

# Association of innate immune activation with latent Epstein-Barr virus in active MS lesions

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## ABSTRACT

**Objective:** To determine whether the activation of innate immune responses, which can be elicited by pathogenic and endogenous triggers, is associated with the presence of Epstein-Barr virus (EBV) infection in the multiple sclerosis (MS) brain.

**Methods:** White matter postmortem MS (n = 10) and control tissue (n = 11) was analyzed for the expression of the proinflammatory cytokine interferon  $\alpha$  (IFN $\alpha$ ) by immunohistochemistry and for EBV by using the highly sensitive method of EBV-encoded RNA (EBER) in situ hybridization.

**Results:** We detected overexpression of IFN $\alpha$  in active areas of white matter MS lesions but not in inactive MS lesions, normal-appearing white matter, or normal brains. The presence of IFN $\alpha$  in macrophages and microglia (expressing human leukocyte antigen class II) is suggestive of local production as part of an acute inflammatory process. Interestingly, EBERs were also specifically detected in areas where IFN $\alpha$  was overexpressed in these preselected active MS lesions. EBER+ cells were also found in CNS lymphoma and stroke cases, but were absent in other control brains. We next addressed a potential mechanism, e.g., the role of EBERs in eliciting IFN $\alpha$  production, and transfected EBERs into human embryonic kidney (HEK) cells. We used HEK cells that stably expressed Toll-like receptor-3, which recognizes double-stranded RNAs, associated with many viral infections. EBERs elicited IFN $\alpha$  production in vitro.

**Conclusion:** These findings suggest that latent EBV infection may contribute to the inflammatory milieu in active MS lesions by activating innate immune responses, e.g., IFN $\alpha$  production. Unraveling the underlying mechanisms may help in uncovering causal pathways and developing better treatment strategies for MS and other neuroinflammatory diseases. *Neurology*® 2012;78:15-23

## GLOSSARY

**ds** = double-stranded; **EBER** = EBV-encoded RNA; **EBNA-1** = EBV nuclear antigen 1; **EBV** = Epstein-Barr virus; **FCS** = fetal calf serum; **H&E** = hematoxylin & eosin; **HEK** = human embryonic kidney; **HLA** = human leukocyte antigen; **HSE** = herpes simplex encephalitis; **IFN $\alpha$**  = interferon  $\alpha$ ; **IgG** = immunoglobulin G; **ISH** = in situ hybridization; **LFB** = Luxol fast blue; **mAb** = monoclonal antibody; **MS** = multiple sclerosis; **OHL** = oral hairy leukoplakia; **PBS** = phosphate-buffered saline; **pDC** = plasmacytoid dendritic cell; **PLP** = protein lipid protein; **SLE** = systemic lupus erythematosus; **SSPE** = subacute sclerosing panencephalitis; **TLR3** = Toll-like receptor-3.

The innate immune response is an essential part of the host response to many infections and type I interferon (IFN) is produced within hours of systemic infection.<sup>1</sup> Our previous findings showed that Toll-like receptor-3 (TLR3) stimulation of human microglia with the viral-mimic polyIC led to IFN $\alpha$  production and downstream Th1 polarization of CD4+ helper T cells,<sup>2</sup> which may impact on CNS immunity.

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Supplemental data at [www.neurology.org](http://www.neurology.org)

Supplemental Data



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Infectious agents are plausible candidates for triggering and perpetuating multiple sclerosis (MS) in genetically susceptible subjects. The strength of its associations renders Epstein-Barr virus (EBV), a herpesvirus with a population prevalence >90%, the outstanding candidate. A history of symptomatic infectious mononucleosis increases the MS risk more than twofold<sup>3</sup> and elevated serum antibody titers to EBV nuclear antigen 1 (EBNA-1) precede the onset of MS, and are associated with MRI activity in established disease.<sup>4,5</sup> Furthermore, EBV infection was found to be a characteristic feature of the MS brain<sup>6</sup>; however, these findings were countered by other studies.<sup>7-9</sup>

EBV infects predominantly B lymphocytes and persists in the blood in rare memory B cells. The gold standard for detecting EBV in tissue is in situ hybridization for the EBERs, 2 small EBV-RNAs (EBER1 and EBER2), abundant in EBV-infected cells ( $10^6$ – $10^7$  copies/infected cell nucleus)<sup>10</sup> with immunomodulatory effects upon secretion and TLR3 ligation on neighboring cells.<sup>11</sup>

Our hypothesis is that active MS lesions carry an IFN $\alpha$  signature, which can be elicited by viral stimuli. We propose that innate activation in these areas, driven or maintained by EBV-encoded RNAs, leads to an antiviral state that contributes to neuroinflammation.

**METHODS Standard protocol approvals, registration, and patient consents.** Postmortem paraffin brain and control tissues were obtained from the Thomas-Willis-Oxford Brain Collection, Oxford, with informed consent and Ethics Committee approval (REC: 08/H0304/7).

**Tissue specimens.** For all MS brains studied, the clinical diagnosis had been made during life and confirmed at autopsy. Brains from patients with cancer and other neurologic and neuroinflammatory diseases were used as controls (table e-1 on the *Neurology*<sup>®</sup> Web site at [www.neurology.org](http://www.neurology.org)). To characterize white matter lesions, Luxol fast blue (LFB), anti-human leukocyte antigen (HLA) class II (macrophages/microglia), and hematoxylin & eosin (H&E) staining was performed to identify the centers and borders of lesions and their activity, and areas of hypercellularity (inflammatory cell infiltration) and hypocellularity (loss of parenchymal cells in inactive lesions). Lesions were classified into 3 groups: 1) acute lesions, rich in dense lymphocytic infiltrates with numerous B cells and macrophages (containing LFB); 2) chronic active lesions, characterized by hypercellular active borders staining strongly for LFB in macrophages and hypocellular demyelinated centers, with very low densities of LFB-negative macrophages; 3) inactive lesions, characterized by demyelination and marked hypocellularity (reflecting loss of parenchymal cells) and only limited numbers of LFB-negative

inflammatory cells. Case 13 is a stroke case with cause of death noted as cerebral hemorrhage with some B-cell infiltration, whereas case 12, without B-cell infiltration, died of pulmonary embolism.

**Immunohistochemistry for IFN $\alpha$ , gp350, LMP-1, and plasmacytoid-dendritic-cell markers.** Paraffin sections (6  $\mu$ m) used in this study were deparaffinized and antigens unmasked as described previously.<sup>12</sup> Sections were blocked with 10% fetal calf serum (FCS) followed by staining with sheep polyclonal anti-IFN $\alpha$  antibody at 1/1,000 dilution in 10% FCS. This antibody was raised against lymphoblastoid IFN $\alpha$  and is thus broadly cross-reactive with IFN $\alpha$  subtypes.<sup>13</sup> Antisheep immunoglobulin G (IgG) antibody (Vector, UK) was used as secondary antibody followed by avidin-biotin peroxidase complex (ABC kit, Vector, UK) and detected with diaminobenzidine substrate (DAKO, UK). B cells in MS lesions were stained with anti-CD79 $\alpha$ , which is part of the B cell receptor complex (JCB117 clone, donated by Prof. Margaret Jones),<sup>14</sup> and developed with Dako Real Envision Detection System as described previously.<sup>12</sup> LMP-1 and gp350/220 were detected with the mouse IgG1 monoclonal antibodies (mAb) OT21C (LMP-1; 1/500) and OT6 (gp350; 1/2,000), both of mouse origin (IgG1), in phosphate-buffered saline (PBS) containing 1% bovine serum albumin.<sup>15</sup> Binding was visualized with labeled polymer HRP (peroxidase-labeled polymer conjugated to goat antimouse and goat antirabbit Igs in Tris-HCl buffer, Dako, UK) for 30 minutes followed by DAB as above. All sections were counterstained with hematoxylin. pDCs staining was done as described previously using BDCA2 clone 124B3.13 (1/50) (Dendritics, France)<sup>16,17</sup>; CD2ap, clone B-4 (1/1,000; Santa Cruz Biotechnology, USA)<sup>18</sup>; and CD123, clone 7G3 (1/50; BD Biosciences, USA).

**Immunofluorescence staining.** For double-labeling, IFN $\alpha$  staining was performed as described above, followed by biotin-antisheep Ig (Vector, UK), visualized with conjugated streptavidin alexa-488 (Invitrogen, Paisley, UK). Slides were incubated overnight with second primary antibodies: rat anti-major histocompatibility complex class II (1/200 dilution; Dako, UK), rat anti-CD3 (1/100; Serotec UK), mouse anti-CD19 (1/50; Serotec, UK), rabbit anti-GFAP (1/1,000; Dako, UK), or mouse anti-protein lipid protein (PLP) (1/200; Serotec). Appropriate secondary antibodies included Alexa 568 anti-rat Ig (1/200; Invitrogen, UK), Alexa 568 antimouse Ig (1/200), and Alexa Fluor 568-antirabbit (1/200) for 3 hours. As negative controls for each second layer, primary antibodies were omitted. No staining was seen in these sections.

**In situ hybridization for the detection of EBERs in brain tissue.** The EBER-in situ hybridization (ISH) was carried out essentially as previously described in detail.<sup>10,19</sup> Two antisense oligonucleotides (30 nucleotides each) corresponding to positions 91–120 and 82–111 of EBER 1 and EBER 2, respectively, were synthesized (Sigma, UK) and end-labeled by tailing with digoxigenin-dUTP using a commercially available kit (Roche Diagnostics, UK), following the manufacturer's instructions. Briefly, 6- $\mu$ m sections on sialinized slides were dewaxed and digested with 100  $\mu$ g/mL proteinase K for 15 minutes in a 37°C incubator. Sections were subsequently washed in water and dehydrated in ethanol. Sections were incubated with hybridization buffer containing 100 ng/mL of EBER-1/EBER-2 probe. The EBER probes and target nucleic acid were denatured using microwave irradiation followed by hybridization overnight at 42°C in a hot air oven. After high-stringency washes in  $0.1 \times$  SSC at 55°C, hybridized probes were detected using mouse

antidigoxin mAb (1/5,000; Sigma, UK) followed by the avidin-biotin complex peroxidase technique (ABC-Elite kit, Vector Laboratories, UK) and DAB as the chromogenic substrate. We examined all lesions blindly, i.e., without knowledge of the other results or the histologic status of the MS lesions. A negative control (using PBS instead of EBER probes) was included with each sample. Hodgkin lymphoma or post-transplant lymphoma (PTLD) sections were included as positive controls with each batch. In our previous studies,<sup>10,19</sup> negative controls included treatment of sections with RNase prior to hybridization with EBER probes and using random probes of the same size and GC content to EBER probes. Our findings consistently showed that our EBER probes were highly specific, giving positive signals only in samples containing EBV.

**EBER-encoding plasmids.** Plasmids encoding EBER1 or EBER2 were prepared by cloning PCR-amplified B95-8 EBV EBER1 or EBER2 DNA digested with *Bgl*II and *Hind*III into pSUPER (OligoEngine Ltd., Seattle) between the *Bgl*II and *Hind*III sites. EBER plasmids were calibrated and found to give transient expression similar to that found in EBV-infected cells as determined by Northern blotting and flow cytometric staining for EBER-RNA.

Primers were as follows:

EBER1 *Bgl* II CCAGATCTCCAGGACCTACGCTGCCCT  
EBER1 *Hind* III CCAAGCTTGGATGCATAAATCCTAA  
EBER 2 *Bgl* II CCAGATCTCCAGGACAGCCGTTGCCCT  
EBER 2 *Hind* III CCAAGCTTGGGTGCAAACTAGCCA

**Transfection of EBER-encoding plasmids into HEK293/TLR3 cells.** HEK293 cells stably transfected with TLR3 (Invivogen, UK) were cultured in Dulbecco modified Eagle medium supplemented with 10% FCS, penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL) and 5  $\mu$ g/mL of blastomycin for selection.

Plasmids encoding EBER1, EBER2, or empty vector were used to transfect HEK293/TLR3 cells, seeded at  $7.5 \times 10^4$ /mL in 24-well plates. Cells were 80% confluent at the time of transfection with Fugene HD (Roche) at a ratio of 0.75  $\mu$ g Fugene: 0.5  $\mu$ g of DNA. A GFP-encoding plasmid (Lonza, UK) was cotransfected as a control and comparable transfection efficiency checked by microscopy. As positive controls, cells were transfected with polyIC at the same ratio. Transfections were performed in duplicate and supernatants were collected after 24 hours of culture; their IFN $\alpha$  content was determined with a human IFN $\alpha$  multi-subtype ELISA kit (PBL Interferon Source, UK).

**Data acquisition and statistical analysis.** The numbers of IFN $\alpha$ + cells/mm<sup>2</sup> were counted in 3 100 $\times$  parenchymal fields per lesion with a total area =  $1.735 \times 10^6 \mu\text{m}^2$  using ImageJ (NIH). Digital images of tissue sections were made with Olympus light microscope BX41 and Zeiss camera (Zeiss, Thornwood, NY). To investigate IFN $\alpha$  protein colocalization, multiple images were acquired using a confocal microscope (BioRad, Hercules, CA). We performed semiquantification by counting of EBER+, LMP-1+, and gp350+ cells in perivascular cuffs and parenchyma of MS lesions with different activity and observations were confirmed blindly by a second pathologist. Statistical analysis was performed using analysis of variance (SPSS, IBM).

**RESULTS IFN $\alpha$  is expressed in active areas of MS lesions by HLA class II-expressing cells.** To investigate the potential role of innate immunity in the pathogenesis of MS, we studied the expression of

IFN $\alpha$  within white matter of MS lesions with different disease activity. Large numbers of cells labeling very strongly for IFN $\alpha$ + were observed consistently in all the acute lesions (figure 1A) and borders of chronic active MS lesions (figure 1B). In contrast, we found  $\sim 5$  times fewer IFN $\alpha$ + cells in the inactive centers of chronic active MS lesions (figure 1C) and in inactive MS lesions (figure 1D). IFN $\alpha$ + cells were rare in normal-appearing white matter (figure 1E) and normal control brain tissue (figure 1F). These differences and their significance are quantified in figure 1J.

The IFN $\alpha$ -producing cells were consistently HLA class II+ in MS lesions, and showed the morphology of macrophages/microglia (figure 1, G–I). In sharp contrast, B cells (CD19+), T cells (CD3+), astrocytes (GFAP+), and oligodendrocytes (PLP+) showed no detectable immunoreactivity for IFN $\alpha$  (figure e-1A).

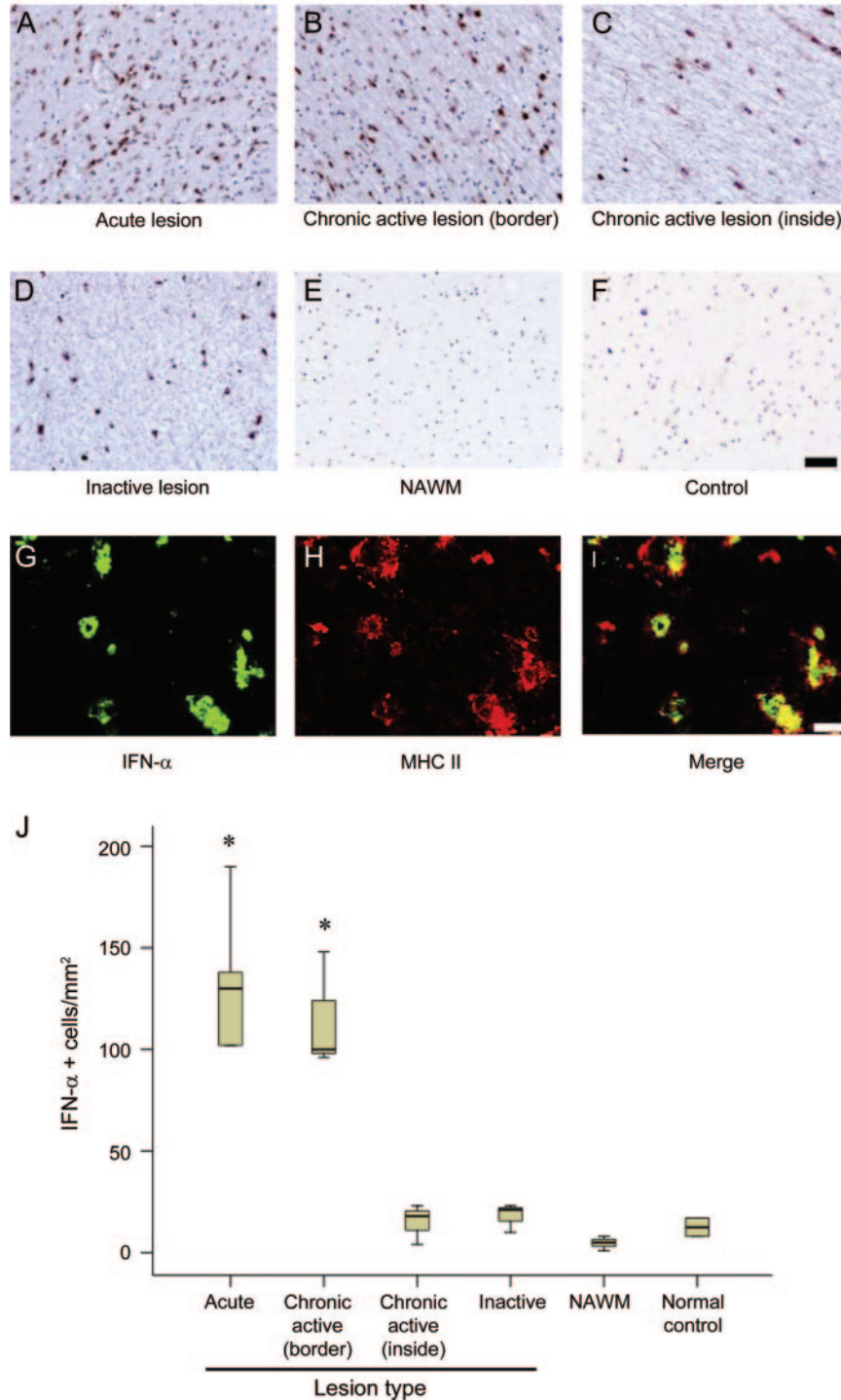
**Detection of EBV RNAs and proteins in active MS lesions that show IFN $\alpha$  signatures.** We next checked for the presence of EBV-infected cells in active MS lesions overexpressing IFN $\alpha$  by EBER-ISH. This approach, therefore, represents a preselection strategy to identify active MS lesions, and increase the possibility of finding EBER+ cells. Positive controls included Hodgkin-, post-transplant-, and AIDS-associated CNS lymphomas and oral hairy leukoplakia (OHL), all of which were known to be EBV+. Our disease controls were 2 cases of stroke and 1 each with subacute sclerosing panencephalitis (SSPE) and herpes simplex encephalitis (HSE), and there were 2 healthy control brains (table e-1).

We observed EBER+ cells in white matter areas of all the active MS lesions (figure 2A), including active areas of chronic-active MS lesions (figure 4A and figure e-2, C). In all cases, the EBER signal was nuclear, as in the Hodgkin lymphoma control (figure 2D). We semiquantified EBER+ cells in perivascular cuffs and categorized cases into (–) no EBER+ cells; (+) 1–5 EBER+ cells; (++) 6–20 EBER+ cells; (+++) more than 20 EBER+ cells; and (++++) more than 20 EBER+ cells in perivascular cuffs and additional dispersed positive cells in parenchyma as shown in table e-1. The perivascular areas of most active MS cases harbored around 20 EBER+ cells.

We next checked for EBV-encoded markers for its latency program II/III (LMP-1) and lytic replication (gp350). The LMP-1 antibody stained cytoplasm and surface membranes in the Hodgkin lymphoma (figure 2E) as previously described.<sup>20</sup> We observed cross-reactivity of LMP-1 with  $\alpha$ -synuclein in brain tissue, which led to strong background staining, particularly in the cortex rather than white

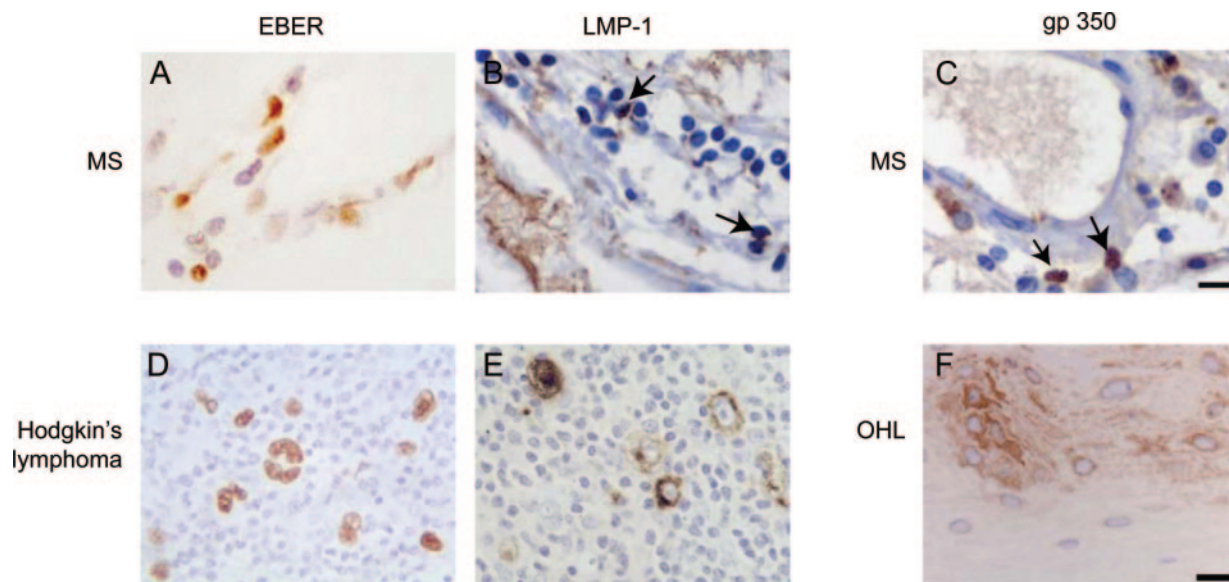


**Figure 1** Interferon  $\alpha$  (IFN $\alpha$ ) is expressed in white matter areas of active multiple sclerosis (MS) lesions by human leukocyte antigen (HLA) class II+ cells



(A-F) IFN $\alpha$  immunostaining (brown signal) in MS lesions of different activity. (A) Acute lesion (case 1). In (B), an active border of a chronic active lesion is seen (case 6A) and in (C), an inactive center of a chronic active lesion (case 6A). (D) Inactive lesion (case 6B), (E) normal-appearing white matter (NAWM) (case 3), and (F) control brain tissue (case 8). All sections were counterstained with hematoxylin. Scale bar = 50  $\mu$ m. In (G), staining of active area of chronic active lesion (case 5) for IFN $\alpha$  (green), and in (H) for major histocompatibility complex (MHC) class II (macrophages/microglia; red). (I) Overlay of IFN $\alpha$  and MHC class II staining (yellow). Scale bar: 10  $\mu$ m. (J) Histogram shows quantitative analysis of density of IFN $\alpha$ + cells in MS lesions of different activity, in NAWM and in control brain (cases 1-9). Significantly higher densities of IFN $\alpha$ + cells were present in acute MS lesions ( $130 \pm 9.4$  cells/mm<sup>2</sup>) and active borders of chronic active MS lesions ( $114.8 \pm 9.7$  cells/mm<sup>2</sup>) compared to inactive MS lesions ( $18.22 \pm 2.8$  cells/mm<sup>2</sup>), NAWM ( $4.4 \pm 1.2$  cells/mm<sup>2</sup>), and control tissue ( $12.25 \pm 2.0$  cells/mm<sup>2</sup>),  $p < 0.0001$ .

**Figure 2** Epstein-Barr virus (EBV) infection is found in active multiple sclerosis (MS) lesions by in situ hybridization and immunohistochemistry

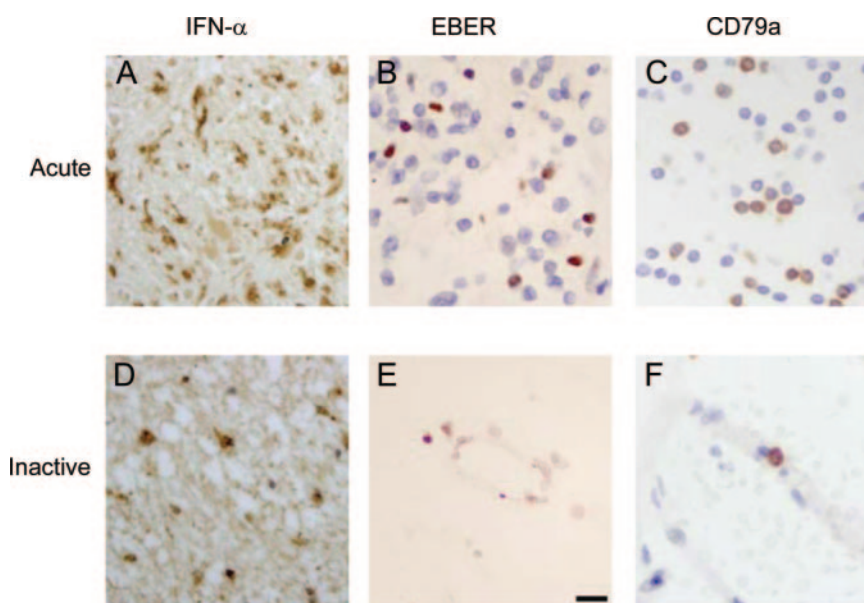


(A, D) Expression of EBV-encoded RNA (EBER) (EBER1/EBER2, brown signal) in active MS lesion (A: case 4B) and Hodgkin lymphoma case (D: case 15). (B, E) Latent membrane EBV protein (LMP-1) was occasionally detected in the active MS lesion (B: case 4B) and Hodgkin lymphoma (E: case 16). (C, F) Lytic virus replication, as assessed by staining with gp350 antibody, was only occasionally detected in the active MS lesion (C: case 4B) but was pronounced in oral hairy leukoplakia (OHL) (F: case 18). A-C: scale bar: 20  $\mu$ m; D-F: scale bar: 30  $\mu$ m.

matter areas.<sup>21</sup> We detected gp350+ cells in OHL tissue (figure 2F). We only found the occasional LMP-1+ and gp350+ cell in EBER+ active MS lesions (figure 2, B and C). The results of this semi-quantification are shown in table e-1. We also

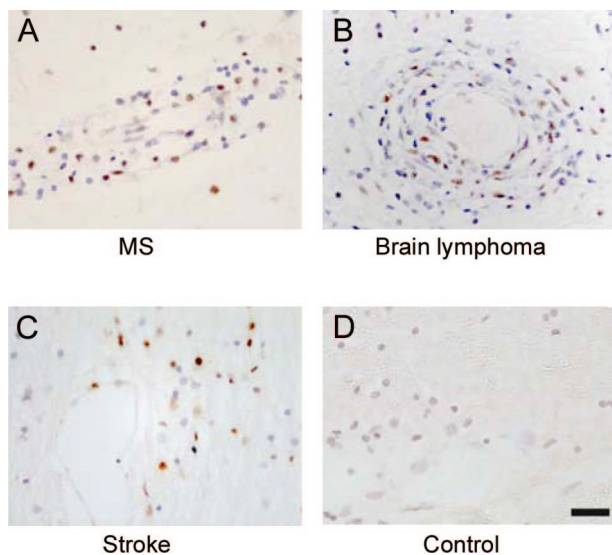
stained consecutive sections for EBERs and IFN $\alpha$ . Indeed, areas of acute MS lesions consistently exhibited a dual IFN $\alpha$ /EBER signature (figure 3, A and B). EBER+ and IFN $\alpha$ + cells were found in perivascular areas, however, IFN $\alpha$ + cells were also promi-

**Figure 3** Active multiple sclerosis (MS) lesions carry an interferon  $\alpha$  (IFN $\alpha$ )/EBV-encoded RNA (EBER)+ signature



IFN $\alpha$ + cells (A) and EBER+ cells (B) in white matter areas of an acute MS lesion (case 3). IFN $\alpha$ + cells (D) and EBER+ cells (E) can only occasionally be found in white matter areas of an inactive MS lesion (case 6B). CD79 $\alpha$ + B cells in acute MS lesion (C) and inactive MS lesion (F). Scale bar: 20  $\mu$ m.

**Figure 4** In situ hybridization for EBV-encoded RNA (EBER) in non-multiple sclerosis (MS) control CNS tissue



(A) Presence of EBER+ cells (brown signal) in perivascular area of active MS lesion (case 3) and (B) in HIV-associated CNS lymphoma (case 14). (C) Perivascular area of stroke case (case 12) and in (D) another neurologic control (case 8). Scale bar: 40  $\mu$ m.

ment in the parenchyma, whereas inactive MS lesions showed only the occasional IFN $\alpha$  or EBER+ cell (figure 3, D and E). The majority of perivascular and parenchymal EBER+ cells had the morphology and distribution of B cells, which correlated with lesion activity (figure 3, B, C, E, and F).

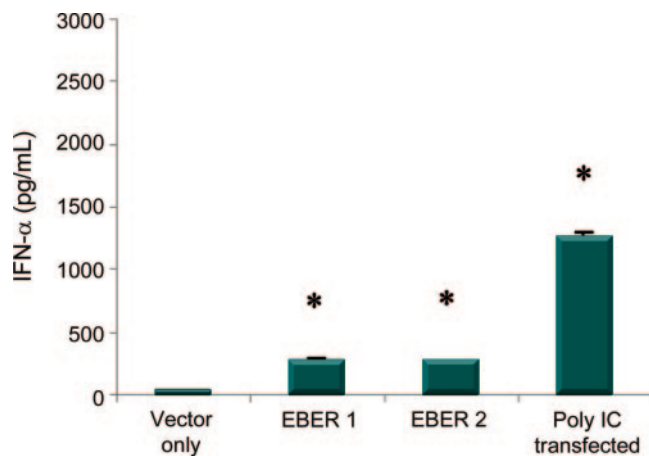
We conclude that EBV infection is present in active white matter MS lesions. Most infected cells harbor latent virus. EBER+ cells were more prominent

than LMP-1+ cells, only occasional cells undergoing lytic replication.

**Is cerebral expression of EBERs restricted to MS?** We next studied brains affected by a variety of other disorders. EBER+ cells were detected in perivascular areas in HIV-associated CNS lymphoma (figure 4B). Stroke is another neurologic disease, where inflammation appears to play an important early role in pathogenesis.<sup>22</sup> Interestingly, we detected EBER+ cells in 2 cases of stroke (figure 4C and figure e-2, E) but not in the white matter of healthy control brains (figure 4D). The EBER+ cells were dispersedly located all over the inflammatory area (both perivascular and in the parenchyma). Evidently, EBER+ cells can be found in other inflammatory conditions such as stroke, which raises interesting questions about their contribution to neuroinflammation in general.

**EBERs elicit IFN $\alpha$  production in TLR3-expressing HEK cell cultures.** Finally, we tested whether EBERs could elicit IFN $\alpha$  production and thus contribute to neuroinflammation in the CNS. EBERs form double-stranded (ds) RNA-like molecules with stem-loop structures.<sup>23</sup> Virally encoded dsRNAs are one of the proximal inducers of type I IFN production, and their effects can be mimicked by treatment of uninfected cells with the synthetic dsRNA, poly(I):poly(C) (polyIC).<sup>24</sup> HEK cells, stably expressing TLR3, were supertransfected with polyIC, which induced significant IFN $\alpha$  production (figure 5). Production of IFN $\alpha$  also increased significantly when these cells were transfected instead with either EBER1 or EBER2 expression plasmids. Thus even latent EBV infection can trigger IFN $\alpha$  production observed in active MS lesions, and therefore contribute to the neuroinflammation.

**Figure 5** Epstein-Barr virus-encoded RNA (EBER)1 and EBER2 transfection into TLR3-expressing human embryonic kidney (HEK)293 cells elicits interferon  $\alpha$  (IFN $\alpha$ ) production



Significant production of IFN $\alpha$  upon transfection of TLR3-expressing HEK293 cells with plasmids encoding EBER1 or EBER2 (EBER1:  $p = 0.0064$ , EBER2:  $p = 0.024$ , vs vector alone) and polyIC ( $p = 0.0118$ ). Data are also shown for cells transfected with empty vector or poly IC. The graph shows mean levels of IFN $\alpha$  production 24 hours post-transfection. Error bars represent the SD of 3 independent experiments.

**DISCUSSION** The MS brain shows characteristic inflammation that targets myelin sheaths and leads to focal destruction and demyelinated plaques in the white matter. Analysis of white matter areas revealed overexpression of the proinflammatory cytokine IFN $\alpha$  in active MS lesions. Notably, we found EBER+ cells in areas of IFN $\alpha$  overexpression. Furthermore, the occasional LMP-1+ and gp350+ cells were also present in EBER+ MS lesions. Whereas we detected no signs of EBV infection in white matter areas of SSPE, HSE, and control brains, EBER+ cells were also present in CNS lymphoma and stroke. We also showed that EBERs can elicit IFN $\alpha$  production by TLR3+ HEK cells, and can thus contribute to the inflammation in active MS lesions. Together with the overexpression of IFN $\alpha$  that we found there too, these findings imply activation of innate immune responses associated with the hitherto unre-



ported EBV infection, consistent with its proposed involvement in MS pathogenesis. Our findings suggest that latent EBV infection may contribute significantly to neuroinflammation in patients with MS and possible others, too.

Production of IFN $\alpha$  is seen during many different systemic virus infections, e.g., EBV,<sup>25</sup> Sendai,<sup>26</sup> and measles viruses.<sup>27</sup> However, there are endogenous stimuli too, as in systemic lupus erythematosus (SLE)<sup>28</sup> and Aicardi-Goutières syndrome.<sup>29</sup> Several CNS disorders such as encephalitis<sup>30</sup> and neuropsychiatric lupus<sup>28</sup> have been linked to dysregulation of IFN $\alpha$  responses. We found HLA class II+ cells with the morphology of microglia and macrophages expressed IFN $\alpha$  in active MS lesions in line with previous study.<sup>31</sup> The presence of plasmacytoid dendritic cells (pDCs) was assessed with 3 pDCs markers (BDCA-2, CD123, CD2ap); pDCs were not detected in active MS lesions in contrast to previous findings<sup>32</sup> (figure e-1B).

Though the possible roles of viruses in the pathogenesis of MS have been discussed for many years,<sup>33–35</sup> there are conflicting recent reports on EBV infection in the MS brain.<sup>6–9</sup> Our findings are in line with previous reports, which describe the presence of EBER+ cells in the MS lesions<sup>6</sup> and the observation that active EBV infection is not a characteristic feature of the MS brain.<sup>7–9</sup> However, our study included a novel preselection strategy and focused only on white matter MS lesions rather than meningeal areas. We believe that there are 2 primary reasons for the discrepancies in previous reports: the sensitivity and specificity of the methodology and the clinical material used. Techniques such as PCR, albeit very sensitive, are dependent on the quality of the extracted DNA, which is generally very poor from formalin-fixed tissues and amplification of larger fragments of EBV (>300 bp) is not consistently possible.<sup>36</sup> In this context, using EBER-ISH is highly favored. However, during the establishment of the EBER-ISH methodology, we have identified that factors such as tissue digestion, probe design, labeling of EBER probes, amount of probe used, microwave heating prior to overnight hybridization, and the stringency washes posthybridization influenced its sensitivity and specificity.<sup>10</sup> Our probe targets EBER1 and EBER2 in contrast to all the other studies.<sup>6–9</sup> With our optimized protocol, we have consistently been able to detect as little as a few EBV-infected lymphocytes in entire sections.<sup>37</sup>

As we found EBER+ cells not only in white matter areas of these active MS lesions but also CNS lymphoma and 2 cases of stroke, we conclude that the presence of EBV may not be unique to the MS brain in contrast to previous findings.<sup>6</sup> Indeed, some

EBER+ cells may well be included in the inflammatory infiltrate, attracted by locally produced chemokines and cytokines. We did not find EBER+ cells in our SSPE and HSE cases. The inflammatory infiltrate may differ in SSPE and HSE. Alternatively these cases may not yet be infected with EBV, as in the teenage SSPE case.

Our data addressing a potential mechanism of action, e.g., the role of EBERs in eliciting IFN $\alpha$  production, were supported by recent findings, which showed active secretion of EBERs and binding to extracellular TLR3 on dendritic cells, which triggered type I IFN production.<sup>11</sup> More recently, expression of EBER1 in lymphoblastoid cell lines was found to regulate IFN $\alpha$ 6,  $\alpha$ 5, and  $\alpha$ 4 genes.<sup>38</sup> In addition, binding of EBERs to cytosolic “receptor” retinoic acid inducible gene 1 (RIG-I) also induced type-I IFN production.<sup>39,40</sup>

Future studies should include more suitable control cases as this will be crucial to understand the role of EBV in MS. Further characterization of EBER+ cells is also warranted as not all cells have the morphology of B cells, which is technically extremely difficult given the few EBV-infected cells and problems in combining immunohistochemistry with in situ hybridization without compromising sensitivity and specificity.

Our study indicates that EBV may be more subtle than we anticipated as active EBV infection is not a characteristic feature of the MS brain. Perhaps that is not too surprising as EBV is a persistent virus with the aim to coexist rather than eradicate the host. Thus our study casts new light on mechanistic interactions of viral RNAs and innate immune activation in the CNS, and may highlight the propensity of latent viral infections to contribute to neuroinflammation in the CNS, not only in MS but also in other neuroinflammatory diseases.

## AUTHOR CONTRIBUTIONS

Dr. Tzartos: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, contribution of vital reagents/tools/patients, acquisition of data. Prof. Khan: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, contribution of vital reagents/tools/patients, acquisition of data, obtaining funding. Dr. Vossenkamper: drafting/revising the manuscript, analysis or interpretation of data, contribution of vital reagents/tools/patients, acquisition of data. Dr. Cruz-Sadaba: drafting/revising the manuscript, analysis or interpretation of data, contribution of vital reagents/tools/patients, acquisition of data. S. Lonardi: drafting/revising the manuscript, acquisition of data. E. Sefia: analysis or interpretation of data, acquisition of data. Dr. Meager: drafting/revising the manuscript, contribution of vital reagents/tools/patients. Dr. Elia: drafting/revising the manuscript, analysis or interpretation of data, acquisition of data. Prof. Middeldorp: drafting/revising the manuscript, analysis or interpretation of data, contribution of vital reagents/tools/patients, data analysis and interpretation. Prof. Clemens: drafting/revising the manuscript, analysis or interpretation of data. Prof. Farrell: study concept or design, analysis or interpretation of data, contribution of vital reagents/tools/patients. Prof. Giovannoni: drafting/revising the manuscript, study concept or design, analysis or interpretation of

data, study supervision, obtaining funding. Dr. Meier: drafting/revising the manuscript, study concept or design, analysis or interpretation of data, contribution of vital reagents/tools/patients, acquisition of data, statistical analysis, study supervision, obtaining funding.

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## DISCLOSURE

Dr. Tzartos has a patent pending re: Methods for detecting the presence of target molecules in a biological sample. Prof. Khan reports no disclosures. Dr. Vossenkamper receives research support from the Medical Research Council, UK. Dr. Cruz-Sadaba receives research support from the Medical Research Council, UK. Dr. Lonardi and E. Sefia report no disclosures. Dr. Meager serves on the editorial board of the *Journal of Interferon and Cytokine Research* and receives publishing royalties for *The Interferons: Characterization and Application* (Wiley-VCH Verlag GmbH & Co. KGaA, 2006). Dr. Elia receives research support from The Wellcome Trust. Prof. Middeldorp reports no disclosures. Prof. Clemens receives research support from The Wellcome Trust. Prof. Farrell serves on the editorial boards of the *Journal of Virology* and the *Journal of General Virology* and receives research support from Leukaemia and Lymphoma Research, UK. Prof. Giovannoni serves on scientific advisory boards for Merck Serono and Biogen Idec and Vertex Pharmaceuticals; served on the editorial board of *Multiple Sclerosis*; has received speaker honoraria from Bayer Schering Pharma, Merck Serono, Biogen Idec, Pfizer Inc, Teva Pharmaceutical Industries Ltd.–sanofi-aventis, Vertex Pharmaceuticals, Genzyme Corporation, Ironwood, and Novartis; has served as a consultant for Bayer Schering Pharma, Biogen Idec, GlaxoSmithKline, Merck Serono, Protein Discovery Laboratories, Teva Pharmaceutical Industries Ltd.–sanofi-aventis, UCB, Vertex Pharmaceuticals, GW Pharma, Novartis, and FivePrime; serves on the speakers bureau for Merck Serono; and has received research support from Bayer Schering Pharma, Biogen Idec, Merck Serono, Novartis, UCB, Merz Pharmaceuticals, LLC, Teva Pharmaceutical Industries Ltd.–sanofi-aventis, GW Pharma, and Ironwood. Dr. Meier receives research support from British Technology Group, ABN/MS Society, Aims2Cure, and the Roan Charitable Trust.

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## Neurologists Needed to Volunteer in Haiti

The AAN is working with Operation Blessing International (OBI) to promote opportunities for neurologists to aid the victims of the January 2010 earthquake in Haiti. For one to two weeks, physician volunteers will care for patients with a variety of needs, and offer neurologic care when necessary. To learn more about the work of OBI and this unique volunteer program, visit [www.ob.org/haitiprojects/volunteer.asp](http://www.ob.org/haitiprojects/volunteer.asp).

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