences in the frequency of EBV-specific CTL between both groups. These data do not support the hypothesis that EBV could play a role in MS by inducing quantitatively altered EBV-specific CTL responses. Other pathogenic mechanisms for EBV in MS remain to be elucidated.

Keywords: Cytotoxic T cells; Elispot; Epstein-Barr virus; Multiple sclerosis; HLA-B7

1. Introduction

Although the etiology of multiple sclerosis (MS), a chronic inflammatory demyelinating disease of the central nervous system (CNS), is as yet unknown, it is widely accepted that MS results from a complex interplay of environmental and genetic factors, triggering a predominantly immune-mediated injury of CNS structures (Noseworthy, 1999). In terms of environmental factors, viruses have repeatedly been implicated in the pathogenesis of MS (Cook et al., 1995; Dalgleish, 1997; Simmons, 2001). Among these, the Epstein-Barr virus (EBV), a human γ-herpesvirus with a marked tropism for B lymphocytes, may play a unique role.

Numerous case-control studies have consistently shown that virtually all patients with MS are seropositive for EBV, compared to an EBV-seroprevalence in the general population of about 90%. This translates into a greater than 10-fold higher risk of developing MS for EBV-infected individuals than for EBV-seronegative individuals (Ascherio and Munch, 2000). Furthermore, serum and cerebrospinal fluid (CSF) antibody titers against certain EBV proteins are increased in patients with MS (Bray et al., 1992; Cepok et al., 2005; Larsen et al., 1985). Of note, four independent studies on serum samples collected before the onset of MS have unanimously demonstrated elevated antibody titers to the EBV nuclear antigen-1 (EBNA1) already several years before the first clinical manifestation of the disease (Ascherio

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et al., 2001; Delorenze et al., 2006; Levin et al., 2005; Sundstrom et al., 2004), leading to the assumption that "past EBV infection is a prerequisite for the acquisition of MS" (Sundstrom et al., 2004). Nevertheless, despite the strong relationship between MS and EBV infection, the pathogenic mechanism(s) by which EBV may be involved in this disease are unknown.

Following primary infection, EBV establishes a latent infection of B lymphocytes resulting in a life-long virus carrier state. Whereas the primary and persistent phases of EBV infection are associated with various antibody reactivities to lytic and latent antigens, evidence suggests that cell-mediated immune responses, in particular those by $CD8^+$ cytotoxic T lymphocytes (CTL), are more important for the control of EBV (Rickinson and Moss, 1997). CTL combat viral infections by recognizing peptides (typically of 9–10 amino acids) produced by proteosomal cleavage of endogenously expressed viral proteins and presented as a complex with human leukocyte antigen (HLA) class I molecules on virus-infected cells (Pamer and Cresswell, 1998). As binding and presentation of such peptides is governed by the specific affinity of the peptide binding groove of the different HLA class I molecules to certain amino acids in the peptide, the choice of a peptide epitope is highly HLA class I allele-specific. Notably, some HLA alleles are associated with presumed autoimmune diseases, e.g. the frequency of the HLA-B7 allele is elevated in patients with MS (Fogdell-Hahn et al., 2000).

CTL have lately gained increasing attention in MS. Besides macrophages, they are the predominant cells in inflammatory MS lesions, outnumbering $CD4^+$ T cells (Bauer et al., 2001). Also, CTL have been demonstrated to undergo clonal expansions in MS lesions (Babbe et al., 2000) and CSF from patients with MS (Jacobsen et al., 2002), while similar clonal expansions were considerably less prevalent in the $CD4^+$ T cell compartment. Although the antigenic target of these clonotypic $CD8^+$ CTL is still enigmatic, $CD8^+$ CTL are currently regarded as important immunologic effector cells in MS (Neumann et al., 2002).

We here hypothesized that one mechanism by which EBV could play a role in MS may involve a quantitatively altered CTL response to EBV antigens in patients with MS. Therefore, we have studied the frequencies of EBV-specific CTL in $HLA\text{-}B7^+$ patients with MS and healthy controls using a panel of seven previously characterized HLA-B7-restricted immunogenic EBV peptides.

2. Materials and methods

2.1. Patients and healthy controls

The study was approved by the ethics committee of the faculty of medicine, University of Würzburg, and participants provided written informed consent. DNA samples from 73 patients with MS and 32 healthy controls (hospital staff) were selected for HLA-B7 genotyping from a local DNA bank collected from patients with MS attending the MS clinic of the Department of Neurology, University of Würzburg, on a regular basis. Clinical data were obtained by review of the medical records. MS was diagnosed according to Poser's criteria (Poser et al., 1983) and categorized as relapsing–remitting (RRMS, $n=48$), secondary progressive (SPMS, $n=15$), or primary progressive MS (PPMS, $n=10$). 22 additional $HLA\text{-}B7^+$ healthy controls were recruited through a database of HLA-typed potential bone-marrow donors, maintained at the Department of Transfusion medicine and Immunohematology, University of Würzburg. A flow-chart of the study is provided in Fig. 1. Enzyme-linked immunospot assay (ELISPOT) data were eventually available for 23 patients with MS and 26 healthy controls. Demographic and clinical characteristics of this population are summarized in Table 1. Patients included in the ELISPOT analysis were either untreated ($n=8$), or treated with Interferon (IFN)-β ($n=6$), glatiramer acetate ($n=5$), or methotrexate ($n=2$). Two patients had received mitoxantrone 3 and 12 months before entering the study. None of the patients had been treated with corticosteroids for at least 2 months before inclusion in the study.

2.2. HLA-B7 genotyping

DNA samples from patients and controls were genotyped for HLA-B7 using an Olerup SSP HLA-B low resolution bulk kit (GenoVision, Vienna, Austria) according to the instructions of the manufacturer. DNA probes were amplified with cyclers from Peqlab Biotechnologie and from Biozym Technik GmbH, and PCR products were visualized by 2% agarose gel electrophoresis with ethidium bromide.

2.3. ELISPOT

About 40 ml of blood was drawn from study subjects by peripheral venous puncture using EDTA-coated tubes. Peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep (Axis Shield, Oslo, Norway) gradient centrifugation. After isolation, cells were washed once in phosphate buffered saline (PBS, Biochrome AG, Berlin, Germany) before further use. ELISPOT experiments were performed with a Mabtech (Nacka Strand, Sweden) IFN-γ ELISPOT-kit according to the instructions of the manufacturer with minor modifications. Multiscreen MAIP 96-well plates (Millipore, Bedford, MA, USA) were coated overnight with 15 μg/ml primary anti IFN-γ antibody. Membranes were then blocked with 1% bovine serum albumine (BSA, Sigma-Aldrich, Munich, Germany) in PBS for 1 h at room temperature (RT) and washed three times with PBS. PBMC were added at 250,000, 125,000, and 62,500 cells per well in a volume of 100 μl of cell culture medium containing RPMI 1640 (Biochrome, Berlin, Germany), 100 U/ml penicilline/streptomycine (Sigma-Aldrich, Munich, Germany), and 5% fetal calf serum (Biochrome AG, Berlin, Germany). Immediately afterwards, 100 μl of the appropriate

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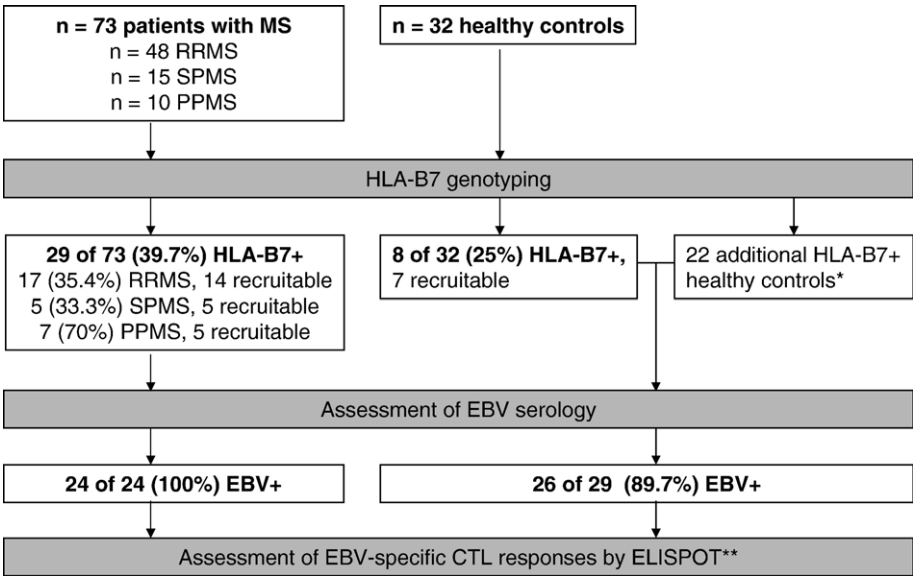

Fig. 1. Flow-chart of the study and results of HLA-B7 genotyping and EBV serology in patients with MS and healthy controls. The frequency of HLA-B7 ($p=0.19$, Fisher's exact test) and seropositivity for EBV ($p=0.24$, Fisher's exact test) were not significantly different between patients and controls. HLA-B7 was significantly more frequent ($p=0.045$, Fisher's exact test) in PPMS patients than in RRMS and SPMS patients. * The 22 additional controls were recruited through a database of HLA-typed potential bone marrow donors. ** One of the 24 patients (RRMS) could not be evaluated for technical reasons.

peptide or antigen diluted in cell culture medium was added to duplicate wells. Peptides were used at 10 μg/ml, as determined in set-up titration experiments (data not shown). Plates were incubated at 37 °C and 5% CO_2 in a humidified incubator for 20 h. In set-up experiments, longer incubation periods did not result in higher sensitivity (data not shown). Cells were then discarded and plates washed five times with PBS. A secondary biotinylated anti-IFN-γ antibody was added at 1 μg/ml and incubated for 2 h at RT. Plates were washed three times and streptavidine-alkaline phosphatase conjugate was added for 1 h at RT. After washing three times, spots were visualized by 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue-tetrazolium (alkaline phosphatase conjugate substrate kit, Bio-Rad, Richmond, CA, USA). After 10 min of incubation, development was stopped with tab water. Plates were sent to CTL ELISPOT scanning services (CTL, Schwäbisch Gmünd, Germany) for scanning, and spots were enumerated using the CTL Immunospot 2 software. In pilot experiments, results calculated from the mean spot number of two samples with 250,000 cells per well highly correlated ($r^2=0.99$, $p<0.0001$) with results obtained by linear regression using the smallest sum of squares method from the means of duplicates from three different cell concentrations (250,000, 125,000, and 62,500 cells per well). Therefore, values were calculated from duplicates of 250,000 cells per well in all further experiments. Frequencies of antigen/peptide-specific CTL were calculated by subtracting the mean spot number of unstimulated control wells from the mean spot number of peptide-stimulated wells. Results are expressed as IFN-γ spots per 10^6 PBMC. CTL responses to a given peptide were considered positive when after subtraction of the spot number in control wells the spot number in peptide-stimulated wells was ≥ 20 per 10^6 PBMC.

2.4. Synthetic peptides and antigens

HPLC-purified EBV peptides checked by mass spectometry analysis were purchased from NMI-Peptides (Reutlingen, Germany) and dissolved in DMSO. The final DMSO concentration per well was 0.5%. The peptide sequences and the corresponding EBV proteins are listed in Table 2. The 7 EBV-specific peptides selected did not show homologies with other viral peptide sequences when analyzed in a BLAST search. In particular, no homologies with proteins of other herpesviruses could be identified. The HLA-B7-restricted HIV nef peptide TPGPGVRYPL and medium containing 0.5% DMSO served as negative controls. Phytohemagglutinin (PHA, 5 μg/ml; Sigma-Aldrich, Munich, Germany) and tetanus toxoid (20 LF/ml; kindly provided by Chiron Behring, Marburg, Germany) were used as positive controls.

2.5. EBV serology

After gradient centrifugation during PBMC isolation, the upper layer consisting of plasma diluted 1:1 in PBS was collected. Samples were stored at −80 °C and subsequently tested at the Institute of Virology and Immunobiology, University of Würzburg, for the presence of EBV-IgG antibodies by enzyme immunoassay using the Enzygnost Anti-EBV-IgG test kit (Dade Behring, Marburg, Germany). In this test, microwell pates are coated with a mixture of EBV antigens, including viral capsid (VCA), nuclear and early antigens. Samples with borderline EBV-IgG results by

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Table 1
Demographic and clinical characteristics of the study population

	Patients with MS ($n=23$)	Healthy controls ($n=26$)	p-value
Female/Male	15/8	13/13	0.39 (Fisher's exact test)
Age (median, range)	39, 25–54	36.5, 19–61	0.54 (unpaired t test)
EDSS[a] (median, range)	4, 1–8.5	n.a.	n.a.

EDSS = extended disability status scale.
n.a. = not applicable.
[a] For one patient EDSS was not available.

immunoassay were further tested for the presence of antibodies against VCA by an immunofluorescence assay using P3HR1 cells.

2.6. Statistics

Statistical significance of differences was determined using two-tailed Fisher's exact probability and unpaired t tests where appropriate. Calculations were performed using graph pad prism 3.0 software and VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). p-values below 0.05 were considered statistically significant.

3. Results

3.1. HLA-B7 genotyping and EBV serology in patients with MS and healthy controls

As expected, HLA-B7 was more common in patients with MS (39.7%) than in controls (25%; Fig. 1) (Fogdell-Hahn et al., 2000), but this difference was not statistically significant ($p=0.19$, Fisher's exact test). Of note, the HLA-B7 frequency was significantly higher in PPMS (70%) than in RRMS and SPMS (~35%; $p=0.045$, Fisher's exact test). Nevertheless, given the rather small number of PPMS patients included, these data should be considered preliminary. In keeping with previous case-control studies (Ascherio and Munch, 2000), all patients with MS had antibodies against EBV, whereas only 89.7% of healthy controls were EBV-seropositive. This finding did, however, not reach statistical significance ($p=0.24$, Fisher's exact test).

3.2. Frequencies of CTL responses to HLA-B7-restricted EBV peptides do not significantly differ between patients with MS and healthy controls

ELISPOT results could be calculated for 13 patients with RRMS (one of the 14 RRMS patients tested could not be evaluated for technical reasons), 5 patients with SPMS, 5 patients with PPMS, and 26 healthy controls, all of them HLA-B7$^+$ and EBV$^+$ (Fig. 1). There were more women in the MS group and patients tended to be older than controls, but both differences were not significant (Table 1).

HLA-B7-restricted EBV-specific CTL responses in both, patients and controls, were highly focussed on the EBNA3A-derived peptide RPPIFIRRL (Table 2). Still, the number of individuals with positive CTL responses against RPPIFIRRL did not significantly differ ($p=0.51$, Fisher's exact test) between the patient (16/23) and the control group (21/26). Also, the absolute frequencies of RPPIFIRRL-specific CTL in patients with MS (68.5+17 spots/10^6 PBMC, mean+SEM) versus healthy controls (94.9+23.8 spots/10^6 PBMC) were not significantly different ($p=0.38$, unpaired t test; Fig. 2A). Analysis of RPPIFIRRL-specific CTL with respect to the clinical course of MS patients revealed a non-significant tendency for higher RPPIFIRRL responses in PPMS compared to RRMS and SPMS (Fig. 2B). There was no relationship between RPPIFIRRL responses and the extended disability status scale (EDSS) score ($r^2=0.002$, $p=0.85$) or the treatment status (treated vs. untreated, $p=0.88$ unpaired t test) of patients with MS. In patients and controls, RPPIFIRRL responses did not significantly differ between males and females ($p=0.31$, unpaired t test) and there was no correlation of RPPIFIRRL responses with age ($r^2=0.074$, $p=0.06$).

In control experiments, PBMC isolated from either EBV$^+$, HLA-B7$^-$ ($n=2$) or EBV$^-$, HLA-B7$^+$ ($n=4$) donors that were stimulated with RPPIFIRRL did not show IFN-γ responses that exceeded those of unstimulated control wells. Representative examples of IFN-γ responses to RPPIFIRRL are shown in Fig. 2C.

Additionally, there were minor responses towards the EBNA1-derived peptide IPQCRLTPL in patients with MS (25+1.7 spots/10^6 PBMC) and one healthy control (48 spots/10^6 PBMC). Although patients with MS showed more

Table 2
Sequences of EBV peptides (all HLA-B7-restricted), corresponding EBV proteins, and number of individuals in the patient and control group with positive CTL responses to individual EBV-peptides

EBV Peptide	EBV Protein	Reference	CTL responses (positives/total number tested)		
			MS	Controls	p-value[a]
RPPIFIRRL	EBNA3A	(Hill et al., 1995)	16/23	21/26	0.51
VPAPAGPIV	EBNA3A	(Rickinson and Moss, 1997)	0/20	0/20	n.a.
QPRAPIRPI	EBNA3C	(Hill et al., 1995)	0/20	1/22	1
LPRAWLQRL	BBLF2/3	(Turcanova and Hollsberg, 2004)	0/20	0/20	n.a.
LPCVLWPVL	BZLF1	(Stuber et al., 1995)	0/20	1/22	1
RPQKRPSCI	EBNA1	(Blake et al., 2000)	0/20	0/22	n.a.
IPQCRLTPL	EBNA1	(Blake et al., 2000)	4/20	1/22	0.18

CTL responses were considered positive when the mean spot number in peptide-stimulated wells minus the mean spot number from control wells was ≥20 per 10^6 PBMC. n.a. = not applicable.
[a] Fisher's exact test.

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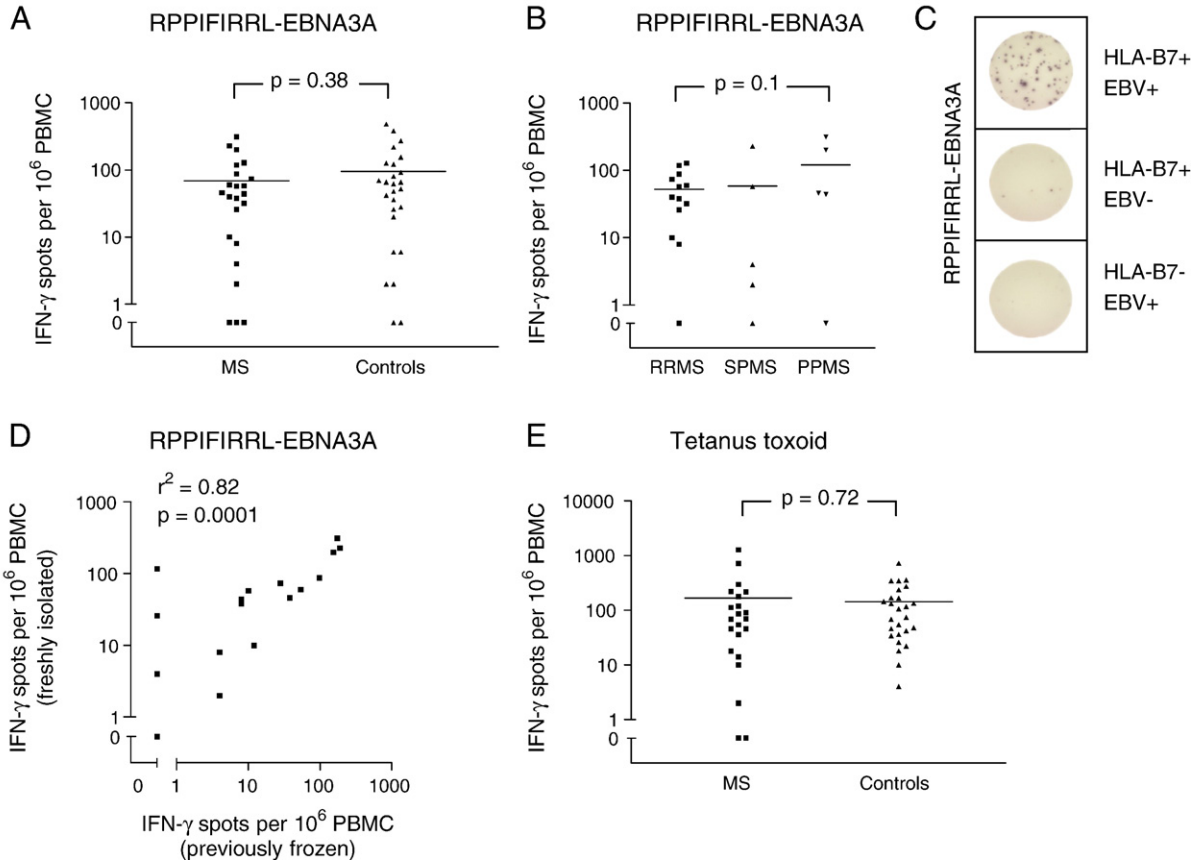

Fig. 2. (A) Frequency of CTL directed against the HLA-B7-restricted EBNA3A peptide RPPIFIRRL in HLA-B7$^+$, EBV$^+$ patients with MS (n=23) and healthy controls (n=26). The difference between both groups was not significant (unpaired t test). (B) Frequency of RPPIFIRRL-specific CTL in the different MS subtypes. RPPIFIRRL-specific responses tended to be higher in PPMS compared to RRMS, however, this was not statistically significant. Also, differences between RRMS and SPMS (p=0.85), and SPMS and PPMS (p=0.42) were not significant (unpaired t test). (C) Representative examples of IFN-γ responses to RPPIFIRRL. IFN-γ spots are clearly visible in RPPIFIRRL-stimulated PBMC from a HLA-B7$^+$, EBV$^+$ donor, whereas no relevant responses are detectable in PBMC from HLA-B7$^+$, EBV$^-$, and HLA-B7$^-$, EBV$^+$ individuals. (D) Frequency of RPPIFIRRL-specific CTL responses in freshly isolated and previously cryopreserved (−80°) PBMC from the same donors (n=17). Although responses correlated quite well, the absolute number of spots in previously frozen PBMC samples was on average 1.7 fold lower than in freshly isolated PBMC. (E) Frequency of tetanus toxoid-specific cellular immune responses in patients with MS (n=22) and healthy controls (n=26). No significant difference could be observed (unpaired t test).

frequently positive responses to this peptide (4/20) than controls (1/22), this again did not reach statistical significance (Fisher's exact test p=0.18). The single control that had a positive response to IPQCRLTPL also responded to the BZLF-1 peptide LPCVLWPVL (188 spots/10^6 PBMC), while a different single control showed IFN-γ spots following stimulation with the EBNA3C peptide QPRAPIRPI (30 spots/10^6 PBMC). All remaining EBV peptides tested did not elicit detectable CTL responses (Table 2). As not all EBV peptides could be investigated in the entire study population, Table 2 lists the number of positive CTL responses per total number of individuals analyzed with each EBV peptide. Overall, at least 20 patients with MS and healthy controls were studied with each EBV peptide.

Stimulation with PHA, used as a positive control, always resulted in strongly positive IFN-γ responses that usually were confluent and to numerous to count, while none of the study subjects responded to the HLA-B7-restricted HIV nef peptide TPGPGVRYPL which served as a negative control (data not shown).

3.3. Analysis of RPPIFIRRL responses in freshly isolated and cryopreserved PBMC

During the course of this study, we also compared CTL responses against RPPIFIRRL in freshly isolated and cryopreserved PBMC (stored at −80 °C) from the same donors. As shown in Fig. 2D, although results obtained from fresh and cryopreserved PBMC correlated quite well (r^2=0.82, p<0.0001), the absolute number of spots in previously frozen PBMC samples was on average 1.7 fold lower than in freshly isolated PBMC. Therefore, only freshly isolated PBMC were used in the present work.

3.4. Cellular immune responses to tetanus toxoid are not significantly different between patients with MS and controls

To exclude potential confounding factors for the interpretation of the ELISPOT results on EBV-specific CTL, we wanted to assure that the overall immunologic responsiveness to antigenic stimulation of PBMC from patients with MS

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compared to healthy controls is not intrinsically different. Therefore, PBMC from patients and controls were challenged with the common recall antigen tetanus toxoid, in parallel to stimulation with EBV peptides. Although individual tetanus toxoid responses were relatively variable from subject to subject, there was no significant inter-group difference in IFN-γ responses following tetanus toxoid stimulation ($p = 0.72$, unpaired t test; Fig. 2E), suggesting that the general immunologic responsivenss of the two groups, at least as analyzed by IFN-γ secretion to a common recall antigen, is similar.

4. Discussion

Strong and consistent evidence accumulated over the last 25 years suggests an association of MS with EBV, and the presently available data seem compatible with the view that EBV infection is a necessary, but not sufficient, prerequisite for the development of MS. Still, the mechanisms by which EBV may be involved in MS are currently elusive. In particular, it remains challenging to explain a disease-specific function of an infectious agent that, even if found in virtually 100% of patients with MS, is also present in about 90% of the normal population. A number of possible pathogenic mechanisms for EBV in MS have been proposed, such as an infection of autoreactive B lymphocytes (Pender, 2003), an EBV-induced expression of a putative autoantigen (van Sechel et al., 1999), or that antibodies against viral (i.e. EBV-encoded) interleukin 10 might crossreact with human interleukin 10 (Tenser, 2003). The most frequently evoked scenario, however, is an autoimmune mechanism where an altered T cell-mediated immune response against EBV is thought to lead to a cross-reactive immune attack against CNS antigens, probably on the background of a genetic susceptibility (Bray et al., 1992; Cepok et al., 2005; Lang et al., 2002; Lunemann et al., 2006).

Assuming that an altered cellular immune response against EBV may be reflected in an increased number of EBV-specific CTL, we here have analyzed the frequency of HLA-B7-restricted EBV-specific CTL in patients with MS and healthy controls using IFN-γ ELISPOT assays and seven previously identified HLA-B7-restricted immunogenic EBV peptides. Overall, the results of our study do not demonstrate significant differences, neither in terms of the absolute numbers of EBV-specific CTL, nor in terms of the targeted EBV antigens, between patients with MS and healthy controls.

In contrast to the wealth of data on humoral immune responses (Ascherio and Munch, 2000; Ascherio et al., 2001; Bray et al., 1992; Cepok et al., 2005; Larsen et al., 1985; Levin et al., 2005; Sundstrom et al., 2004), few studies have so far addressed cellular immune responses to EBV in MS (Cepok et al., 2005; Hollsberg et al., 2003; Lunemann et al., 2006). In the work of Hollsberg et al., EBV-specific CTL against five HLA-A2- and one HLA-B7-restricted EBV peptide were investigated by IFN-γ ELISPOT. Frequencies of CTL directed against two of these peptides, HLA-A2/CLGGLLTMV and HLA-B7/RPPIFIRRL, were found to be significantly higher among patients with MS. Although the design of the present work is comparable to that of the study by Hollsberg et al., our findings on RPPIFIRRL-specific CTL do not confirm their results. Similarly, in a very recent report by Lunemann et al., no significant differences in the frequencies of $CD8^+$ CTL against a panel of EBV peptides (including RPPIFIRRL) were found using a short-term *ex vivo* intracellular cytokine staining assay. Possible explanations for the diverging results on RPPIFIRRL-specific CTL could be differences in demographic, genetic, or clinical characteristics of the study populations. Moreover, the frequency of EBV-specific CTL may be affected by further factors, e.g. unrelated viral infections (Clute et al., 2005; Khan et al., 2004; Wakiguchi et al., 1999). Also, the magnitude of CTL responses to EBV peptides presented by a given HLA class I allele can be influenced by other co-expressed HLA class I alleles (Hollsberg, 2002). Inter-group differences in EBV-specific CTL responses to single EBV peptides may, therefore, also be related to the history of previously encountered viruses or different co-expressed HLA class I alleles in the individuals studied.

Circumventing the need for HLA-typing, Cepok et al. have recently analyzed total immune responses of PBMC subsets to autologous EBV-transformed B cell lines by intracellular cytokine staining for IFN-γ. In this study, no differences in EBV-specific immune responses were found in the $CD4^+$ T cell compartment, but there was a non-significant trend for higher responses in the $CD8^+$ T cell compartment, and a significantly higher frequency of EBV-specific $CD8^+$ $CD28^+$ T cells in patients with MS (Cepok et al., 2005), suggesting that MS-associated differences in EBV-specific CTL responses may only be detectable in a subset of the $CD8^+$ population. Because in the present ELISPOT approach no subset-specific analysis of $CD8^+$ T cells could be performed, we cannot exclude that such differences might have escaped detection. Along this line, the data generated by IFN-γ ELISPOT provide information concerning the quantity of CTL responses only, whereas it also seems possible that there may exist disease-associated differences in functional properties of EBV-specific CTL, such as in the status of $CD8^+$ T cell differentiation or activation.

In general, ELISPOT assays detect $CD8^+$ T cells that produce IFN-γ on *in vitro* stimulation with the appropriate exact peptide antigen. Of note, not all of these cells are necessarily cytotoxic, and not all antigen-specific T cells will secrete IFN-γ upon peptide stimulation (Maggi et al., 1994). Alternative approaches to determine the frequency of antigen-specific CTL are intracellular cytokine staining for IFN-γ, which basically relies on the same principle as the ELISPOT assay, or staining with tetrameric MHC-peptide complexes (tetramer staining), which directly detect the expression of specific T cell-receptors on the cell surface. In a comparative study, values of EBV-specific $CD8^+$ T cells obtained from tetramer staining were thus on average 4.4-fold higher than those obtained from ELISPOT assays (Tan et al.,

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1999). Nevertheless, despite the lower sensitivity of the ELISPOT assay, the correlation between both techniques was good (Tan et al., 1999).

In view of the converging data on elevated humoral immune responses to EBNA1 in MS (Bray et al., 1992; Cepok et al., 2005; Levin et al., 2005; Sundstrom et al., 2004), we were especially interested in CTL responses against the two EBNA1 peptides included in our analysis. In accordance with the well-recognized rarity of EBNA1-specific CTL in EBV-seropositive donors (Blake et al., 2000), responses against one of these peptides (IPQCRLTPL) were detectable in only few study subjects, but tended to be more frequent in patients with MS than in healthy controls (Table 2). Because elevated antibody titers against EBNA1 indicate a more severe or more recent primary infection or reactivation of EBV infection (Levin et al., 2005), such phenomena could perhaps also be responsible for the, albeit non-significant, somewhat more frequent CTL responses against EBNA1 in MS.

In sum, our present data do not support the hypothesis that EBV could play a role in MS by inducing quantitatively altered EBV-specific CTL responses in patients with MS as compared to healthy controls. These results obviously do not rule out the possibility that EBV may act in MS by triggering a cross-reactive autoimmune response. Other mechanisms by which EBV may contribute to the pathogenesis of MS are, however, conceivable and remain to be elucidated. Indeed, it has been argued that beyond T cell-mediated immune mechanisms, EBV could also exert its role in MS through the interaction with (infectious) co-factors (Christensen, 2006). These might e.g. be other herpesviruses, a reinfection by a EBV strain different from that originally carried by the host (Delorenze et al., 2006), but also (endogenous) retroviruses (Haahr et al., 1992). In this respect, we have recently suggested that the MS-asociated retroviral element (MSRV) may be a particularly interesting candidate (Ruprecht and Perron, 2005).

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