

Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis

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MS is a chronic inflammatory and demyelinating disease of the CNS with as yet unknown etiology. A hallmark of this disease is the occurrence of oligoclonal IgG antibodies in the cerebrospinal fluid (CSF). To assess the specificity of these antibodies, we screened protein expression arrays containing 37,000 tagged proteins. The 2 most frequent MS-specific reactivities were further mapped to identify the underlying high-affinity epitopes. In both cases, we identified peptide sequences derived from EBV proteins expressed in latently infected cells. Immunoreactivities to these EBV proteins, BRRF2 and EBNA-1, were significantly higher in the serum and CSF of MS patients than in those of control donors. Oligoclonal CSF IgG from MS patients specifically bound both EBV proteins. Also, CD8⁺ T cell responses to latent EBV proteins were higher in MS patients than in controls. In summary, these findings demonstrate an increased immune response to EBV in MS patients, which suggests that the virus plays an important role in the pathogenesis of disease.

Introduction

MS is a chronic inflammatory disease of the CNS that leads to demyelination and neurodegeneration (1). Although the cause of MS is still unknown, it is widely accepted that the acquired immune response plays a key role in disease onset and progression (2, 3). Several findings suggest that the local immune response in the CNS is highly focused in MS. CD8⁺ T cells clonally accumulate at large numbers in the lesions and cerebrospinal fluid (CSF) of MS patients (4, 5). B cells, which are found in the CNS compartment, display a limited heavy chain repertoire and also contain dominant clonotypes. These cells show extensive replacement mutations in the B cell receptor genes compatible with repeated antigenic challenges (6–10). Oligoclonal IgG bands (OCBs) are observed in the lesions and CSF but either to a much lesser extent or not at all in the serum of these MS patients (11–14). The pattern of OCBs in the CSF of MS patients is usually stable over time (15). Dominant B cell clonotypes persist over time in the CNS compartment (16). All of these findings are compatible with a focused and temporally stable humoral immune response in the CNS of MS patients.

Intrathecal IgG responses and OCBs are also found in subacute and chronic infectious CNS disorders, such as subacute sclerosing panencephalitis, human T cell lymphotropic virus-associated myelopathy, neurosyphilis, and neuroborreliosis. In all of these disorders, the intrathecal IgG-antibody response is specific to the underlying infectious agent (17–21). Therefore,

it is conceivable that the persistent IgG response in MS targets disease-relevant antigens.

Several studies have addressed the specificity of the intrathecal antibody response in MS. Approaches involving phage display and expression libraries were used to dissect the IgG antibody specificity in the CSF (22–25). These studies identified possible target peptides in single patients. However, immune responses to these peptides did not differ between patients and controls when larger groups were analyzed (23). In addition, these proteins did not specifically bind oligoclonal IgG in the CSF of MS patients.

Here, we applied a large-scale protein expression clone array combined with epitope mapping techniques to decrypt the specificity of the CSF IgG in MS patients.

Results

Dissecting the antibody repertoire in MS patients by a human cDNA protein-expression array. To investigate the antibody specificity of IgG antibodies from the CSF of MS patients, we applied a novel protein array. The array was generated from a human brain cDNA expression library comprising 37,000 expression clones. CSF samples from 12 MS patients and 5 controls were adjusted to 1 mg IgG/l and each applied to a separate protein array. Immunoreactivity was visualized by HRP-conjugated anti-IgG antibodies. From 0 to 10 expression clones that specifically stained above background were identified in each patient (Figure 1A). After comparing the staining pattern between MS patients and controls, we selected expression clones that showed strong reactivity in MS patients but not in controls.

Identification and characterization of primary immunoreactivity in MS. Among the arrayed expression clones, a total of 54 clones were selected. cDNA inserts were sequenced and the corresponding AA sequences determined in all of them. Forty-two unique sequences were defined; these comprised proteins expressed in the correct reading frame but also sequences which were expressed out of frame generating artificial protein sequences. The 21 expression clones that showed the strongest reactivity in 2 or more MS patients were

Nonstandard abbreviations used: CSF, cerebrospinal fluid; EBNA-1, Epstein-Barr nuclear antigen-1; GST, glutathione-S-transferase; IEF, isoelectric focusing; IPTG, isopropyl-β-D-thiogalactopyranoside; MAZ, Myc-associated zinc finger protein; NIND, noninflammatory neurological disease; OCB, oligoclonal IgG band; OIND, other inflammatory neurological disease; PBS-T, PBS containing 0.05% Tween-20; RZPD, Deutsches Ressourcenzentrum für Genomforschung GmbH; TBS, Tris-buffered saline.

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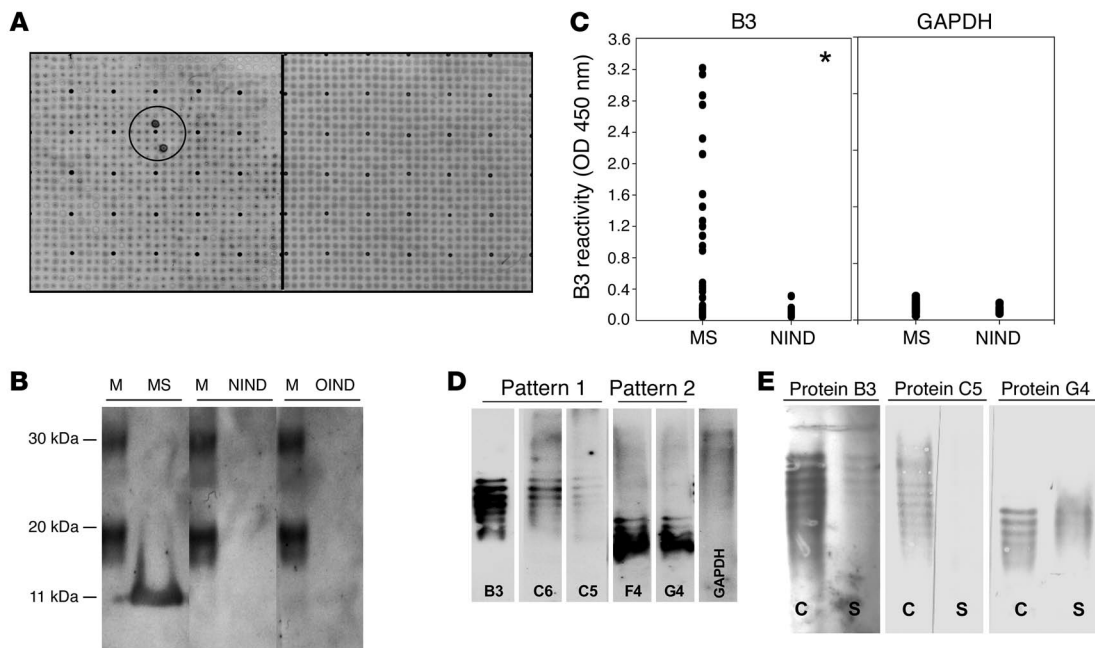


Figure 1

Analysis of CSF IgG immunoreactivity in MS patients by protein expression arrays. **(A)** Incubation of the protein expression array with CSF from a representative MS patient (left) and a control donor (right). A 3 cm × 3 cm section of the 24 cm × 24 cm array is shown. IgG immunoreactivity of the MS CSF to the expression clone B3 (spotted in duplicate) is marked by a circle. IgG concentration was adjusted to 1 mg/l IgG in MS and control CSF. **(B)** Western blot with purified protein B3. Immunoreactivity was observed in the CSF of a representative MS patient (left) but not the NIND (middle) or OIND (right) control donors. All CSF samples were adjusted to 10 mg/l IgG. IgG binding was developed with ECL. M, molecular weight marker. **(C)** Analysis of immunoreactivity to B3 protein (left) and control protein GAPDH (right) with CSF (1:5 diluted) of 132 MS patients and 125 NIND patients by ELISA. Antibody titers were significantly higher in MS patients. Dot points represent the OD of a single CSF sample. Cut-off point, OD > 0.3 (mean ± 6 SEM). **(D)** CSF from a patient with high immunoreactivity to patterns I and II proteins was separated by IEF and blotted against proteins B3, C6, C5, F4, and G4 as well as the control protein GAPDH. Binding of proteins from both patterns was similar with little overlap between the patterns. Similar results were obtained with CSF from another patient (data not shown). **(E)** IEF immunoblot with CSF (C) and serum (S) from 3 MS patients (adjusted to 10 mg IgG/l) was performed to compare IgG binding patterns to proteins B3, C5, and G4. A stronger and more focused immune response to the proteins was observed in the CSF of 10 patients analyzed in total. **P* < 0.001, Fisher's exact test.

selected for further analyses (Table 1). All of these proteins, irrespective of whether they were expressed in the correct frame (10 clones) or not (11 clones), and the control protein GAPDH were purified for further analyses. The size of all expressed proteins was verified by SDS-PAGE. Specific binding of CSF IgG from MS patients was confirmed by Western blot analysis (Figure 1B). ELISAs were established for all proteins. In initial experiments, a group of 46 MS patients and 28 controls were analyzed for CSF reactivity to the proteins. If a higher immunoreactivity was observed in the MS group by ELISA, additional samples from MS patients and controls were evaluated. For certain proteins, a significant difference with regard to antibody reactivity was observed between MS patients and controls. While more than 13% of the 132 MS patients had CSF antibodies against the expression product of clone B3, no such antibody responses were detected in any of the controls. In contrast, MS patients and controls showed similar immunoreactivity to the control protein GAPDH (Figure 1C). A summary of immunoreactivities to the 21 proteins and GAPDH in patients and controls as determined in the initial ELISA experiments is provided in Table 1.

Next, the immunoreactivity to the selected 21 proteins in individual MS patients was compared. Interestingly, immunoreactivities to certain sets of distinct proteins occurred consistently in different patients. Patients with IgG reactivity against protein B3 usually also had antibodies against C5, C6, D6, and F3 (pat-

tern I; Table 1), suggesting the presence of a similar epitope that was targeted by the IgG response of the patients. All 5 proteins of this pattern represent artificial products that were expressed out of frame. Three other expression clones (G4, F4, and H5), which contained different cDNA fragments of *Myc-associated zinc finger protein (MAZ)*, also showed high and largely overlapping immunoreactivities (pattern II; Table 1).

To demonstrate that 1 single epitope was responsible for the immunoreactivity observed within the 2 groups, we used isoelectric focusing (IEF) to separate CSF IgG antibodies to OCBs from patients with immunoreactivity to proteins of the 2 patterns. After separation, IgGs were blotted onto membranes coated with the different proteins of patterns I and II as well as the control protein GAPDH (Figure 1D). We found that proteins in each group always bound the same OCBs. In contrast, little overlap was observed between the immune responses to pattern I and II proteins. As expected, no binding to GAPDH was observed.

Finally, we compared the extent of antibody reactivity against selected proteins of each pattern by IEF and immunoblot using the serum and CSF of MS patients, adjusted to the same IgG concentration. We found a qualitatively or quantitatively enhanced CSF antibody reactivity against proteins from both patterns in the majority of patients (Figure 1E), which confirmed the existence of an intrathecal response to these antigens in MS.



Table 1
Summary of immunreactivities to expression clones identified by protein array

	RZPD clone	Protein ID	Identity	% MS	% Control
Pattern I	N04524	B3	Out-of-frame expression	10.9	0
	L04523	F3	Out-of-frame expression	10.9	0
	E21555	C5	Out-of-frame expression	8.7	0
	G17581	D6	Out-of-frame expression	15.2	0
	H13574	C6	Out-of-frame expression	13	0
Pattern II	E06542	G4	MAZ	23.9	3.6
	H07541	F4	MAZ	15.6	3.6
	M18568	H5	MAZ	17.1	3.6
	L17507	D3	ASPI	9.3	3
	A07545	G2	Out-of-frame expression	19.6	0
	A03592	H1	TCOF1	6.5	0
	C06553	D1	Out-of-frame expression	4.3	0
	L02568	G5	RNA-binding-motif protein 5	4.3	0
	O17528	H3	Out-of-frame expression	4.3	3.6
	M05594	A7	Mitochondrial ribosomal	6.5	0
	A11524	A1	Out-of-frame expression	2.2	0
	J23564	D5	Out-of-frame expression	6.5	0
	A02550	A5	Hemoglobin α 3 chain	2.2	0
	H15589	E1	Chaperon containing TCRP1 CCT5	0	0
	F22533	B1	Out-of-frame expression	1.9	0
	C10601	B7	RED protein	2.2	0
		GAPDH	GAPDH	0	0

RZPD hEx1 clone identifiers (without prefix MPMGp800), protein name used throughout the manuscript (ID), protein identity, and % MS patients and % controls with CSF immune reactivity determined by ELISA are displayed. The ELISA cut-off point was 0.3 OD.

Identification of 2 EBV proteins as targets of the CSF IgG response in MS. Epitopes responsible for the immunoreactivity to patterns I and II were mapped by peptide-scan analysis. Short 13-mer peptides with 11-mer overlappings covering the entire sequence of the smallest proteins from each pattern were synthesized onto membranes. CSF from patients with immunoreactivity to these proteins was used to screen for binding to the peptides. We identified IgG binding to the same 2 linear 8-AA minimal epitopes in 3 patients with CSF antibodies against pattern I and pattern II (Figure 2A). Then we performed a fine mapping of both identified peptide sequences by substitution analysis in which 20 AAs were substituted for each position of the 8-mer minimal epitope sequence. Again, CSF from patients with reactivity to the identified proteins was applied to define the best binding AA at each position (shown for pattern I in Figure 2B). In all patients studied, we noted highly similar binding motifs of CSF IgG antibodies. The motifs were identified in the proteins in patterns I and II. However, a complete match was not observed. Therefore, the Swiss-Prot database (<http://au.expasy.org/sprot/>) was searched for full matches using the identified motifs. We found 10 matches with the first and 13 with the second motif. Among the matches were some irrelevant proteins, as they were from nonpathogenic organisms, but also epitopes derived from human, bacterial, or viral proteins (Figure 2, C and D). We determined binding of CSF IgG to 13-mer peptides derived from proteins that were found by database search. CSF antibody staining to all peptides was observed but was strongest to the 2 peptides, Epstein-Barr nuclear antigen-1 (EBNA-1) and BRRF2, derived from EBV, as determined in 3 patients (Figure 2D). Interestingly, the genes of both EBV proteins are located adjacently in

the EBV genome. One, EBNA-1, is known to be expressed in the latent phase of EBV infection. The function of the other, BRRF2, has not been characterized. Recently, this protein was shown to be present in the EBV virion as a putative part of the tegument (26). To ensure that the gene is expressed, we studied transcript expression in latently EBV-infected and EBV-transformed cell lines (Figure 3A). We confirmed transcript expression of both partial and full-length BRRF2 RNA, demonstrating that the genes are transcribed in latently EBV-infected and EBV-transformed cell lines (Figure 3B).

Increased antibody responses to EBV, EBNA-1, and BRRF2 in MS patients. EBV peptides comprising the dominant epitope were synthesized and the CSF IgG response determined in a large group of MS patients and controls. We found a significant difference in the response to EBNA-1 and BRRF2 peptides between MS patients and controls (data not shown). Therefore, protein fragments of EBNA-1 (AAs 302–641) and BRRF2 containing the peptide sequence of interest were used to investigate the antibody response. For BRRF2, we established a recombinant expression as full (AAs 1–537) and partial (AAs 385–537) protein. Western blotting confirmed specific binding of CSF IgG to the recombinant BRRF2 proteins (Figure 3C). ELISAs with the recombinant proteins confirmed that MS patients had higher levels of IgG reactivity to EBNA-1 in CSF

and serum compared with control donors who had other inflammatory neurological diseases (OINDs) or noninflammatory neurological diseases (NINDs). A significant difference was also observed for BRRF2 in serum. In CSF, the difference was only statistically significant between patients with MS and those with NINDs but not between patients with MS and OIND patients (Figure 3D). To determine whether antibodies to BRRF2 are intrathecally produced, CSF and sera of MS and OIND patients were adjusted to the same IgG concentration, and BRRF2 antibody titers were determined. In most MS patients, higher titers of antibodies against BRRF2 were detected in CSF than in serum, indicating an intrathecal antibody response to the protein (Figure 4A). In contrast, only 1 OIND patient had an intrathecal IgG response to BRRF2. The number of patients with intrathecal synthesis was significantly higher in MS patients than in controls ($P = 0.0054$).

Finally, we investigated how antibody titers relate to other clinical and laboratory parameters. Besides a weak correlation of EBNA-1 serum antibody titers with age in MS patients ($P = 0.0261$, $r = -0.1959$; Pearson test without Bonferroni adjustment, $n = 129$), no other correlations between BRRF2 and EBNA-1 serum antibody levels and clinical or other laboratory parameters were observed. To rule out the possibility of nonspecific EBV reactivation following CNS damage and inflammation, we determined the EBNA-1 antibody levels in 9 stroke patients. No change in EBNA-1 antibody titers was observed 4 weeks ($n = 9$) and 8 weeks ($n = 3$) after onset of stroke (data not shown).

EBNA-1 and BRRF2 bind oligoclonal IgG from the CSF of MS patients. To further investigate the role of the humoral immune response against EBV in MS patients, we needed to clarify whether OCBs in

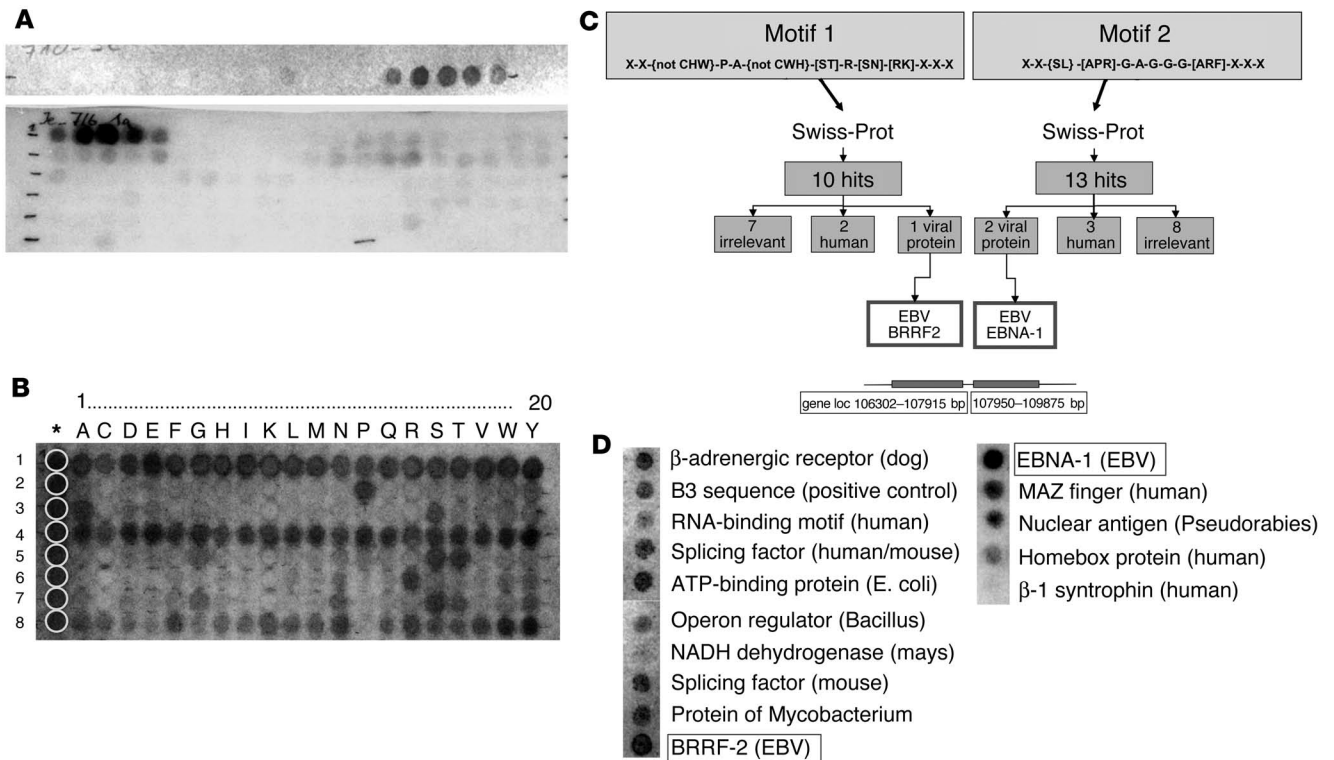


Figure 2

Identification of the CSF IgG-binding epitope. **(A)** Peptide scan analysis with 13-mer peptides that overlapped 11 AAs, covering the entire sequence of protein B3 of pattern I (upper membrane) and protein H5 of pattern II (lower membrane), was used to define the epitopes. Membranes were incubated with CSF (in 1:100 dilution) from MS patients immunoreactive to B3 or H5. Binding of IgG was visualized by anti-human IgG-HRP and TMB substrate. The minimal peptide epitopes were EPARSRSR for motif 1 and EAGAGGGA for motif 2. Similar results were obtained with CSF from 2 additional patients. **(B)** Substitution analysis was performed in order to define the optimal binding motif for the 8 AA epitopes defined in **A**. Binding of IgG was visualized by anti-human IgG-HRP and TMB substrate. A representative example for pattern I is shown. 1–8, the substituted AA-positions of the minimal epitope; 1–20, the 20 naturally occurring acids A–Y; *original peptide sequence. Similar results were obtained with 2 additional CSF samples from MS patients. **(C)** Definitions of 2 consensus motifs were based on the epitope mapping in 3 MS patients. These motifs were used to search the Swiss-Prot database. Database searching revealed 10 proteins matching with motif 1 and 13 proteins with motif 2. Two identified EBV proteins and the genomic locations according to <http://www.ncbi.nlm.nih.gov> are displayed. **(D)** Qualitative comparison of CSF IgG binding to peptides matching motif 1 (left) and motif 2 (right). Antibody binding was quantified by gel densitometry (highest signal and integrated density), which revealed the strongest binding to the 2 EBV epitopes BRRF2 and EBNA-1. The analysis was performed with similar results in 2 additional patients.

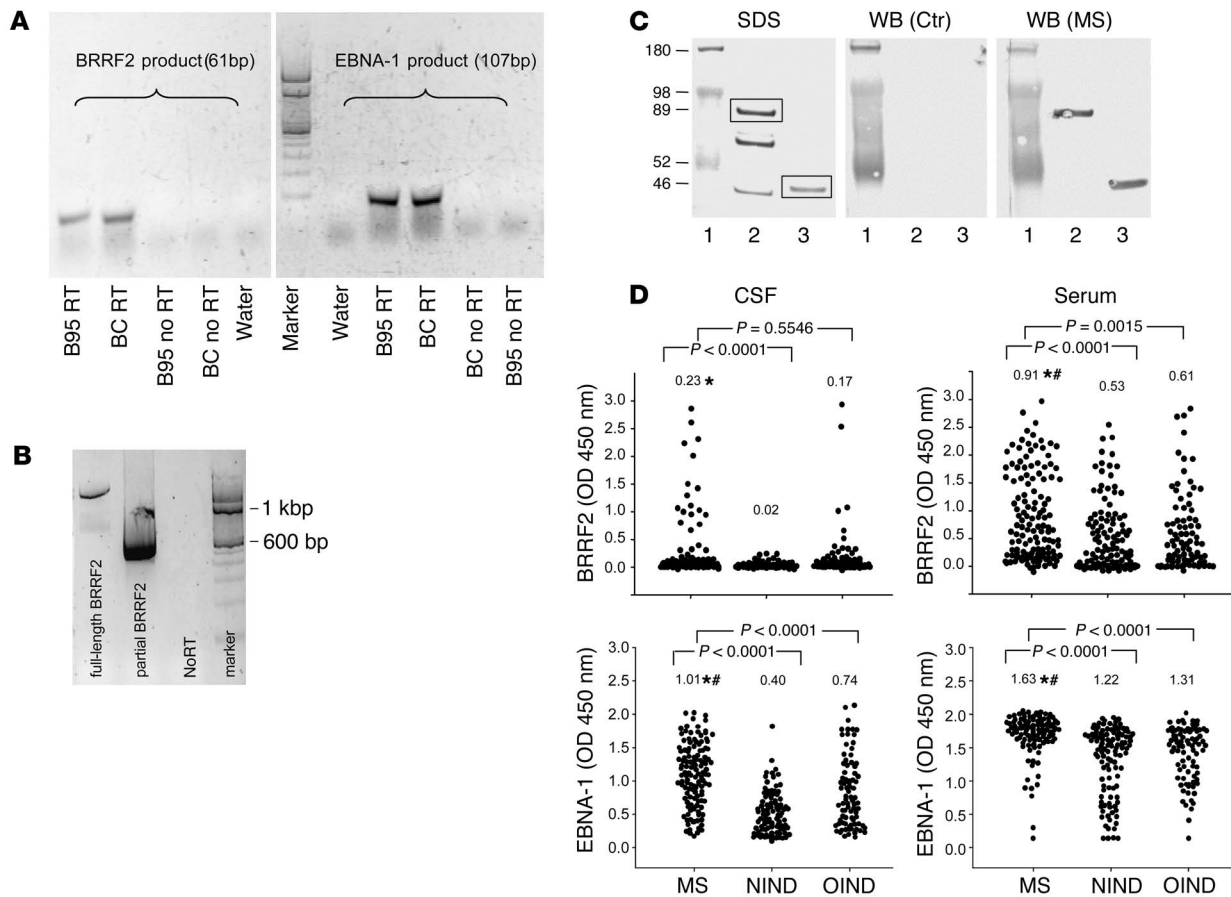
CSF bind specifically to these proteins. For this purpose, IgG antibodies in CSF were separated by IEF and blotted onto membranes precoated with the 2 EBV proteins. We found oligoclonal IgG patterns binding to both proteins in MS patients who were antibody positive in ELISA experiments (Figure 4B). The OCB reactivity of BRRF2 and EBNA-1 corresponded to the binding pattern of the initial antigens found on the protein array (compare left panels of Figures 1D and 4B). Furthermore, we demonstrated that oligoclonal IgG antibodies binding BRRF2 and EBNA-1 correspond to the OCBs that are observed by regular IEF immunoblot of CSF IgG (shown for 1 patient with BRRF2 in Figure 4C). In 3 patients with high EBNA-1 antibody titers in CSF, part of the oligoclonal bands were absorbed by preincubation of CSF with EBNA-1 but not GAPDH (Figure 4D). Finally, we confirmed the specificity and high affinity of EBV-specific CSF antibodies by solution phase assays, in which EBNA-1 and BRRF2 reactivity could be competed with the EBV proteins in soluble phase (Figure 4E).

Increased T cell responses to latent EBV protein in MS patients. To determine the extent of T cell responses to latent EBV pro-

teins, we transformed B cells from MS and control donors with EBV. The transformed cell lines expressed latent EBV proteins, including EBNA-1 and BRRF2 (Figure 3A), in the context of the autologous HLA molecules. These cell lines, which process the endogenous proteins into the HLA class I pathway, were used to determine the CD8⁺ T cell response to EBV proteins expressed in infected and transformed human B cells. Peripheral blood mononuclear cells of each patient were incubated with autologous EBV-transformed B cell lines, and the number of EBV-specific T cells in the CD4, CD8, and CD28 T cell subsets was determined by intracellular cytokine staining (Figure 5). We found a higher number of EBV-specific CD8⁺ T cells in MS patients than in controls. In particular, a significant difference ($P = 0.005$) was found for the CD8⁺CD28⁺ T cells. In contrast, no difference was observed for CD8⁺CD28⁻ and CD4⁺ T cells.

Discussion

Despite intense research efforts over the last decades, the cause of MS remains unknown. Although many findings support the

**Figure 3**

Expression and immunoreactivity to EBNA-1 and BRRF2. **(A)** BRRF2 and EBNA-1 RNA transcripts were detected in the B95 cell line (B95) and the EBV-transformed B cell line (BC) by BRRF2- (61 bp) and EBNA-1-specific (107 bp) RT-PCR. To exclude contamination with residual DNA, a control sample without reverse transcription (no RT) was included in the experiment. **(B)** Expression of full-length and partial BRRF2 RNA in the B95 cell line verified by RT-PCR. **(C)** BRRF2 expression in *E. coli*. The BRRF2 protein was cloned as partial (16 kDa) and full-length (58 kDa) protein with a 30-kDa GST tag resulting in 46-kDa partial and 88-kDa full-length band on SDS-PAGE (SDS) after Coomassie staining (left). Western blot (WB) and immunostaining with CSF of a control donor (Ctr; middle) and of a representative MS patient (right) confirmed the specific binding of CSF IgG to the BRRF2 proteins. **(D)** Immunoreactivity to recombinant BRRF2 (upper panels) and EBNA-1 (lower panels) was investigated by ELISA in CSF (left, 1:5 dilution) and serum (right, 1:100 dilution) of 130 MS patients compared with 115 NIND and 85 OIND patients. The immunoreactivities for each sample are given as OD values. Mean ODs and *P* values comparing the extent of immunoreactivity by Student's *t* test are displayed above each group. Fisher's exact test was applied to compare the frequencies of reactive patients: *Significant compared with NIND patients; #significant compared with OIND patients. RT, reverse transcription.

view of a highly selective acquired immune response in the CNS of MS patients, it has not been possible to determine the target(s) of this localized immune response within the CNS. In the current study, we used a broad screening approach to investigate the specificity of IgG antibodies in the CSF of MS patients. Starting from a human cDNA expression library, we identified several protein sequences that showed higher reactivity in MS patients than in controls. The 2 epitopes that showed the highest IgG reactivity in MS patients in comparison with those of control patients were mapped to obtain high-affinity ligands. In both cases, we identified peptide sequences from EBV proteins. The genes that coded for the 2 proteins are adjacently located in the EBV genome and expressed in latently EBV-infected cells. Humoral immune responses to these proteins were elevated in the serum and CSF of MS patients compared with that of controls. Intrathecal synthesis and binding to the OCBs were confirmed for both proteins. The relevance of this finding was further strengthened by the obser-

vation that MS patients show increased CD8 T cell responses to EBV proteins expressed in latently infected cells. These responses were mainly found in the CD8⁺CD28⁺ population, which is most relevant for the control of latent infection of EBV (27, 28). Since CD28⁺ memory cells are dominant in the CSF compartment in MS and other infectious diseases, it is conceivable to assume that this population of cells plays an important role in the CNS immune response (5, 29, 30).

Our findings are in line with previous epidemiological studies on the role of EBV in the pathogenesis of MS (31). EBV titers in serum are higher in MS patients than in control donors. Almost all MS patients are seropositive whereas between 5 and 10% of the general population are seronegative. Children who develop MS differ from controls by their higher immunoreactivity to EBV but not to other common viral pathogens (32). MS patients more frequently have a history of mononucleosis than controls (33). A 2- to 3-fold increased risk of developing MS after late EBV

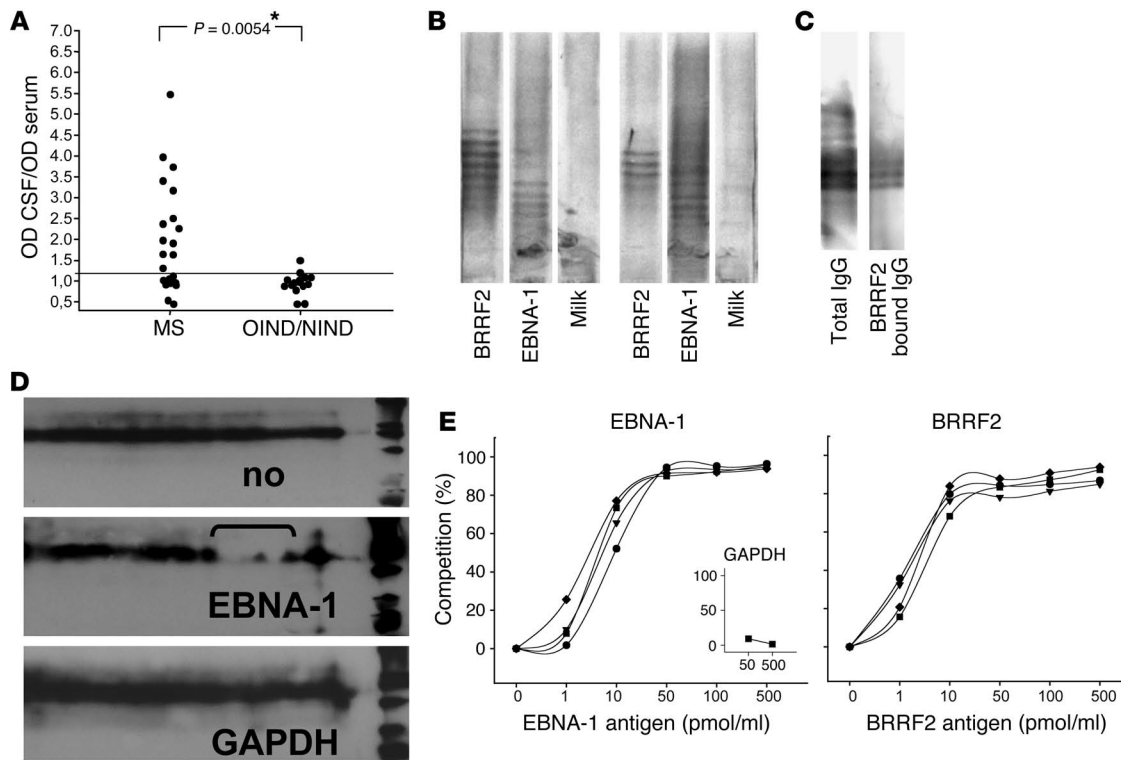


Figure 4

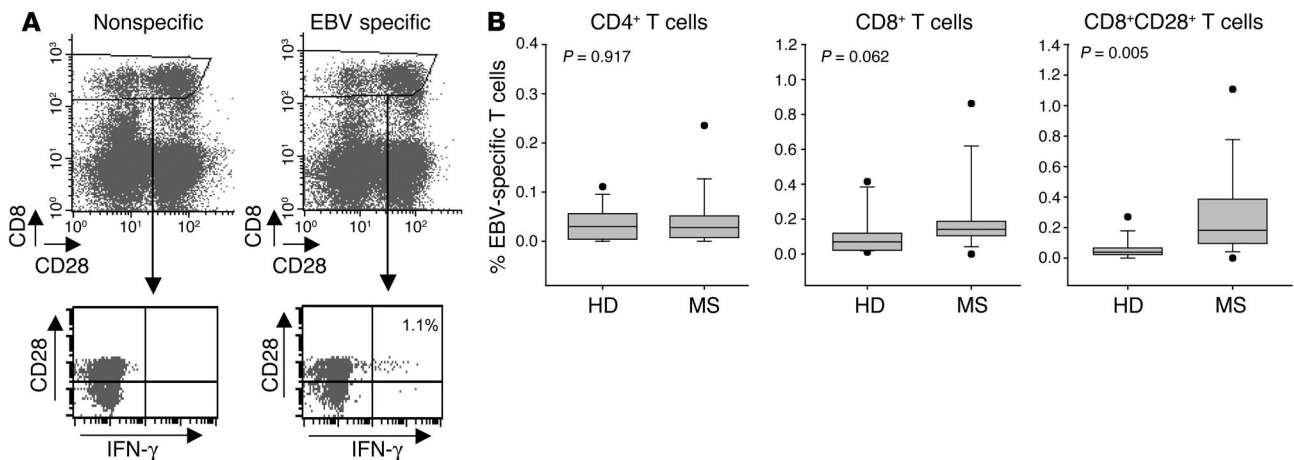
Specific intrathecal IgG response to EBV proteins. (A) Intrathecal IgG response to BRRF2 proteins in MS patients and control donors detected by ELISA. CSF and serum were adjusted to 10 mg/l IgG and the ratio of OD CSF/OD serum determined. Ratios above 1.2 indicate intrathecal synthesis. MS patients showed intrathecal BRRF2-specific IgG synthesis more frequently than controls. (B) IEF-immunoblot demonstrating specific binding of CSF oligoclonal IgG bands from 2 MS patients to the EBV proteins. The membranes were coated with BRRF2, EBNA-1, or a solution containing 10% milk alone as indicated. Affinity-blotted IgG was detected with anti-human IgG-HRP and visualized by TMB substrate. (C) IEF-immunoblot for BRRF2-specific OCBs (right) and total OCB pattern (left). Detection of bound IgG was performed as described in B. BRRF2-specific OCBs correspond to some of the major bands in the OCB pattern of the MS patient. (D) Loss of OCBs by preabsorption with EBNA-1 but not GAPDH. 2D-electrophoresis and IgG immunoblot of CSF from a patient with EBNA-1 immunoreactivity. No, no preabsorption of CSF-IgG was performed with EBNA-1 or GAPDH. (E) Solution phase assays demonstrate high affinity and specificity of CSF antibodies to EBNA-1 (left) and BRRF2 (right). Soluble EBV proteins at different dilutions were incubated with CSF and, subsequently, the remaining immunoreactivity measured by ELISA. Competition by soluble antigens is displayed. No competition was measured with GAPDH protein. For each assay, 4 MS patients were analyzed. *Fisher's exact probability test.

infection has been observed (34, 35). Studies have demonstrated that immunoreactivity to EBV was higher in donors who later developed MS than in those who did not (36, 37). Latent proteins have been suggested as bearing primary responsibility for the differences in immunoreactivity to EBV (38). Recently, an association between EBV reactivation and disease activity was reported whereas no association was found with other herpes viruses (39, 40). Another study found elevated EBV DNA levels in the blood of MS patients during relapses (41).

While our study provides evidence for increased humoral and cellular immunity to EBV in MS, the mechanisms through which EBV contributes to the pathogenesis of MS remains uncertain. Theoretically, 3 scenarios can be envisioned to explain the role of EBV in MS. First, EBV may persist in a niche in the central nervous system and periodically become reactivated. Glia cells, which seem to carry the EBV entry receptor CD21, can be infected with the virus under certain conditions (42–44). However, EBV RNA has not been detected in the lesions of MS patients by hybridization methods (45); this argues against permanent CNS infection. Second, the virus may

periodically spread from infected B cells to CNS tissue, resulting in a local antiviral immune response. In this case, EBV may only be detectable in the CNS during a narrow window preceding the appearance of inflammatory lesions. Third, EBV-infected peripheral B cells may trigger a crossreactive autoimmune response against CNS antigens. Reactivation of the virus in infected B cells that are potent antigen-presenting cells may periodically boost an autoimmune response in the CNS that involves antibodies and HLA-class I restricted T cell responses. Indeed, CD8⁺ T cells dominate MS lesions and are more often clonotypic than CD4⁺ T cells in MS tissue and CSF (4, 5). T cell cross-recognition between virus (e.g., EBV peptides) and myelin antigens was demonstrated on the cellular (46, 47) and structural levels (48). Cross-reactivity with viral antigens can trigger autoimmunity in animal models of MS (49). Given the life-long persistence of EBV in immunocompetent cells and its periodic reactivation, the virus has all the features which are required for a sustained cross-reactive autoimmune response.

Although we provide strong evidence for the role of EBV in the pathogenesis of MS, we are far from understanding the mechanisms

**Figure 5**

Frequency and phenotype of EBV-specific T cells in MS patients and healthy donors. **(A)** Strategy for detection of EBV-specific T cells in PBMC samples by intracellular IFN- γ staining and flow cytometry. PBMCs and autologous EBV-transformed B cell lines were either cultured separately (nonspecific activation, left graph) or in short-term coculture (EBV-specific activation, right graph) before staining. Shown are analyses of CD8 $^+$ T cells in the CD28 $^+$ and CD28 $^-$ compartments of an MS patient. One percent of CD8 $^+$ CD28 $^+$ T cells were EBV-specific in this patient. **(B)** Frequency of EBV-specific CD4 $^+$ and CD8 $^+$ T cells in 11 MS patients and 14 healthy donors (HD). No significant differences were observed in the CD4 $^+$ T cell compartments, whereas a higher frequency of EBV-specific CD8 $^+$ T cells was observed in MS patients. Further characterization revealed a significantly higher frequency of EBV-specific CD8 $^+$ CD28 $^+$ in MS patients compared with healthy controls. Student's *t* test was applied.

leading to disease development and progression. Further studies are necessary to clarify whether targeting EBV or the immune response against the virus may become therapeutic options in MS.

Methods

Patients and controls. MS patients and controls were recruited at the Philipps-University Department of Neurology. The MS patients were diagnosed according to the McDonald or Poser criteria (50). CSF was obtained by lumbar puncture. Since most MS patients received CSF analysis during the initial diagnostic workup, the MS group consisted predominantly of untreated MS patients. Control groups consisted of patients with NIND and OIND. The study was approved by the ethics committees at Philipps-University and Heinrich-Heine University.

Protein arrays. Protein arrays were developed at the Max Planck Institute for Molecular Genetics (Berlin, Germany) (51). Briefly, a human fetal brain cDNA library, hEx1, was directionally cloned into an *Escherichia coli* expression vector that allows isopropyl- β -D-thiogalactopyranoside-inducible (IPTG-inducible) expression of His $_6$ -tagged fusion proteins. The expression clones were detected by monoclonal anti-RGS-His antibodies, and the positive clones were arrayed onto high-density filters. Random analyses showed that about 66% of these clones contained inserts within a correct reading frame. Other clones were out of frame and expressed random AA sequences. For our study, hEx1 protein arrays were obtained from the Deutsches Ressourcenzentrum für Genomforschung GmbH (RZPD) (<http://www.rzpd.de/>) and were utilized for high-throughput antibody screening against 37,000 different expression clones, which were spotted in duplicates onto PVDF filter membranes (2 parts, each 26 cm \times 26 cm in size).

Proteins and peptides. Peptides and peptide membranes were synthesized with a soluble BRRF2-peptide (Jerini Ag) and membrane-bound peptides (Jerini Ag and MultiSynTech). The peptides for peptide scan and substitutional analysis were synthesized at a concentration of 100 μ g per spot. The EBV protein EBNA-1 (WAK - Chemie Medical GmbH) consisted of the last 340 AAs of the EBNA protein, including a part of the glycine-alanine rich region. The BRRF2 protein was not commercially available, and we

therefore recombinantly expressed this protein in *E. coli*, using the cloning approach described below.

Identification and purification of recombinant proteins. For screening with CSF antibodies, the protein array filters were first incubated with blocking buffer (3% nonfat milk in Tris-buffered saline [TBS]) for 2 hours and then incubated with the patient's CSF (adjusted to 1 mg/l IgG in blocking buffer) for 14 hours. After extensive washing with TBS containing 0.05% Tween-20 and 0.5% Triton X-100 (TBS-T), filters were incubated with HRP-conjugated anti-human IgG (1:2000 diluted in blocking buffer) for 1 hour. After several washings, the filters were developed by stabilized tetramethylbenzidine-blotting (TMB-blotting) substrate (Pierce) for 5 minutes. Blue spots in duplicates indicated specific binding of CSF antibodies to recombinant proteins.

The corresponding expression clones were obtained from the RZPD and were cultured in LB Broth Base medium (Invitrogen Corp.) supplemented with ampicillin. Their plasmids were isolated, and the cDNA inserts were sequenced for identification of the proteins. Subsequently, the expression of recombinant proteins was induced by 1 mM IPTG added to LB medium. The recombinant His-tag fusion proteins were then purified by immobilized metal ion-affinity chromatography. SDS-PAGE and Western blot analysis confirmed the binding of CSF antibodies to the purified proteins.

ELISA and Western blot. For ELISA experiments, 96-well ELISA plates (Dynex Technologies) were coated with 100 μ l of 10 μ g/ml purified recombinant protein in 0.05 M bicarbonate buffer (pH 9.6) and kept overnight at room temperature. Wells were then washed with PBS containing 0.05% Tween-20 (PBS-T) and blocked for 2 hours at room temperature with blocking buffer (2% nonfat milk in PBS). After washing, the plates were incubated with 100 μ l of diluted samples (CSF, 1:5 diluted and serum, 1:100 diluted in blocking buffer) for 2 hours at room temperature. After several washings with PBS-T, wells were incubated with 100 μ l of a 1:2000 dilution of HRP-conjugated anti-human IgG in blocking buffer for 1 hour. After washing, 100 μ l of TMB-developing solution (KPL; 1:1 TMB/hydrogen peroxide) was added to each well, which was then incubated at room temperature in the dark for 5 to 10 minutes. The reaction was



stopped by the addition of 100 μ l of 1 M H_2SO_4 and read at 450 nm. For negative controls, some wells were not incubated with sample, and some were not coated with antigen but were incubated with sample. To exclude bias, the samples from MS patients and control donors were placed randomly on the plates. In the initial experiments, the immunoreactivity was considered positive if the absorption reached a value of 0.3 or above (mean and 6 SD from mean of control donors).

For Western blot analysis, the proteins were separated on 4–12% SDS gels (Invitrogen Corp.) by electrophoresis. Gels were stained with Coomassie blue or transferred to nitrocellulose membranes, which were then processed as described for protein arrays except that the incubation time with CSF was shortened to 2 hours. The transfer of proteins was controlled by a positive ponceau staining.

IEF immunoblot. To determine the binding of oligoclonal CSF IgG bands to the antigens, an IEF affinity immunoblot was performed. Briefly, agarose IEF gels (pH 3–10) were loaded with 6 μ l undiluted CSF samples and the IEF was performed according to the manufacturer's instructions (Titan Gel Electrophoresis Kit; Helena BioSciences). After focusing the CSF IgG by IEF according to the isoelectric point, appropriate gel lanes were blotted on nitrocellulose membranes for 45 minutes using a 1.5 kg weight. Before blotting, the nitrocellulose membranes were cut to strips the size of gel lanes and precoated with 50 μ g/ml of each antigen in 0.1 M carbonate buffer (pH 9.5) for 4 hours or overnight. To block nonspecific binding, the membrane strips were subsequently incubated in a solution containing 10% low-fat milk powder for 2 hours at room temperature. After blotting, specifically bound IgG was detected by incubation with anti-human IgG HRP-antibody. The membranes were developed either with stabilized TMB substrate or with the more sensitive ECL detection system (Amersham Biosciences). For control experiments, a strip was precoated only with 10% milk or left completely uncoated for determination of total IgG pattern. For the comparison between CSF and serum OCB pattern, samples were diluted to 10 mg/l IgG.

Analysis of peptide reactivity. Immunoreactivity to peptides that were spotted at a concentration of 100 μ g on the membrane was quantified by Gelscan Standard software (BioSciTec) for the highest signals and the absolute and differential integrated density of each spot. Highest values reflect the best binding of antibody.

2D immunoblot electrophoresis and absorption. CSF was incubated several times for 20 minutes in wells coated with 100 μ g/ml EBNA-1 or the control protein GAPDH to absorb specific antibodies. 2D-electrophoresis with IPGRunner System (Invitrogen Corp.) was performed according to the manufacturer's instructions. Briefly, 20 μ l of absorbed and nonabsorbed CSF was diluted with dehydration buffer, and each sample was loaded on IPG strips (pH range 3–10), equilibrated, and run. Second dimension electrophoresis was done on 2–20% tris-glycine gel, and the proteins were blotted on nitrocellulose membranes. IgG was visualized by anti-human IgG and ECL detection.

RNA expression of BRRF2 and EBNA-1 in latently infected cells. RNA was isolated from EBV-transformed B cell lines and the B95 cell line with the RNeasy Mini Kit (QIAGEN) followed by digestion of genomic DNA by DNase (Ambion). Subsequently, a portion of isolated RNA was reverse transcribed to cDNA with SuperScript II RT (Invitrogen Corp.); the remainder was not reverse transcribed and served as a control. Both templates were used for amplification of BRRF2 and EBNA-1 genes to clarify whether these proteins are expressed. The following primers were used for amplification of BRRF2: the forward primer 5'-GAATTTGAGGCCTGGCTGAG-3' and the reverse primer 5'-TCGACGCCCGGAAAACCTC-3' and for amplification of EBNA-1: the forward primer 5'-AAGCCCGCTCTACTCTGCAA-3' and the reverse primer 5'-GCGGACGCCCTTCCA-3'. PCR consisted of 33 cycles at 94°C for 30 seconds, an annealing step at 60°C for 30 seconds, and an

amplification step at 72°C for 1 minute. To exclude contamination with residual DNA, control samples without reverse transcription were included in the experiment.

Cloning of BRRF2. Partial and full-length sequences of BRRF2 were amplified from DNA derived from EBV-infected B95 cell line. For cloning, forward primers were modified by addition of the CACC sequence at the 5' end, and reverse primers were modified with a TTA sequence at the 5' end. PCR products were cloned into the pENTR plasmid vector using the directional TOPO cloning kit (Invitrogen Corp.) and TOP10 *E. coli* cells. Subsequently, inserts were cloned into the pGEX-glutathione-S-transferase (pGEX-GST) vector by LR clonase reaction (Invitrogen Corp.) according to the manufacturer's instructions. GST-BRRF2 fusion protein was expressed and purified with glutathione columns (Pierce). Western blot analyses confirmed the expression of BRRF2 proteins. A truncated construct of BRRF2 (AAs 385–537, including the epitope sequence PARSRS) was used for ELISA and Western blot analysis.

Measurement of EBV-specific T cells. PBMCs from 11 MS patients and 15 healthy donors were purified by Ficol-Paque density centrifugation (Amersham Pharmacia). B cells from each donor were transformed with virus containing supernatant from the B95 cell line. Stable B cell lines were used as antigen-presenting cells. For measurement of EBV-specific T cells, 2×10^5 PBMCs were cocultured with 1×10^5 autologous EBV-transformed B cells in a round-bottom 96-well plate in a humidified incubator at 37°C and 5% CO_2 for 8 hours. For the last 2 hours, the cells were incubated in the presence of brefeldin A (5 μ g/ml) to enhance intracellular accumulation of IFN- γ . At the same time, PBMCs and EBV-transformed B cells from each donor were cultured separately but otherwise under the same conditions to determine the percentage of nonspecifically activated cells. The cells were combined directly before the staining procedure. Cocultured and separately cultured cells were washed and stained extracellularly using a mixture of monoclonal antibodies of CD8-PerCP, CD4-APC, and CD28-FITC (BD Biosciences – Pharmingen). Subsequently, cells were permeabilized with FACS permeabilization solution (BD Biosciences) and stained intracellularly with anti-IFN- γ -PE (Beckman Coulter–Immunotech) antibodies. IFN- γ -producing cells in each T cell subset were identified by 4-color flow cytometry on a FACSCalibur (BD Biosciences). The percentage of IFN- γ -producing cells in the coculture assay were subtracted from the number of IFN- γ -producing T cells in the control assay to determine the number of EBV-specific T cells in each subset.

Statistical analysis. Comparisons between patient groups were performed by Fisher's exact probability test and Student's *t* test where appropriate. *P* values below 0.05 were considered statistically significant.

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