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Strong EBV-specific CD8+ T-cell response in patients with early multiple sclerosis

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Epstein-Barr virus (EBV) has been associated with multiple sclerosis (MS), however, most studies examining the relationship between the virus and the disease have been based on serologies, and if EBV is linked to MS, CD8+ T cells are likely to be involved as they are important both in MS pathogenesis and in controlling viruses. We hypothesized that valuable information on the link between MS and EBV would be ascertained from the study of frequency and activation levels of EBV-specific CD8+ T cells in different categories of MS patients and control subjects. We investigated EBV-specific cellular immune responses using proliferation and enzyme linked immunospot assays, and humoral immune responses by analysis of anti-EBV antibodies, in a cohort of 164 subjects, including 108 patients with different stages of MS, 35 with other neurological diseases and 21 healthy control subjects. Additionally, the cohort were all tested against cytomegalovirus (CMV), another neurotropic herpes virus not convincingly associated with MS, nor thought to be deleterious to the disease. We corrected all data for age using linear regression analysis over the total cohorts of EBV- and CMV-infected subjects. In the whole cohort, the rate of EBV and CMV infections were 99% and 51%, respectively. The frequency of IFN-γ secreting EBV-specific CD8+ T cells in patients with clinically isolated syndrome (CIS) was significantly higher than that found in patients with relapsing–remitting MS (RR-MS), secondary-progressive MS, primary-progressive MS, patients with other neurological diseases and healthy controls. The shorter the interval between MS onset and our assays, the more intense was the EBV-specific CD8+ T-cell response. Confirming the above results, we found that EBV-specific CD8+ T-cell responses decreased in 12/13 patients with CIS followed prospectively for 1.0 $\pm$ 0.2 years. In contrast, there was no difference between categories for EBV-specific CD4+ T cell, or for CMV-specific CD4+ and CD8+ T-cell responses. Anti-EBV-encoded nuclear antigen-1 (EBNA-1)-specific antibodies correlated with EBV-specific CD8+ T cells in patients with CIS and RR-MS. However, whereas EBV-specific CD8+ T cells were increased the most in early MS, EBNA-1-specific antibodies were increased in early as well as in progressive forms of MS. Our data show high levels of CD8+ T-cell activation against EBV—but not CMV—early in the course of MS, which support the hypothesis that EBV might be associated with the onset of this disease.

Keywords: Epstein-Barr virus; multiple sclerosis; cellular immune response; humoral immune response; CD8+ T cells

Abbreviations: EBV = Epstein-Barr virus; MS = multiple sclerosis; EBNA-1 = EBV-encoded nuclear antigen-1; EA = early antigen; LMP2 = latent membrane protein-2; CMV = cytomegalovirus; CIS = clinically isolated syndrome; RR-MS = relapsing-remitting MS; SP-MS = secondary-progressive MS; PP-MS = primary-progressive MS; MRI = magnetic resonance imaging; IEF = isoelectric focusing; HLA = Human leucocyte antigen; PBMC = peripheral blood mononuclear cells; ICS = intracellular cytokine staining assay; ELISPOT = enzyme linked immunospot; PHA-L = phyto-hemagglutinin lectin; SFC = spot-forming cells; PAs = proliferation assays; SI = stimulation index; cpm = counts per minute; ELISA = enzyme-linked immunosorbent assay; AU = arbitrary units; MBP = myelin basic protein; MOG = myelin oligodendrocyte glycoprotein

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Introduction

The γ-herpesvirus Epstein-Barr virus (EBV) has consistently been associated with multiple sclerosis (MS) (Ascherio and Munger, 2007). Seroepidemiological studies have demonstrated that about 100% of adult MS patients are infected with EBV in contrast to 95.8% of age-matched healthy control subjects and two independent studies found a significantly higher rate of EBV infection (about 88%) in children with MS as compared to healthy age-matched children (about 50%) (Alotaibi *et al.*, 2004; Pohl *et al.*, 2006). Cepok and colleagues detected the presence of anti-EBV-encoded nuclear antigen-1 (EBNA-1) and anti-BRRF2 IgG in the CSF of MS patients, but not of patients with other neurological diseases (Cepok *et al.*, 2005). In addition, meta-analyses have shown that patients suffering from EBV-related infectious mononucleosis carry a higher risk of developing MS—in average 10 years after infection—compared to asymptomatic EBV-infected carriers (Goldacre *et al.*, 2004; Thacker *et al.*, 2006; Ascherio and Munger, 2007).

Elevated titers of anti-EBNA-1-specific IgG have been associated with an increased risk of subsequent development of MS (Ascherio *et al.*, 2001; Sundstrom *et al.*, 2004; Levin *et al.*, 2005; DeLorenze *et al.*, 2006). Others have found that there is a correlation between EBV reactivation—as measured by early antigen (EA)-specific antibodies—and disease activity of MS patients, as estimated by the number of relapses and progression of Kurtzke Expanded Disability Status Scores (Wandinger *et al.*, 2000). Thus, although current knowledge supports an association between EBV and MS, it is not clear whether the virus is in fact a trigger for the disease.

Most studies examining the relationship between EBV and MS have been based on serologies. Studies on the cellular immune response are scarce: EBV latent protein-specific T cells (EBNA-1-specific CD4+ and EBNA-3A- and latent membrane protein-2 (LMP2)-specific CD8+) were detected more often in the blood of MS patients as compared to healthy EBV-infected controls (Hollsberg *et al.*, 2003; Cepok *et al.*, 2005; Lunemann *et al.*, 2006). CD4+ T cells from the CSF of MS patients recognized autologous B cells transformed with EBV (Holmoy and Vartdal, 2004). Yet, if EBV plays a role in the pathogenesis of MS, one has to postulate that EBV-specific CD8+ T cells are likely to be involved. Indeed, these cells are important in controlling viruses infections, often appearing earlier than virus-specific antibodies (Barouch and Letvin, 2001), and increasing rapidly after viral reactivation (Du Pasquier *et al.*, 2004).

In parallel, several elements suggest that CD8+ T cells are involved in MS (Hemmer *et al.*, 2002; Friese and Fugger, 2005). CD8+ T cells are more numerous than CD4+ T cells in MS plaques and they exhibit an oligoclonal expansion which is noticeable in MS plaques (Babbe *et al.*, 2000) and in the CSF of MS patients (Jacobsen *et al.*, 2002). There is an enrichment of highly differentiated (CCR7-) CD8+ T cells in the CSF of patients with early MS (Jilek *et al.*, 2007*b*), suggesting that these cells are activated and antigen-driven.

Since both EBV and CD8+ T cells are involved in MS, we decided to study the EBV-specific CD8+ T cells in MS on a large cohort of patients at different stages of the disease, as well as patients with other neurological diseases and healthy subjects. To avoid a bias, we performed a comprehensive study of the virus-specific cellular immune response in this cohort, testing for CD4+ as well as for CD8+ T cells. In order to discriminate between a specific response against EBV and an aspecific immune hyperactivation phenomenon in relation with MS, we tested, in the same cohort, the responses against cytomegalovirus (CMV), which, like EBV, is a herpes virus that can establish latent infections, and is neurotropic. However, contrary to EBV, it has neither convincingly been associated with MS, nor is thought to play a deleterious role in the disease (Ascherio *et al.*, 2001; Buljevac *et al.*, 2005).

Material and Methods

Patients and study subjects

Since June 2004, we enrolled a total of 164 study subjects, including 35 patients with a clinically isolated syndrome (CIS), 31 patients with relapsing–remitting MS (RR-MS), 24 with secondary-progressive MS (SP-MS), 18 with primary-progressive MS (PP-MS), 35 patients with other neurological diseases and 21 healthy control subjects. Patients with MS and those with other neurological diseases were treated in our Department of Neurology. They were enrolled consecutively and were followed up clinically to December 31, 2007. This study was accepted by our institution's ethical commission and all subjects gave their written consent according to review board guidelines. At the time of enrolment, the diagnosis of MS was made using the criteria of McDonald (McDonald *et al.*, 2001; Polman *et al.*, 2005).

CIS was defined by the presence of: a first neurological event consistent with MS; compatible brain/spinal magnetic resonance imaging (MRI); compatible CSF; exclusion of an alternate diagnosis. To rule out other neurological disease than MS, all CIS patients had complete blood count, electrolytes, kidney and renal function, erythrocyte sedimentation rate, C-reactive protein, TSH, B12 and folates, serology for syphilis, Lyme disease and HIV, immunological markers (anti-nuclear antibody, rheumatoid factor, anti-nucleoprotein antibodies and anti-neutrophil cytoplasmic antibodies and protein electrophoresis). CIS patients also had a CSF evaluation including leukocyte count and differentiation, glucose level and blood-brain barrier function. To detect the presence of a putative intrathecal synthesis of oligoclonal IgG, we performed isoelectric focusing (IEF) on the CSF and homologous serum on agarose gels followed by immunoblotting. If an infectious disease of the CNS was suspected, microbial serologies and PCR were performed in the blood and the CSF to rule it out.

Brain MRIs were performed in all CIS patients, and spinal cord MRI or visual evoked potentials in selected cases. During follow-up of CIS patients, MS (RR form) was confirmed in 18/35. However, since at the time of our assays, these 18 patients presented with a CIS, they were kept in the CIS category. Of the 17 remaining CIS patients, 10/15 had an intrathecal synthesis of

Table 1 Clinical data of the 164 patients enrolled

	Age at blood draw in years[a]	Delay between disease onset and study entrance in years[a,b]	EDSS score[a]	Patients in relapse	Number of MS diagnosis subsequently confirmed
Inflammatory MS ($n = 66$)					
CIS (35)	39 ± 14	0.4 ± 1.2	2 ± 0.8	9	18 (follow-up 2.4 ± 0.9 years)
RR-MS (31)	41 ±7	8.2 ± 6.7	2.5 ± 0.8	19	n/a
Progressive MS ($n = 42$)					
SP-MS (24)	57 ± 16	14.9 ± 14.3	6 ± 1.9	2	n/a
PP-MS (18)	55 ± 7	5.6 ± 5.4	3 ± 0.8	0	n/a
Control subjects ($n = 56$)					
OND (35)	39 ± 20	0.4 ± 1.0	n/a	n/a	n/a
HC (21)	35 ± 10	n/a	n/a	n/a	n/a

[a]Numbers represent the median ± inter-quartile range. [b]Study entrance corresponded to the diagnostic procedure including drawing of blood sample. OND = other neurological diseases. HC = healthy controls.

IgG (two missed the LP), 13/17 displayed typical MRI lesions of MS and eight fulfilled Barkhof criteria (Barkhof *et al.*, 1997). The five patients without intrathecal synthesis of IgG had brain lesions typical of MS. Out of 35 control patients with other neurological diseases, 25 had a lumbar puncture, which was inflammatory in four ($\geqslant 5$ cells/μl).

Inflammatory mechanisms predominate in the pathogenesis of CIS and RR-MS, whereas neuro-degenerative ones are more important in SP- and PP-MS (Noseworthy *et al.*, 2000; Vukusic and Confavreux, 2003). Thus, we analysed some of our results by grouping CIS and RR-MS patients on one side and SP- and PP-MS on the other side, under the names of 'inflammatory MS' and 'progressive MS', respectively.

None of the study subjects had received corticosteroids within the 3 months prior to enrolment. Out of the 31 RR-MS patients, 10 were on interferon-β treatment and 9 of these 10 patients were relapsing at the time of the assay. One SP-MS was on interferon-β treatment and relapsing at the time of the assay. Patients were considered as relapsing if a relapse had started <4 weeks prior to the blood sample draw. Human leucocyte antigen (HLA) class I (A and B) typing was performed in 126/164 (77%) study subjects. Clinical data of our cohort of 164 study subjects are given in Table 1.

Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated by density gradient centrifugation on Ficoll-Hypaque (Amersham Biosciences, Otelfingen, Switzerland) and either resuspended in RPMI (Invitrogen, Basel, Switzerland) for immediate use or frozen in freezing medium, as composed of 90% fetal calf serum (FCS; Invitrogen) and 10% dimethylsulfoxide (Sigma, Buchs, Switzerland), and stored in liquid nitrogen for further analysis.

Virus-specific cellular immune response: distinction between virus-specific CD4+ and CD8+ T-cell responses

To elicit virus-specific CD4+ or CD8+ T-cell responses, PBMC were stimulated with purified viral lysates (ABI Inc., Columbia, MD, USA) or pools of known immunodominant eight- to fifteen-mer peptide viral epitopes (SynPep Corporation, Dublin, CA, USA), respectively. For EBV, there were 18 nine- or ten-mer peptide epitopes, known to elicit CD8+ T cells, restricted by 10 HLA class I alleles, and including epitopes of lytic as well as latent proteins. For CMV, there were 34 eight- to fifteen-mer peptide epitopes, known to elicit CD8+ T cells, restricted by 13 HLA class I alleles, including epitopes of the pp65, pp150 and IE1 proteins (Supplementary Table 1).

To verify that viral lysates and CD8+-restricted T-cell peptide epitopes indeed elicited CD4+ and CD8+ T cells, respectively, we performed intracellular cytokine staining assay (ICS) in a subset of 24 study subjects, including eight patients with inflammatory MS (CIS or RR-MS); eight with progressive MS (SP- or PP-MS); four with other neurological diseases and four healthy controls. Two million cells were resuspended in RPMI-10% fetal calf serum (FCS), stimulated for 18 h with viral lysate or viral CD8+-restricted peptide epitope pools at 1 μg/ml and stained with CD3-FITC, CD4-PerCP-Cy5.5, CD8-Pacific Blue and IFN-γ -APC (all from Becton Dickinson, Franklin Lakes, NJ, USA). Data were acquired on a LSRII flow cytometer and analysed using FlowJo Software (Tree Star Inc., Ashland, OR, USA).

Detection of viral-specific interferon-γ-secreting T cells

To detect EBV- or CMV-specific activated T cells secreting IFN-γ, we used an enzyme linked immunospot (ELISPOT) assay, such as we and others have previously described it (Dutoit *et al.*, 2005; Jilek *et al.*, 2007*a*). Briefly, 200 000 PBMC were incubated in quadruplicates in the presence of viral lysates or pools of viral CD8+-restricted peptide epitopes at a concentration of 1 μg/ml. Peptide-free medium and phyto-hemagglutinin lectin (PHA-L) (5 μg/ml) served as negative and positive controls, respectively. Responses were expressed as net spot-forming cells (SFC) per 10^6 PBMC. The assay was considered experimentally valid if the SFC, in the absence of peptide, was lower than 50 per 10^6 cells and if, in the presence of peptide, the SFC was higher that 50 per 10^6 cells and at least 3-fold higher than the background levels. Background values corresponding to medium-stimulated PBMC were subtracted from the data before analysis.

Detection of virus-specific proliferating T cells

To determine the presence of T cells proliferating specifically against a given viral antigen, we performed proliferation assays (PAs) (Jilek *et al.*, 2007*a*). Briefly, upon isolation, 200 000 PBMC were plated in quadruplicate in 96-well plates (Corning Life Science, Schiphol-Rijk, Netherland) in RPMI-5% human AB serum (Inotech, Dottikon, Switzerland) and stimulated with viral lysates or pools of viral CD8+-restricted peptide epitopes at a concentration of 1 μg/ml. Peptide-free medium served as negative control and PHA-L (Calbiochem, Dietikon, Switzerland) at 5 μg/ml as positive control. Cells were incubated for 5 days at 37°C and then pulsed overnight with 1 μCi/well methyl-[3H]thymidine (Hartmann Analytic, Braunschweig, Germany). After harvesting, nuclear incorporation of radioactivity was measured in a scintillation beta counter (Topcount, Zurich, Switzerland). Proliferation responses were calculated as stimulation index (SI), as determined by the mean ratio of antigen-stimulated counts per minute (cpm) over background cpm. Patients with SI lower than 4.0 were considered as non-responding.

Analysis of the data of the cellular immune assays

We performed the eight cellular immune response assays (namely, PA with EBV/CMV lysate; PA with pools of EBV/CMV CD8+-restricted T-cell peptide epitopes; ELISPOT with EBV/CMV lysates; ELISPOT with pools of EBV/CMV CD8+-restricted T-cell peptide epitopes) in as many study subjects as possible. However, some of these subjects could not have all those eight assays, which was due either to limited amount of PBMC or to a mismatch between HLA class I typing of the study subject and alleles represented in the pool of viral CD8+-restricted T-cell peptide epitopes, such as explained hereafter.

If a given patient had not, at least, one HLA class I allele in common with the alleles represented in the pool of viral CD8+ T cell-restricted peptide epitopes, we excluded this patient from the analyses for the corresponding assay. Since there were some differences in the HLA class I-restriction between the pools of EBV and CMV (Supplementary Table 1), some patients could have determination of their CD8+ T-cell response for one virus, but not for the other, depending on their HLA class I typing.

In 38/164 (23%) study subjects, HLA class I typing could not be determined. In these 38 subjects, the results of viral lysate stimulation could still be validated since viral lysates elicit CD4+ and not CD8+ T cells. Regarding stimulation with the pools of CD8+-restricted peptide epitopes, in order to be able to gather information on these patients, we applied the following procedure: (i) if both PA and ELISPOT assay were negative for a given virus as tested by the corresponding pool of CD8+-restricted peptide epitopes, we did not count this result as it was not possible to determine whether this negative result was due to HLA class I mismatch between the patient and the pool of viral peptide epitopes or due to a true absence of CD8+ T-cell response against the virus in question; (ii) however, if for a given virus, PA and/or ELISPOT were positive, the results of both assays were validated, as this positivity provided evidence that there was at least one patient HLA class I allele represented in the pool of CD8+-restricted peptide epitopes.

Viral-specific humoral immune response

To determine EBV and CMV seropositivity in our cohort, 2-fold diluted plasma in phosphate-buffered saline from patients were analysed for the presence of IgG against the EBV antigens viral capsid antigen (VCA) and EBNA-1 and against the CMV antigen pp65. Classical enzyme-linked immunosorbent assay (ELISA) was performed according to manufacturer instructions (Biotest, Dreieich, Germany). Samples were considered to be positive for (i) EBV VCA and EBNA-1 IgG if the OD measured was greater than negative control +0.2 OD; (ii) CMV antigen pp65 IgG if the OD measured was greater than negative control +0.3 OD.

To obtain a more comprehensive profile of the EBV-specific humoral immune response in a selected subset of MS patients, we used a bead-based multiplexed immunoassay [Luminex®; (Vignali, 2000)] and the Athena Multi-Lyte® IgM and IgG test system (Teomed AG, Greifensee, Switzerland). We have recently observed in a comparison study that this assay is more sensitive than an immunofluorescence assay (EBNA ACIF) for anti-EBNA-1 detection (P. Meylan, unpublished data). Specifically, we checked for anti-VCA IgM and anti-EA IgG, markers of primo-infection or recent reactivation (Buisson *et al.*, 1999), and anti-VCA IgG and anti-EBNA-1 IgG, markers of past EBV infection. Data were expressed as arbitrary units (AU)/ml. Samples were considered as positive when the resulting measure was $\geqslant$120 AU/ml. We studied 40 patients, including 25 patients with an inflammatory form of MS and 15 with a progressive one, who were chosen among those with a high (22), respectively a low (18) IFN-γ secreting EBV-specific CD8+ T-cell response as measured by IFN-γ secretion, 200 SFC/10^6 cells being the cut-off.

Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). We corrected all the data for age, using linear regression analysis over the total cohorts of EBV- and CMV-infected subjects (MS and controls). Indeed, in this study, the subjects were not matched for age (Table 1). Another reason to perform this correction was that EBV- and CMV-specific T-cell responses have been shown to increase with age (Khan *et al.*, 2002; Stowe *et al.*, 2007).

Subsequent analysis were performed on these age-corrected data sets. The differences among the six groups of study subjects were tested using Kruskal–Wallis test for non-normally distributed variables. The difference between two groups was tested using the non-parametric Mann–Whitney ranked test, whereas paired samples were tested with the non-parametric Wilcoxon ranked test. Correlations were analysed using the Spearman's rank test. r_s denotes the correlation coefficient of Spearman's rank test. A $P<0.05$ was considered significant.

Results

The rate of viral infection in the whole cohort is higher for EBV than CMV

We found that 139/159 (87%) and 63/149 (42%) study subjects displayed evidence of a cellular immune response against EBV and CMV, respectively, as revealed by proliferation and/or IFN-γ secretion by CD4+ and/or CD8+ T cells (Fig. 1, left panels). Thus there were 20/159 (13%) and 86/149 (58%) study subjects who had no EBV- or CMV-specific T-cell responses of any sort, respectively. In order to determine if the absence of virus-specific cellular immune response in those study subjects was due

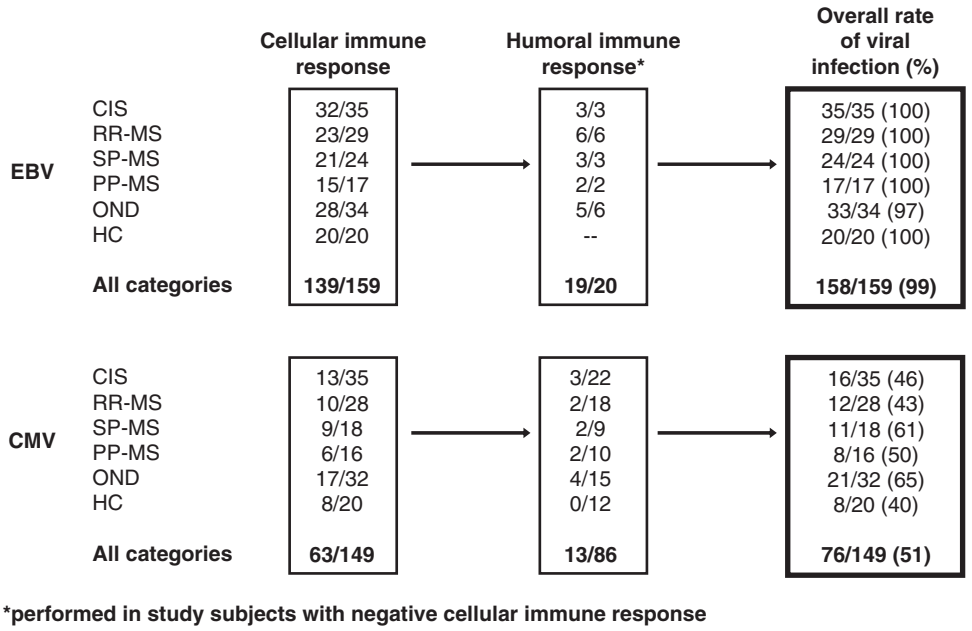

Fig. 1 Viral-specific cellular and humoral immune response and overall rate of infection in the cohort. The cellular immune response was assessed by PA and ELISPOT, while the humoral response was determined by detecting EBV- or CMV-specific antibodies such as described in Material and Methods section.

to hyporesponsiveness or genuine seronegativity, we performed an ELISA to detect anti-EBV (VCA and EBNA-1) and anti-CMV (pp65) IgG responses. We found that among study subjects with a negative viral response as assessed by the 'cellular assays' (PA/ELISPOT), 19/20 patients had EBV-specific antibodies, contrasting with only 13/86 study subjects who had CMV-specific antibodies (Fig. 1, middle panels). Thus, we could determine that, overall, the rate of CMV infection was much lower (76 out of 149; 51%) than the rate of EBV infection (158 out of 159; 99%, $P<0.0001$), in accordance with literature (Harari *et al.*, 2004; Haahr and Hollsberg, 2006). However, for a given virus, there was no difference in the rate of infection between categories of study subjects (Fig. 1, right panels).

ICS performed in 24 study subjects confirmed that viral lysates and pools of 8- to 15-mer peptide epitopes elicited CD4+ and CD8+ T cells, respectively (Supplementary Figure 1).

High activation of EBV-specific CD8+ T cells in patients with CIS

Using ELISPOT assay, we found that the stimulation of PBMC with pools of CD8+-restricted T-cell peptide epitopes of EBV—but not CMV—induced an age-corrected higher frequency of IFN-γ secreting CD8+ T cells in patients with CIS as compared to all other categories of study subjects ($P=0.002$), i.e. patients with RR-MS ($P=0.02$), SP-MS ($P=0.0001$), PP-MS ($P=0.009$), patients with other neurological diseases ($P=0.003$) and control subjects ($P=0.002$) (Fig. 2A).

In contrast, the magnitude of CMV-specific IFN-γ secreting CD8+ T cells was similar between the groups of study subjects ($P>0.9$, Fig. 2B). Moreover, contrary to what we had observed with viral-specific CD8+ T cells, PBMC stimulation with EBV lysate induced no difference in terms of IFN-γ secretion by CD4+ T cells between categories ($P>0.5$). The same was true for CMV ($P>0.1$, results not shown).

Looking at the proliferative capacities of EBV- and CMV-specific T cells, we found that CD4+ T cells were always more prone to proliferate than CD8+ T cells, whichever was the stimulating virus. There was no difference in the SIs between the six groups of study subjects, either for EBV or CMV, and either for CD4+ or CD8+ T cells ($P>0.3$, results not shown).

The more recent the clinical MS onset, the higher the level of IFN-γ secretion by EBV-specific CD8+ T cells

Performing a linear regression analysis over the cohort of all MS patients, including CIS, we found that the EBV-specific IFN-γ secreting CD8+ T-cell response was inversely proportional to the interval between disease onset and our assay: the shorter the interval, the higher the response ($r_s=-0.30$, $P=0.003$, Fig. 2C). Confirming the above results, we found that EBV-specific CD8+ T-cell responses decreased in 12/13 patients with CIS followed prospectively for 1.0 ± 0.2 years ($P=0.0024$; Fig. 2D). However, when we compared, among the category of patients with inflammatory MS, the results of relapsing versus remitting patients,

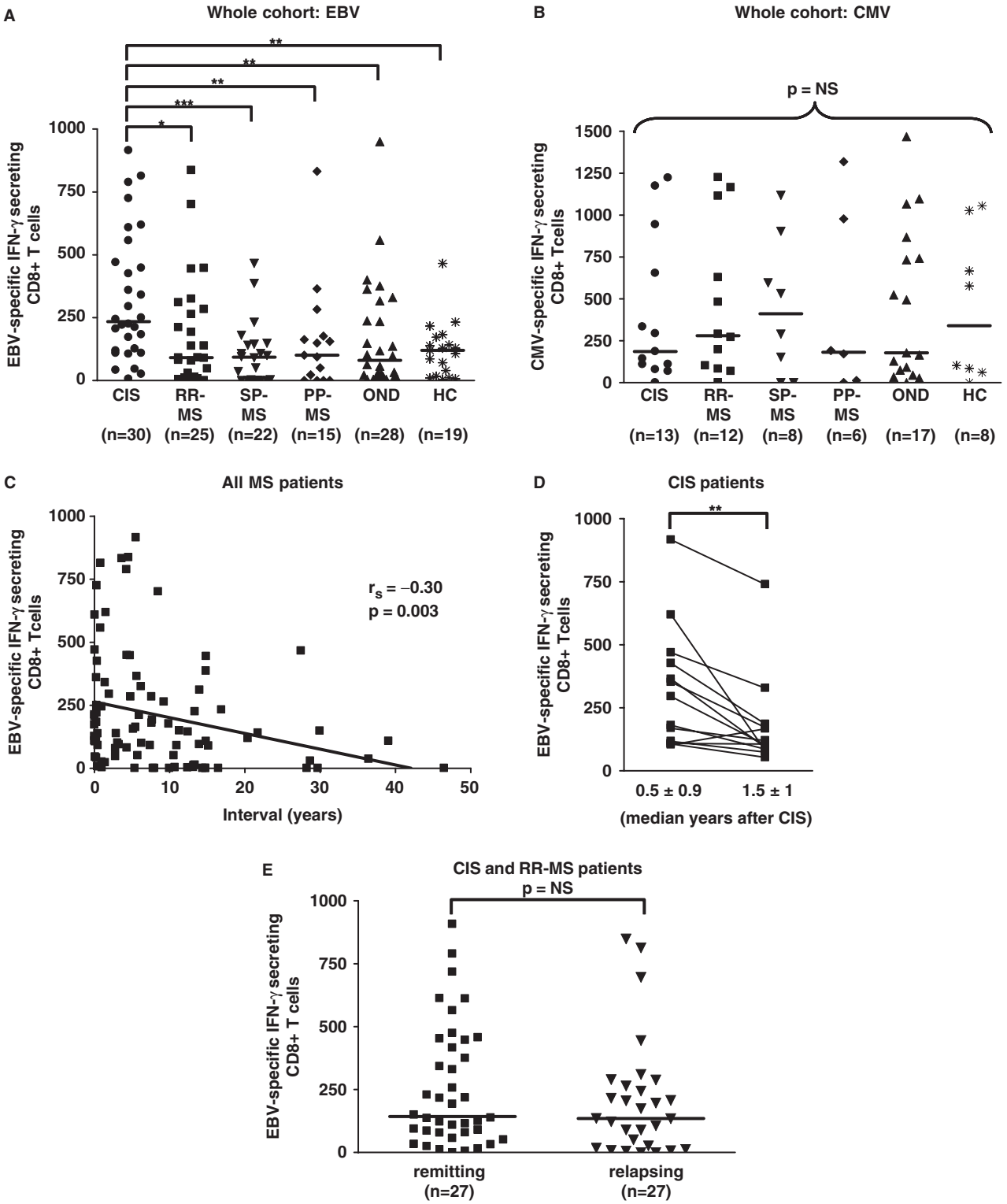

Fig. 2 Elevated frequency of IFN-γ secreting EBV-specific effector CD8+ T cells in patients with CIS. IFN-γ secretion was assessed by ELISPOT and the magnitude of responding CD8+ T cells calculated as SFC/10^6 PBMC. (**A**) EBV- and (**B**) CMV-specific CD8+ T-cell responses are shown. Each dot represents one patient and the horizontal bars correspond to the median values. (**C**) In the MS patient cohort (92 patients; CIS, RR-, SP-, PP-MS), the magnitude of EBV-specific CD8+ T-cell response was inversely correlated with the interval between MS onset and assay. (**D**) In these 13 patients with CIS, the secretion of IFN-γ by EBV-specific CD8+ T cells was assessed on two time-points separated by a median of 1.0 ± 0.2 years. (**E**) Patients with inflammatory MS (CIS and RR-MS) were divided into relapsing ($n = 27$) and remitting patients ($n = 27$). EBV-specific CD8+ T cell responses were plotted accordingly. Virus-specific CD8+ T cell responses were expressed as spot forming (i.e. IFN-γ secreting) cells (SFC)/10^6 PBMC. SFC; r_s, Spearman's coefficient r; NS, $P > 0.05$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

we found no difference between both groups, suggesting that the degree of activation of EBV-specific CD8+ T cells was not dependent on the degree of activity of MS ($P > 0.3$, Fig. 2E).

Correlation between anti-EBNA-1 antibodies and EBV-specific CD8+ T cells

Using the flow cytometry-based multiplexed quantitative serological assay [Luminex® Technology (Vignali, 2000)], we found that anti-VCA IgG and anti-EBNA-1 IgG, but not anti-VCA IgM, and only rarely anti-EA IgG, were detectable in MS patients, suggesting that the increased EBV-specific CD8+ T-cell response was not attributable to recent infection or reactivation (Fig. 3A). Nevertheless, six MS patients had elevated anti-EA IgG, suggesting that in these patients a recent reactivation of EBV was possible. Interestingly, all these six MS patients had a strong EBV-specific CD8+ T-cell response (>150 SFC/10^6 cells). The magnitude of anti-EBNA-1, but not anti-VCA IgG, response was moderately correlated with the one of EBV-specific CD8+ T cells secreting IFN-γ in patients with inflammatory MS ($r_s = 0.38$, $P = 0.016$, Fig. 3B), but not in patients with chronic MS ($P > 0.4$). Finally, in contrast to what we had found for IFN-γ-secreting CD8+ T cells, there was no correlation between the level of anti-EBNA-1 or anti-VCA IgG and the interval between MS onset and our assay.

Discussion

We found that there was a higher EBV-specific CD8+ T-cell response, IFN-γ-mediated, in patients with CIS as compared to patients with all other forms of MS, patients with other neurological diseases and control subjects. In contrast, no difference between categories of study subjects was found in terms of EBV-specific CD4+ T-cell response. We did not find either any difference in the CMV-specific cellular immune response or the different categories of study subjects for both CD4+ and CD8+ T cells. These results suggest that the increased EBV-specific CD8+ T-cell response observed in patients with CIS may reflect a specific role of EBV in MS rather than be a mere surrogate marker of non-specific immune hyperactivation. In both the cross-sectional and longitudinal parts of this study, we found that the more recent was the clinical onset of MS, the higher was the EBV-specific CD8+ T-cell response. However, there was no difference between relapsing and remitting patients with inflammatory MS (CIS and RR-MS). Several reports have consistently shown that viral infections are associated with relapses (Edwards *et al.*, 1998; Buljevac *et al.*, 2002; Correale *et al.*, 2006). In this context, the role of EBV is debated. Indeed, an increase of anti-EA antibodies and detection of serum EBV DNA has been shown in relapsing MS patients by some authors (Wandinger *et al.*, 2000), but not by others (Buljevac *et al.*, 2005). Altogether, our data suggest that if EBV plays a role in the pathogenesis of MS, it would rather be as a trigger of the disease itself than of relapses.

The higher EBV-specific CD8+ T-cell response we found in patients with CIS is in accordance with the trend reported by Lunemman *et al.* in a smaller group of MS patients (Lunemann *et al.*, 2006). In contrast, Gronen *et al.* did not find different IFN-γ secretion in CD8+ T cells between MS patients and control subjects (Gronen *et al.*, 2006). Several points can be raised in order to explain these differences. First, in our study none of the CIS patients received immunomodulatory treatment that may potentially modify viruses-specific cellular immune responses. Second, we used a pool of 18 different EBV peptide epitopes restricted by 10 HLA class I alleles, while Gronen *et al.* focused on HLA B7-restricted CD8+ T-cell peptide epitopes (Gronen *et al.*, 2006), thus missing the CD8+ T-cell responses mediated by other HLA class I alleles. Third, we enrolled a large proportion of patients with CIS, a category which was not included in Gronen's study. Yet, it is precisely in this group that we found the highest EBV-specific CD8+ T-cell response.

To determine their stage of EBV infection, we performed serologies in a large subset of our MS patients. Using the sensitive Luminex® technology, we found anti-VCA and anti-EBNA-1 IgG, but not anti-VCA, and only rarely anti–EA IgG, a pattern indicating that the EBV primary infection was remote, such as it has already been reported by others (Goldacre *et al.*, 2004). Of interest, such as reported by Buljevac and colleagues, we found elevated anti-EA IgG in a small subgroup of patients, suggesting a recent EBV reactivation (Buljevac *et al.*, 2005). Of note, these patients also had a strong CD8+ T-cell response. However, this subgroup of patients was too small to draw definitive conclusions on the reactivation of EBV in these MS patients. Furthermore, while anti-EBNA-1-specific antibodies (but not anti-VCA antibodies) correlated moderately with EBV-specific CD8+ T cells in patients with inflammatory MS, EBV-specific CD8+ T cells were a better marker of early MS than anti-EBNA-1-specific antibodies as the latter were also increased in progressive forms of MS.

Whereas the overall rate of EBV infection in our whole cohort was 99%, it was only 51% for CMV, without differences between MS patients and control subjects. These findings do not support a specific relationship between MS and CMV—as opposed to MS and EBV—and are in agreement with previous work from Buljevac *et al.* These studies showed that, whereas there was a higher percentage of antibodies specific for EBV EA in MS patients (48%) as compared to controls (25%), CMV-specific antibodies were detected at a similar rate in both categories (Buljevac *et al.*, 2005). Zivadinov *et al.* even suggested that CMV might have a protective role as CMV-positive MS patients were found to have lower T2-lesion loads and higher parenchymal brain fraction than CMV-negative MS patients (Zivadinov *et al.*, 2007).

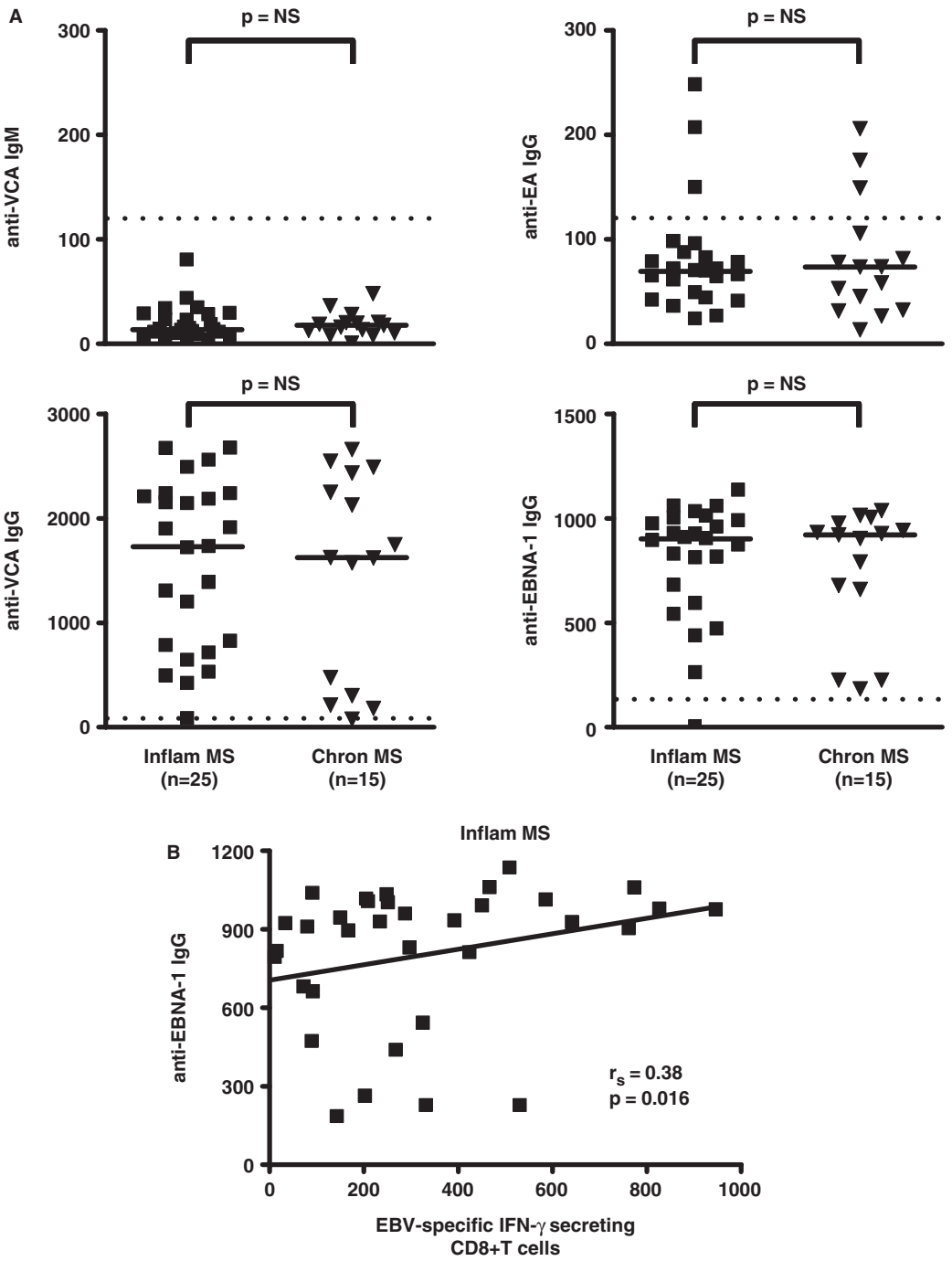

Fig. 3 Anti-EBNA-1 IgG are correlated with the secretion of IFN-γ by CD8+ T cells in inflammatory MS patients. (**A**) Sera from 40 selected patients were collected and assayed for anti-VCA IgM, anti-EA IgG, anti-VCA IgG and anti-EBNA-1 IgG as described in Material and Methods section. Data are presented as arbitrary units/ml. Each dot represents one patient, the horizontal bars correspond to the median values and a dotted line determines the threshold for positive responses. (**B**) In patients with inflammatory MS (CIS/RR-MS), anti-EBNA-1 IgG were moderately correlated with the IFN-γ secretion by EBV-specific CD8+ T cells. r_s, Spearman's coefficient r. NS, $P > 0.05$.

An infectious etiology of MS, in particular a viral one, has been evoked a long time ago (Marie, 1886), but the Koch postulate has never been fulfilled for any studied microbes in MS (Lipton *et al.*, 2007). Nevertheless, even if MS may not be an infectious disease, strictu sensu, an infectious agent might still play an important role in the pathogenesis of this disease. Precisely, it has been hypothesized that, in genetically predisposed individuals carrying a potentially harmful virus, the anti-viral immune response can cross-react against auto-antigen epitopes (Correale *et al.*, 2006). In particular, EBV-specific CD4+ T cells present in the blood or the CSF of MS patients can

cross-react with myelin basic protein (MBP) (Lang *et al.*, 2002; Holmoy *et al.*, 2004). We reported recently that an acute EBV infection could trigger a strong myelin oligodendrocyte glycoprotein (MOG)-specific cellular and humoral immune response in a patient suffering from a severe post-EBV encephalopathy (Jilek *et al.*, 2007*a*). These data suggest that a mechanism of molecular mimicry between EBV and myelin antigen might take place in patients infected with EBV. Another recent paper adds an interesting piece to the story of MS and EBV: Serafini and colleagues have found EBV-infected B cells in the meninges, in the perivascular space and in the demyelinated lesions of patients with different categories of MS, inflammatory as well as progressive ones. Such EBV-infected B cells were not found in patients with inflammatory other neurological diseases (Serafini *et al.*, 2007). An expansion of cytotoxic CD8+ T cells, as demonstrated by the expression of IFN-γ, perforin and CD107a, was present at the sites of EBV-infected B cells, strongly suggesting that the latter cells were recognized and attacked by activated CD8+ T cells in the CNS. Our data showing that there is an increased secretion of IFN-γ by EBV-specific CD8+ T cells in patients with early MS are consistent with these findings. The decline in the frequency of EBV-specific CD8+ T cells in the blood observed over time may reflect either a control of EBV reactivation or a selective migration of CD8+ T cells in the site of MS lesions (Serafini *et al.*, 2007). In this regard, it has been shown a preferential concentration of CD8+ T cells in MS lesions (Babbe *et al.*, 2000). The possibility that this decrease in frequency of EBV-specific CD8+ T cells result from virus-induced T cell exhaustion and/or dysregulation should also be taken into account (Klenerman and Hill, 2005).

Supplementary material

Supplementary material is available at *Brain* online.

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References

Alotaibi S, Kennedy J, Tellier R, Stephens D, Banwell B. Epstein-Barr virus in pediatric multiple sclerosis. JAMA 2004; 291: 1875–9.

Ascherio A, Munger KL. Environmental risk factors for multiple sclerosis. Part I: the role of infection. Ann Neurol 2007; 61: 288–99.

Ascherio A, Munger KL, Lennette ET, Spiegelman D, Hernan MA, Olek MJ, et al. Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. JAMA 2001; 286: 3083–8.

Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, et al. Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J Exp Med 2000; 192: 393–404.

Barkhof F, Filippi M, Miller DH, Scheltens P, Campi A, Polman CH, et al. Comparison of MRI criteria at first presentation to predict conversion to clinically definite multiple sclerosis. Brain 1997; 120: 2059–69.

Barouch DH, Letvin NL. CD8+ cytotoxic T lymphocyte responses to lentiviruses and herpesviruses. Curr Opin Immunol 2001; 13: 479–82.

Buisson M, Fleurent B, Mak M, Morand P, Chan L, Ng A, et al. Novel immunoblot assay using four recombinant antigens for diagnosis of Epstein-Barr virus primary infection and reactivation. J Clin Microbiol 1999; 37: 2709–14.

Buljevac D, Flach HZ, Hop WC, Hijdra D, Laman JD, Savelkoul HF, et al. Prospective study on the relationship between infections and multiple sclerosis exacerbations. Brain 2002; 125: 952–60.

Buljevac D, van Doornum GJ, Flach HZ, Groen J, Osterhaus AD, Hop W, et al. Epstein-Barr virus and disease activity in multiple sclerosis. J Neurol Neurosurg Psychiatry 2005; 76: 1377–81.

Cepok S, Zhou D, Srivastava R, Nessler S, Stei S, Bussow K, et al. Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis. J Clin Invest 2005; 115: 1352–60.

Correale J, Fiol M, Gilmore W. The risk of relapses in multiple sclerosis during systemic infections. Neurology 2006; 67: 652–9.

DeLorenze GN, Munger KL, Lennette ET, Orentreich N, Vogelman JH, Ascherio A. Epstein-Barr virus and multiple sclerosis: evidence of association from a prospective study with long-term follow-up. Arch Neurol 2006; 63: 839–44.

Du Pasquier RA, Kuroda MJ, Zheng Y, Jean-Jacques J, Letvin NL, Koralnik IJ. A prospective study demonstrates an association between JC virus-specific cytotoxic T lymphocytes and the early control of progressive multifocal leukoencephalopathy. Brain 2004; 127: 1970–8.

Dutoit V, Ciuffreda D, Comte D, Gonvers JJ, Pantaleo G. Differences in HCV-specific T cell responses between chronic HCV infection and HIV/HCV co-infection. Eur J Immunol 2005; 35: 3493–504.

Edwards S, Zvartau M, Clarke H, Irving W, Blumhardt LD. Clinical relapses and disease activity on magnetic resonance imaging associated with viral upper respiratory tract infections in multiple sclerosis. J Neurol Neurosurg Psychiatry 1998; 64: 736–41.

Friese MA, Fugger L. Autoreactive CD8+ T cells in multiple sclerosis: a new target for therapy? Brain 2005; 128: 1747–63.

Goldacre MJ, Wotton CJ, Seagroatt V, Yeates D. Multiple sclerosis after infectious mononucleosis: record linkage study. J Epidemiol Community Health 2004; 58: 1032–5.

Gronen F, Ruprecht K, Weissbrich B, Klinker E, Kroner A, Hofstetter HH, et al. Frequency analysis of HLA-B7-restricted Epstein-Barr virus-specific cytotoxic T lymphocytes in patients with multiple sclerosis and healthy controls. J Neuroimmunol 2006; 180: 185–92.

Haahr S, Hollsberg P. Multiple sclerosis is linked to Epstein-Barr virus infection. Rev Med Virol 2006; 16: 297–310.

Harari A, Zimmerli SC, Pantaleo G. Cytomegalovirus (CMV)-specific cellular immune responses. Hum Immunol 2004; 65: 500–6.

Hemmer B, Archelos JJ, Hartung H-P. New concepts in the immunopathogenesis of multiple sclerosis. Nat Rev Neurosci 2002; 3: 291–301.

Hollsberg P, Hansen HJ, Haahr S. Altered CD8+ T cell responses to selected Epstein-Barr virus immunodominant epitopes in patients with multiple sclerosis. Clin Exp Immunol 2003; 132: 137–43.

Holmoy T, Kvale EO, Vartdal F. Cerebrospinal fluid CD4+ T cells from a multiple sclerosis patient cross-recognize Epstein-Barr virus and myelin basic protein. J Neurovirol 2004; 10: 278–83.

Holmoy T, Vartdal F. Cerebrospinal fluid T cells from multiple sclerosis patients recognize autologous Epstein-Barr virus-transformed B cells. J Neurovirol 2004; 10: 52–6.

Jacobsen M, Cepok S, Quak E, Happel M, Gaber R, Ziegler A, et al. Oligoclonal expansion of memory CD8+ T cells in cerebrospinal fluid from multiple sclerosis patients. Brain 2002; 125: 538–50.

Jilek S, Kuhle J, Meylan P, Reichart MD, Pantaleo G, Du Pasquier RA. Severe post-EBV encephalopathy associated with myelin oligodendrocyte glycoprotein-specific immune response. J Neuroimmunol 2007a; 192: 192–7.

Jilek S, Schluep M, Rossetti AO, Guignard L, Le Goff G, Pantaleo G, et al. CSF enrichment of highly differentiated CD8+ T cells in early multiple sclerosis. Clin Immunol 2007b; 123: 105–13.

Khan N, Shariff N, Cobbold M, Bruton R, Ainsworth JA, Sinclair AJ, et al. Cytomegalovirus Seropositivity drives the CD8 T cell repertoire toward greater clonality in healthy elderly individuals. J Immunol 2002; 169: 1984–92.

Klenerman P, Hill A. T cells and viral persistence: lessons from diverse infections. Nat Immunol 2005; 6: 873–9.

Lang HL, Jacobsen H, Ikemizu S, Andersson C, Harlos K, Madsen L, et al. A functional and structural basis for TCR cross-reactivity in multiple sclerosis. Nat Immunol 2002; 3: 940–3.

Levin LI, Munger KL, Rubertone MV, Peck CA, Lennette ET, Spiegelman D, et al. Temporal relationship between elevation of Epstein-Barr virus antibody titers and initial onset of neurological symptoms in multiple sclerosis. JAMA 2005; 293: 2496–500.

Lipton HL, Liang Z, Hertzler S, Son K-N. A specific viral cause of multiple sclerosis: one virus, one disease. Ann Neurol 2007; 61: 514–23.

Lunemann JD, Edwards N, Muraro PA, Hayashi S, Cohen JI, Munz C, et al. Increased frequency and broadened specificity of latent EBV nuclear antigen-1-specific T cells in multiple sclerosis. Brain 2006; 129: 1493–506.

Marie P. Sclerose en plaques et maladies infectieuses. Progr Med 1886; 12: 287–9.

McDonald WI, Compston A, Edan G, Goodkin D, Hartung HP, Lublin FD, et al. Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 2001; 50: 121–7.

Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. N Engl J Med 2000; 343: 938–52.

Pohl D, Krone B, Rostasy K, Kahler E, Brunner E, Lehnert M, et al. High seroprevalence of Epstein-Barr virus in children with multiple sclerosis. Neurology 2006; 67: 2063–5.

Polman CH, Reingold SC, Edan G, Filippi M, Hartung HP, Kappos L, et al. Diagnostic criteria for multiple sclerosis: 2005 revisions to the 'McDonald Criteria'. Ann Neurol 2005; 58: 840–6.

Serafini B, Rosicarelli B, Franciotta D, Magliozzi R, Reynolds R, Cinque P, et al. Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. J Exp Med 2007; 204: 2899–912.

Stowe RP, Kozlova EV, Yetman DL, Walling DM, Goodwin JS, Glaser R. Chronic herpesvirus reactivation occurs in aging. Exp Gerontol 2007; 42: 563–70.

Sundstrom P, Juto P, Wadell G, Hallmans G, Svenningsson A, Nystrom L, et al. An altered immune response to Epstein-Barr virus in multiple sclerosis: a prospective study. Neurology 2004; 62: 2277–82.

Thacker EL, Mirzaei F, Ascherio A. Infectious mononucleosis and risk for multiple sclerosis: a meta-analysis. Ann Neurol 2006; 59: 499–503.

Vignali DAA. Multiplexed particle-based flow cytometric assays. J Immunol Methods 2000; 243: 243–55.

Vukusic S, Confavreux C. Primary and secondary progressive multiple sclerosis. J Neurol Sci 2003; 206: 153–5.

Wandinger K, Jabs W, Siekhaus A, Bubel S, Trillenberg P, Wagner H, et al. Association between clinical disease activity and Epstein-Barr virus reactivation in MS. Neurology 2000; 55: 178–84.

Zivadinov R, Nasuelli D, Tommasi MA, Serafin M, Bratina A, Ukmar M, et al. Positivity of cytomegalovirus antibodies predicts a better clinical and radiological outcome in multiple sclerosis patients. Neurol Res 2007; 28: 262–9.