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EBV Dysregulation Is Associated With Immune Imbalance in Multiple Sclerosis

Evidence From Integrated Viral and Host Analyses

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Abstract

Background and Objectives

Epstein-Barr virus (EBV) infection is a prerequisite for the development of multiple sclerosis (MS), yet whether EBV acts merely as a trigger at disease onset or also contributes to immune dysregulation and disease progression remains unclear. To explore potential mechanisms linking EBV to immune alterations, we performed a comprehensive analysis of EBV markers and immune-related gene expression in peripheral blood samples from therapy-naïve persons with MS (PwMS) and healthy donors (HD) and assessed EBV transcripts in CSF cells to explore compartment-specific viral activity.

Methods

Peripheral blood mononuclear cells (PBMCs) and serum from PwMS (n = 77) and HD (n = 40) were analyzed. EBV serology, DNA load, and RNA expression were assessed by ELISA, droplet digital PCR, and preamplified real-time RT-PCR, respectively. EBV RNA was also evaluated in PwMS CSF cells. Gene expression profiling of 47 immune-related genes selected for their relevance to MS was also performed in PBMCs. Data were analyzed using univariate and multivariate statistical approaches also considering demographic, clinical, and radiologic information. Exploratory factor analysis (EFA) was used to identify transcriptional signatures associated with MS.

Results

Anti-EBNA1 IgG titers were higher in PwMS. In addition, EBV RNA and DNA were more frequently detected, and viral load was increased compared with HD. Notably, EBV transcripts associated with latency II/III (LMP1, LMP2A, EBNA1, EBNA3A) and lytic reactivation (BZLF1, gp350/220) were more prevalent in PwMS. Although viral RNA was detected in only 7% of CSF samples, all positive cases showed profiles consistent with viral reactivation. Immune gene expression analysis revealed broad upregulation of cytotoxic effectors, type I interferon pathways, and chemokine signaling in PwMS. EFA identified a significantly different gene signature linking BZLF1 expression with inflammatory genes, type I interferon responses, and chemokines involved in immune cell migration, in PwMS.

Discussion

Our findings support the hypothesis that EBV latency disruption and lytic reactivation contribute to immune dysregulation in MS. The association between EBV transcriptional activity and immune gene alterations may uncover potential peripheral biomarkers of EBV-driven pathology. These molecular signatures may provide insights into novel therapeutic avenues and peripheral biomarkers for MS monitoring.

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Supplementary Material

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Glossary

BAFF = B-cell activating factor; **BZLF1** = BamHI Z leftward fragment 1; **ddPCR** = droplet digital polymerase chain reaction; **EBNA** = Epstein-Barr nuclear antigen; **EBV** = Epstein-Barr virus; **EDSS** = Expanded Disability Status Scale; **EFA** = exploratory factor analysis; **ELISA** = enzyme-linked immunosorbent assay; **GAPDH** = glyceraldehyde 3-phosphate dehydrogenase; **Gd+** = gadolinium-enhancing; **gp350/220** = EBV envelope glycoprotein gp350/220; **HD** = healthy donors; **IFN** = interferon; **IgG** = immunoglobulin G; **IL** = interleukin; **IRF** = interferon regulatory factor; **LCL** = lymphoblastoid cell line; **LMP** = latent membrane protein; **MS** = multiple sclerosis; **NAMPT** = nicotinamide phosphoribosyltransferase; **NK** = natural killer; **OAS1** = 2′-5′-oligoadenylate synthetase 1; **PBMCs** = peripheral blood mononuclear cells; **PwMS** = persons with multiple sclerosis; **RT-PCR** = reverse transcription polymerase chain reaction; **TLR** = toll-like receptor; **TNF** = tumor necrosis factor.

Background

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS with a pathogenesis shaped by both genetic susceptibility and environmental exposures.[1] Infection with Epstein-Barr virus (EBV) has emerged as a critical factor. Compelling evidence shows that EBV seroconversion precedes MS onset by several years, supporting a causal role in disease initiation.[2] EBV infects over 95% of the adult population and typically persists lifelong in a latent state within B cells under tight immune control.[3] However, in genetically susceptible individuals, EBV may escape immune surveillance and contribute to autoimmunity, malignancy, or chronic inflammation.[4] Despite the strong epidemiologic association, the mechanisms by which EBV contributes to MS pathogenesis remain incompletely understood.[5,6] Given the complex interaction between host genetic susceptibility (mainly involving immune-related genes) and persistent viral activity, integrated molecular analyses may help clarify the mechanisms by which EBV contributes to MS immunopathology.[7] Currently, 2 non-mutually exclusive hypotheses are considered to explain how EBV contributes to MS pathogenesis: EBV may act as a trigger, initiating autoimmunity through molecular mimicry, or as a driver, sustaining chronic immune activation and CNS inflammation via the persistence of infected B cells.[8] The molecular mimicry model posits that EBV, primarily through the viral antigen EBNA1, elicits cross-reactive immune responses targeting CNS self-antigens.[9] Several candidate autoantigens have been proposed, including myelin peptides, GlialCAM, Anoctamin-2, and alpha-crystallin B, but no definitive target has been identified to date.[6,10,11] On the other hand, the driver hypothesis is supported by the detection of EBV-infected B cells in MS brain lesions[12-15] and the accumulation of EBV-specific $CD8^+$ T cells in the CSF and brain tissue of MS patients.[16-18] In addition, increased frequencies of EBV-specific T cells targeting latent and lytic antigens have been documented in PwMS,[19-22] further suggesting that altered control of EBV may contribute to MS. Understanding whether EBV acts primarily as a trigger and/or a driver remains a critical open question with direct therapeutic implications.[23] Among serologic markers, anti-EBNA1 IgG titers are consistently elevated in PwMS,[2,24] although their relationship with disease activity and treatment response remains uncertain.[25] Studies assessing EBV DNA levels in blood or CSF have yielded inconsistent results, often showing no significant differences between MS patients and healthy controls.[19,26-28] However, increased EBV lytic gene expression, associated with a proinflammatory phenotype, has recently been observed in spontaneously outgrowing EBV-positive lymphoblastoid cell lines (LCLs) derived from PwMS,[29] suggesting a potential link between viral reactivation and immune activation in MS. This raises the question of whether EBV transcriptional activity is altered in vivo in PwMS and whether such activity correlates with immune gene expression changes. Despite its potential relevance, EBV gene expression in MS has been rarely explored in clinical samples.[28,30] To address this gap, we conducted an integrated molecular analysis in therapy-naïve PwMS and matched healthy donors, combining EBV DNA/RNA quantification, serology, and immune transcript profiling in peripheral blood. In MS patients, we also assessed EBV RNA expression in CSF cells to examine viral activity in the CNS compartment. Our goal was to define the molecular interplay between EBV and host immune dysregulation in MS and to identify biomarkers of EBV-driven pathology and potential therapeutic targets.

Methods

Sample Collection

Participants included 77 therapy-naïve PwMS and 40 healthy donors (HD) from 3 Italian MS centers. Inclusion criteria, ethical approvals, and clinical definitions are detailed in the "Results" section. Peripheral blood and CSF samples were collected from PwMS as part of routine diagnostic procedures and in accordance with ethical guidelines, with both samples obtained on the same day. Peripheral blood samples from HD were obtained from volunteers with no history of neurologic or autoimmune disorders.

For peripheral blood mononuclear cell (PBMC) isolation, 10 mL of blood were drawn into EDTA tubes. PBMCs were isolated and then preserved in RNAlater (Qiagen, Hilden, Germany) to ensure optimal RNA integrity and subsequently frozen at −80°C.

Serum samples were prepared by centrifuging whole blood at 3,000 rpm for 15 minutes at 20°C. Supernatants were collected in 500 μL aliquots and stored at −80°C.

CSF samples, ranging from 7 to 15 mL (median = 9.5 mL), were obtained by lumbar puncture using an atraumatic

needle. Within 30 minutes of collection, CSF samples were centrifuged at 1,200 rpm for 10 minutes at room temperature. Cell pellets were aliquoted and stored at −80°C in RNAlater (Qiagen). Eight CSF cell samples were excluded from EBV RNA analysis due to low RNA content (GAPDH Ct > 28), leaving 69 samples for evaluation.

Determination of Anti-EBV Antibodies in Serum

Serum levels of EBNA1 IgG and VCA-IgG were assessed using Serion ELISA commercial kits (Virion/Serion, Würzburg, Germany), following the manufacturer's instructions.

Quantification of EBV DNA Load

DNA was extracted from PBMC samples (5×10^5 cells each) and from 3 CSF cell samples with high cellularity (>100,000 cells) using QiAmp DNA mini kit (Qiagen) following the manufacturer's instructions. EBV DNA load in PBMCs of PwMS and HD was assessed using Droplet Digital PCR (ddPCR). Two hundred fifty nanograms of DNA were analyzed for the detection of EBV BamHI W repeat region (sequence in eTbale1) by ddPCR, using Biorad QX200 System (Bio-Rad, Hercules, CA). Human EIF2C2 (Bio-Rad) was used to normalize data. EBV-positive Namalwa cells and EBV-negative BJAB cells were used as positive and negative controls, respectively.

Preamplification Real-Time RT-PCR

Total RNA was extracted from PBMCs (from 5×10^5 cells) and CSF cell samples using RNeasy micro kit (Qiagen, Hilden, Germany). For CSF samples, RNA was extracted from the available number of cells per sample, ranging from 8,400 to 156,000 (median: 35,200). This variability reflects the inherent differences in CSF cellularity between samples. Genomic DNA was depleted using the RNase free DNase set (Qiagen). RNA was quantified using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA), and 500 ng was reverse transcribed using the High Capacity reverse transcription kit with RNase inhibitor (Life Technologies, Carlsbad, CA). The expression of 7 EBV genes (sequences in eTable 1) was assessed using PreAmp real-time RT-PCR as previously described.[30] In brief, cDNA was preamplified (14 Cycles) using the TaqMan PreAmp Master Mix and a pool of EBV assays and the housekeeping gene GAPDH (Life Technologies). The preamplified products were analyzed by real-time PCR. The specificity and sensitivity of the assays were tested using EBV-negative and positive cell lines.[30] EBV gene expression was independently assessed in 2 laboratories, Istituto Superiore di Sanità (ISS) and CRESM (Centro di Riferimento Regionale SM, AOU San Luigi, Orbassano), using real-time PCR platforms (ABI PRISM 7500 and StepOne Plus, Life Technologies). Both laboratory results followed an identical preamplified RT-PCR protocol with the same primer and probe sets. The analyses yielded fully concordant results across the 2 laboratories, thus supporting the reproducibility and robustness of the data set.

Immune-Related Gene Expression Analysis

The expression of 47 immune-related genes selected for their involvement in antiviral defense, inflammation, cytotoxicity, and other immune pathways relevant to MS was evaluated using the BioMark real-time PCR nanofluidic platform with a 48.48 Dynamic Array IFC chip (Fluidigm, San Francisco, CA, USA). To increase sensitivity, cDNA was preamplified (14 cycles) using the TaqMan PreAmp Master Mix and a pool of assays specific for the selected immune-related genes and GAPDH used as reference. The inventoried IDs for all TaqMan assays are listed in eTable 2. Negative controls were included in all steps to rule out cross-contamination.

Statistical Analyses

Statistical analyses were performed using STATA (v 16.0). Group comparisons for continuous variables (e.g., EBV DNA load, antibody titers, gene expression levels) were performed using the nonparametric Mann-Whitney *U* test. Categorical data (e.g., frequency of EBV RNA detection) were compared using the Fisher exact test. Correlations between variables were assessed with Spearman rank correlation coefficient. A p value <0.05 was considered statistically significant. For multivariate analysis, Exploratory Factor Analysis (EFA) based on the principal factors extraction method with orthogonal varimax rotation was applied to normalized expression data of immune-related genes and EBV parameters to identify patterns of co-expression and to reduce data dimensionality. Factors with eigenvalues >1 were retained, and variables with loading coefficients >0.5 in absolute value were considered major contributors to each factor. Internal consistency of EFA-derived factors was assessed using Cronbach alpha, with values above 0.70 indicating adequate reliability. Post hoc Bonferroni correction (n = 6, based on EFA-derived factors) was applied where appropriate, e.g., in group comparisons of immune gene expression.

Sample size was determined by the available therapy-naïve PwMS/HD as well as by the number of high-quality/analyzable samples. Potential confounders such as age, sex, clinical status at sampling (relapse vs remission), and MRI activity were considered in stratified analyses, but these variables did not significantly affect the associations observed.

Standard Protocol Approvals, Registrations, and Patient Consents

All patients with MS were enrolled in IRB-approved natural history study. Both HD and PwMS provided informed consent before participation.

Data Availability

All data related to this article are provided in the main text or as supplementary material.

Results

Study Participants

Seventy-seven therapy-naïve PwMS were prospectively enrolled at the time of diagnosis from 3 specialized MS centers

in Italy, namely, CRESM biobank of the University Hospital San Luigi Gonzaga, Orbassano (n = 36), the University of Cagliari (n = 16), and the University of Florence (n = 25). Diagnosis was established according to the 2017 revised McDonald criteria.[31] All patients were sampled before initiating immunomodulatory/immunosuppressive therapy. None had received corticosteroids in the previous 3 months. Forty HD were recruited as volunteers at the University Hospital San Luigi Gonzaga (n = 23) and the University of Cagliari (n = 17). All participants provided written informed consent, and the study was approved by the ethics committees of participating centers and the Istituto Superiore di Sanità (Protocol ID: PRE-C 165/15). Clinical data (age, sex, disease duration, EDSS, and MRI activity) were standardized across centers. Disease onset was defined as the first episode of neurologic dysfunction consistent with MS. Relapses were defined as new or recurrent neurologic symptoms lasting ≥24 hours and not attributable to fever or infection. At the time of sampling, 28 PwMS (36.4%) were experiencing a clinical relapse, while 49 were in remission. Brain MRI scans, including T1-weighted (pregadolinium and postgadolinium) and T2-weighted sequences, were performed either on the same day or within a median interval of 15 days (range: 0–143 days) from blood and CSF collection. MRI activity, defined as the presence of gadolinium-enhancing lesions, was documented in 31 patients (40.2%). For each subject, peripheral blood was collected for serologic and molecular analyses. In PwMS, paired CSF samples were obtained on the same day during diagnostic lumbar puncture. Of the 77 PwMS, CSF was successfully collected from 69. Eight samples were excluded from EBV RNA analysis due to insufficient RNA yield (GAPDH >28 Ct). The characteristics of the study population are summarized in eTable 3.

EBV Serology in PwMS and HD

Serologic analysis revealed 100% seropositivity for both EBNA1 IgG and VCA IgG in both PwMS and HD of this cohort, confirming the near-universal prior exposure to EBV in both groups. This finding is consistent with the high prevalence of EBV infection in adult populations worldwide and supports the relevance of evaluating not just EBV exposure, but differences in immune responses to the virus in MS. Consistent with previous studies,[2,24,27] we observed that EBNA1 IgG titers were significantly higher in PwMS compared with HD (p = 0.04) (Figure 1, left). In this cohort, VCA-IgG levels did not significantly differ between PwMS and HD (Figure 1, right). To explore potential associations with disease activity or clinical phenotype, we stratified the PwMS cohort by clinical condition (relapse vs remission), MRI activity (active vs inactive), and sex. No statistically significant differences in EBV-specific IgG levels were observed across these subgroups.

EBV DNA Load in PBMCs From PwMS and HD

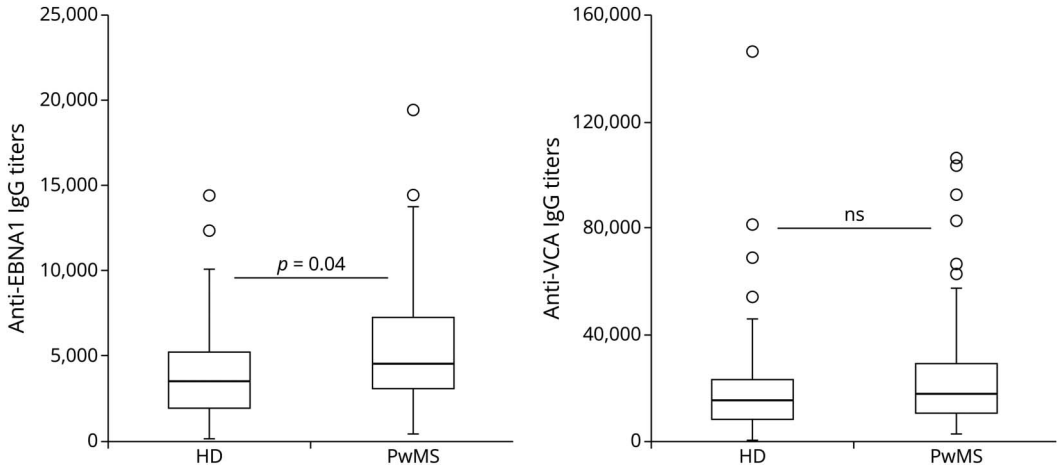
We next assessed the burden of EBV DNA in PBMCs using ddPCR, a highly sensitive and quantitative method targeting the BamHI-W repeat region of the EBV genome. This region was selected due to its high copy number per viral genome, which enhances the sensitivity of detection even in low viral load settings.[32] EBV DNA was detected in 35% of HD (14 of 40) and 45.5% (35 of 77) of PwMS (Figure 2A). This difference was not statistically significant by using the Fisher exact test but suggested a higher prevalence and an easier detection of EBV DNA in PBMCs from PwMS compared with HD. Quantitative analysis showed higher EBV DNA levels in PBMCs from PwMS compared with HD (p = 0.05; Figure 2B). Notably, the median viral load in both groups was zero, reflecting the high proportion of samples without detectable EBV DNA. However, a subset of PwMS exhibited markedly elevated viral loads, contributing to a statistically significant difference between the two groups. To further clarify this observation, we analyzed only the subset of samples with detectable EBV DNA. Within this subgroup (PwMS n = 35; HD n = 14), the viral load was markedly and significantly elevated in PwMS (p < 0.001, Mann-Whitney U test; Figure 2C), indicating that when EBV is detectable, it is present at higher copy numbers in MS patients. No association was found between EBV DNA load and sex, clinical status, or MRI activity.

Viral Gene Expression in PBMCs From PwMS and HD

To investigate the transcriptional activity of EBV beyond DNA quantification, we analyzed the expression of 7 key viral transcripts involved in both latency (EBER1, EBNA1, EBNA3A, LMP1, LMP2A) and lytic (BZLF1, gp350/220) phases of the EBV life cycle. To this aim, we used a real-time RT-PCR method that included a targeted preamplification step to improve detection sensitivity, particularly for low-abundance transcripts in bulk cell populations.[13,30] As shown in Figure 3A, EBV RNA was detected in a significantly higher proportion of PwMS (38%; 29/77) than HD (20%; 8/40; p = 0.039, Fisher exact test). Interestingly, in addition to a higher detection frequency, the transcriptional profiles differed markedly between the 2 groups (p = 0.008; Fisher exact test). This variation is shown in Figure 3A-B and detailed in Table 1. Among the 8 EBV RNA-positive HD samples, 75% (6/8) expressed only EBER1, consistent with a latency 0 program and minimal viral activity. Only 2 HD samples (25%) also expressed LMP1, suggesting limited engagement of latency II gene expression. By contrast, PwMS exhibited a broader and more active EBV transcriptional landscape. Among the 29 EBV RNA-positive samples from PwMS, 80% (23/29) expressed transcripts associated with latency II/III (LMP1, LMP2A, EBNA1, EBNA3A) and/or lytic reactivation (BZLF1, gp350/220), while only 20% expressed EBER1 alone.

This distribution is visualized in Figure 3A as a stacked bar plot, where the red segment indicates latency II/III and lytic gene expression, and the blue segment corresponds to latency 0 (EBER1 only). Figure 3B shows the individual expression patterns for each detected EBV transcript. In comparison with HD, the PwMS RNA samples display greater diversity and frequency of expression across multiple EBV genes, especially those related to latency II/III and lytic cycle.

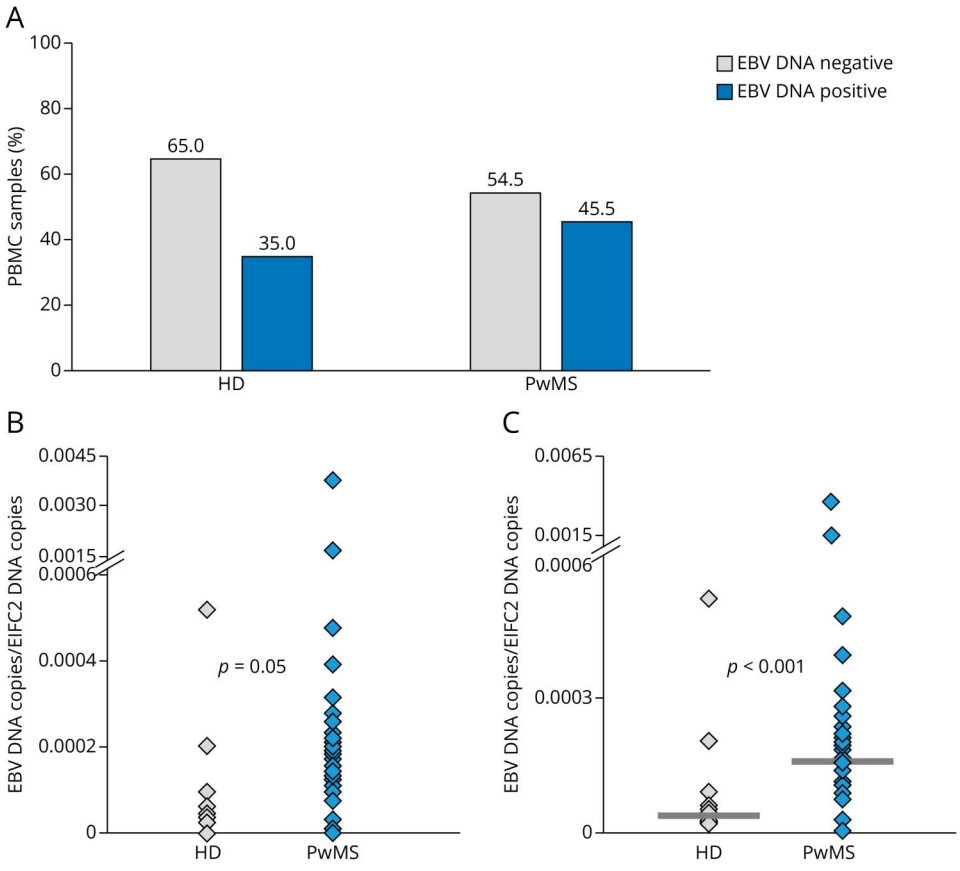
Figure 1 Serum Levels of EBV-Specific Antibodies in PwMS and HD



Serum titers of anti-EBNA1 IgG and anti-VCA IgG were measured by a commercially available ELISA kit in PwMS (n = 77) and HD (n = 40). All participants were seropositive for both EBV antigens. Anti-EBNA1 IgG titers were significantly higher in PwMS compared with HD ($p = 0.04$, Mann-Whitney *U* test), while anti-VCA IgG levels did not significantly differ between the groups. The lines inside the boxes represent the median value; boxes extend from the 25th to the 75th percentile, covering the interquartile range (IQR), and whiskers extend from the 25th percentile−1.5 IQR to the 75th percentile +1.5 IQR. Maximum outliers outside the whiskers are represented by individual marks. n.s = not statistically significant. EBV = Epstein-Barr virus; HD = healthy donors; PwMS = persons with multiple sclerosis.

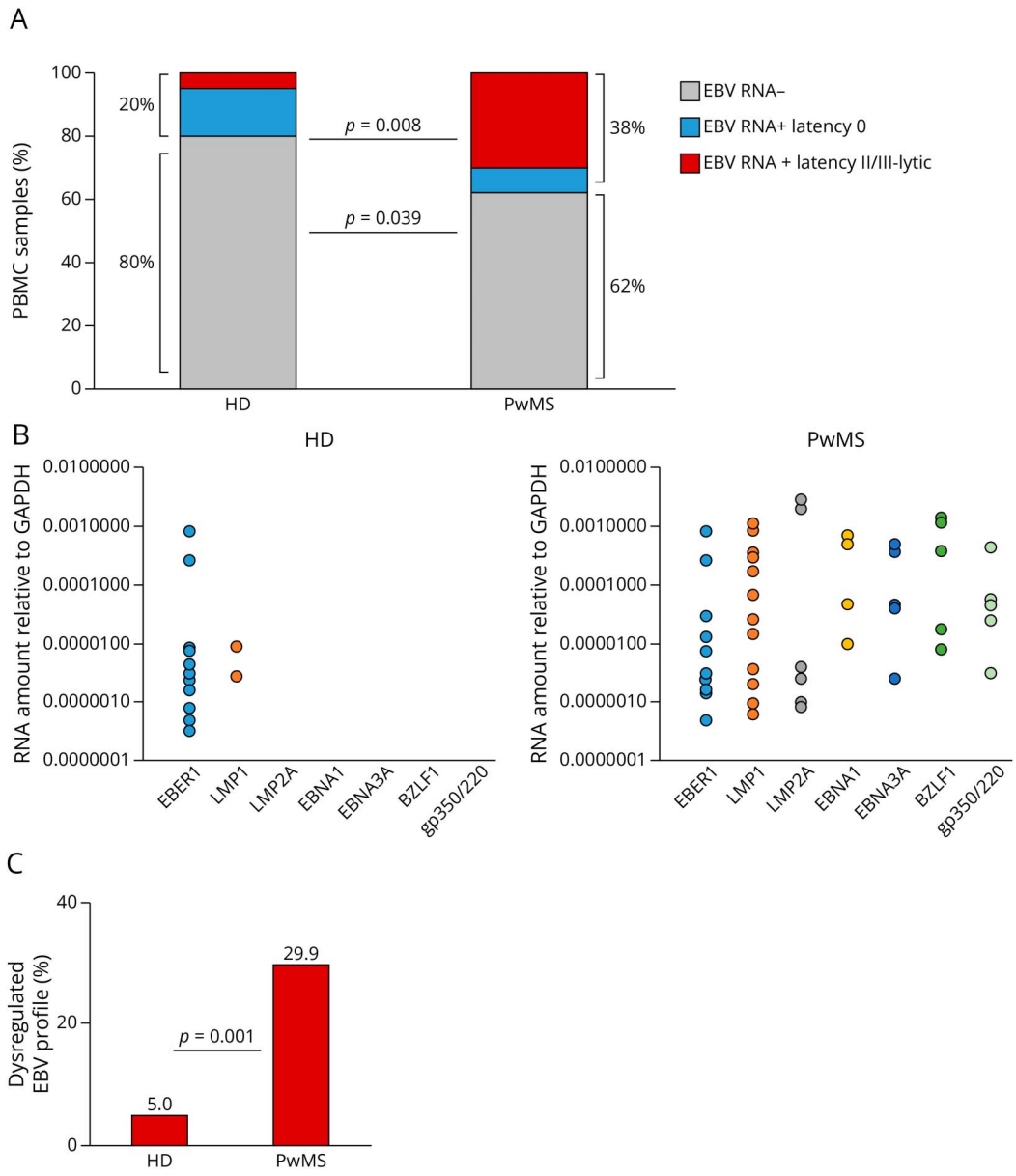
Overall, transcripts indicative of latency disruption, defined as atypical coexpression of latency II/III genes together with occasional lytic transcripts, were significantly more frequently detected in PwMS (23/77; 29.9%) than in HD (2/40; 5%) ($p = 0.001$ with Fisher exact test; Figure 3C). These data support the presence of transcriptionally active EBV reservoirs in the

Figure 2 EBV DNA Load in PBMCs From PwMS and HD



(A) Proportion of EBV DNA-positive PBMC samples detected by ddPCR in HD and PwMS; (B) Quantitative EBV DNA load (expressed as EBV BamHI-W copies normalized to EIF2C2) in all individuals. PwMS showed significantly higher EBV DNA levels than HD ($p = 0.05$). The median value in both HD and PwMS was zero due to a large proportion of undetectable samples. (C) EBV DNA load in the subset of samples with detectable viral DNA. In this subset, the median EBV DNA load (indicated by the horizontal bar) was significantly higher in PwMS compared with HD ($p < 0.001$, Mann-Whitney *U* test). EBV = Epstein-Barr virus; HD = healthy donors; PBMC = peripheral blood mononuclear cell; PwMS = persons with multiple sclerosis

Figure 3 EBV RNA Expression Patterns in PBMCs From HD and PwMS



(A) Bar chart shows the distribution of EBV RNA expression profiles in PBMCs. Samples were classified as EBV RNA-negative (gray), EBV RNA-positive with expression of EBER1 only (blue; latency 0), or EBV RNA-positive with expression of additional latency II/III or lytic transcripts (red). The frequency of EBV RNA-positive samples was significantly higher in PwMS compared with HD ($p = 0.039$), and the overall transcriptional profile significantly differed between the 2 groups with PwMS showing higher frequency of transcripts associated with latency disruption/lytic cycle reactivation ($p = 0.008$; Fisher's exact test). (B) Dot plots displaying the relative expression levels of individual EBV transcripts (normalized to GAPDH) in PBMCs from HD and PwMS. While HD samples predominantly expressed EBER1 alone, PwMS samples showed a broader range of viral transcripts related to EBV dysregulation. (C) Bar plot highlighting the proportion of individuals with dysregulated EBV expression profiles (i.e., expression of latency II/III or lytic genes). PwMS showed a significantly higher proportion of dysregulated EBV profiles compared with HD ($p = 0.001$, Fisher's exact test). EBV = Epstein-Barr virus; HD = healthy donors; PBMC = peripheral blood mononuclear cell; PwMS = persons with multiple sclerosis

peripheral blood of PwMS, potentially contributing to immune dysregulation.

Stratification of PwMS by EBV RNA status revealed that RNA-positive individuals displayed significantly higher viral DNA loads than RNA-negative ones (Figure 4), consistent with increased viral activity. Importantly, the median EBV DNA load in RNA-negative PwMS was zero, indicating that in most cases, the absence of RNA coincided with undetectable DNA (Figure 4). This close association underscores the consistency of our findings and reinforces the link between EBV transcriptional activity and viral DNA burden. However, the presence and pattern of EBV transcripts showed no association with sex, clinical status, or MRI activity in PwMS.

Viral Gene Expression in CSF Cell Samples From PwMS

To explore whether EBV dysregulation observed in peripheral blood was also detectable in the CNS compartment, we assessed EBV RNA expression in CSF cells from 69 PwMS.

Table 1 Clinical, Radiologic, and Virologic Features of PwMS and HD

						PBMC								CSF						
Code	**Clinical cond**	**EDSS**	**MRI**	**Cellule CSF/ul**	**IgG index**	**EBER1**	**LMP1**	**LMP2A**	**EBNA1**	**EBNA3A**	**BZLF1**	**gp350/ 220**	**EBV DNA**	**EBER1**	**LMP1**	**LMP2A**	**EBNA1**	**EBNA3A**	**BZLF1**	**gp350/ 220**
MS_ 01	REMISSION	1	INACTIVE	3	0.5	0.000028									0.0117		0.0048		0.013	
MS_ 08	REMISSION	1	INACTIVE	27	1.44		0.001002	0.001857	0.00001		0.001332		0.00012	0.00058	0.001	0.0019			0.0003	0.0019
MS_ 010	RELAPSE	3	INACTIVE	1.2	0.74		0.00034													
MS_ 11	REMISSION	1	INACTIVE	1.2	0.71		0.000026													
MS_ 16	RELAPSE	1	INACTIVE	5	0.87	0.000013						0.00006	0.00377							
MS_ 19	RELAPSE	1.5	INACTIVE	4	0.91	0.0000014														
MS_ 20	REMISSION	2.5	ACTIVE	3	0.7										0.0004					
MS_ 21	REMISSION	1	INACTIVE	2	0.73	0.0000019				0.0000025			0.00013							
MS_ 25	REMISSION	2	INACTIVE	3	0.94	0.0000025	0.0000006					0.000025	0.00018							
MS_ 28	REMISSION	1	INACTIVE	4	0.6			0.000001					0.00003							
MS_ 29	REMISSION	1	ACTIVE	2	0.63		0.000002						0.00008	0.000042	0.0001	0.00012		0.0004		0.0001
MS_ 31	RELAPSE	1.5	ACTIVE	3	0.58							0.000003	0.00014							
MS_ 32	REMISSION	2	ACTIVE	2	0.68	0.000007		0.000004												
MS_ 34	RELAPSE	2.5	ACTIVE	5	1	0.0000017							0.00001			0.00001				
MS_ 36	RELAPSE	1.5	ACTIVE	3	0.83	0.0000005														
MS_ 39	REMISSION	1	INACTIVE	12	1.71	0.0000015	0.000002	0.0000025					0.00015							

Continued

Table 1 Clinical, Radiologic, and Virologic Features of PwMS and HD *(continued)*

						PBMC								CSF						
Code	**Clinical cond**	**EDSS**	**MRI**	**Cellule CSF/ul**	**IgG index**	**EBER1**	**LMP1**	**LMP2A**	**EBNA1**	**EBNA3A**	**BZLF1**	**gp350/220**	**EBV DNA**	**EBER1**	**LMP1**	**LMP2A**	**EBNA1**	**EBNA3A**	**BZLF1**	**gp350/220**
MS_42	REMISSION	0	INACTIVE	9	0.85	0.0000023														
MS_47	RELAPSE	2	INACTIVE	3	0.61	0.000007														
MS_51	RELAPSE	2	ACTIVE	1	0.84						0.00036		0.00164							
MS_56	REMISSION	0	ACTIVE	4	0.59		0.000001						0.000075							
MS_61	REMISSION	1.5	ACTIVE	1.9	0.64	0.0008	0.000847	0.002741	0.00065	0.00035	0.00113	0.00044	0.00039							
MS_62	REMISSION	2	ACTIVE	5	0.46	0.00025	0.00006	0.000001	0.000045	0.00005	0.000008	0.000044	0.00026							
MS_65	REMISSION	2	INACTIVE	2.5	0.5				0.00048	0.0005			0.00028							
MS_67	REMISSION	2	ACTIVE	3.7	1.06		0.000014				0.000017									
MS_70	REMISSION	1.5	INACTIVE	24	1.04		0.000291													
MS_71	REMISSION	1.5	INACTIVE	4.3	0.9	0.000003	0.000065													
MS_73	REMISSION	1	INACTIVE	5.6	0.89		0.000164						0.00019							
MS_74	REMISSION	2.5	INACTIVE	4.4	0.52		0.001079						0.0002							
MS_75	REMISSION	1.5	INACTIVE	1.9	0.56		0.000035						0.00022							
MS_76	RELAPSE	3.5	ACTIVE	3.8	0.55					0.000038										
HD_01						0.0000026							0.00006							
HD_04						0.0000008							0.00002							

Continued

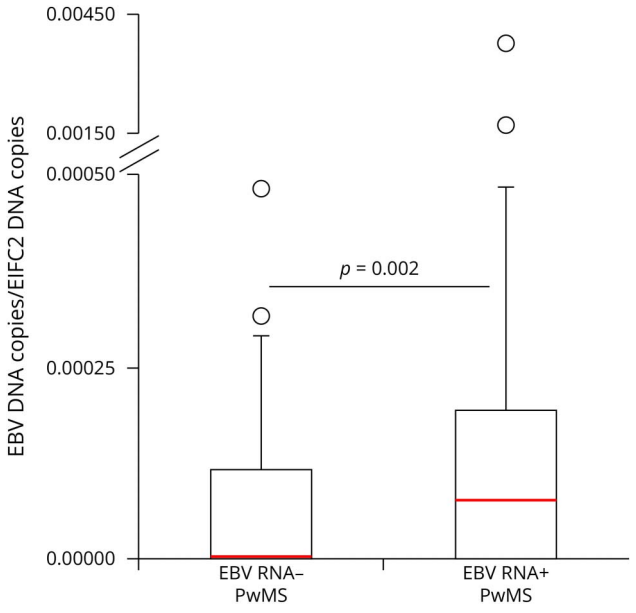
Table 1 Clinical, Radiologic, and Virologic Features of PwMS and HD *(continued)*

						PBMC								CSF						
Code	**Clinical cond**	**EDSS**	**MRI**	**Cellule CSF/ul**	**IgG index**	**EBER1**	**LMP1**	**LMP2A**	**EBNA1**	**EBNA3A**	**BZLF1**	**gp350/220**	**EBV DNA**	**EBER1**	**LMP1**	**LMP2A**	**EBNA1**	**EBNA3A**	**BZLF1**	**gp350/220**
HD_09						0.000004														
HD_14						0.00000032														
HD_16						0.0000028							0.000095							
HD_23						0.000008	0.0000087						0.0002							
HD_26						0.000002							0.00052							
HD_35							0.0000026													

This table summarizes the demographic, clinical, radiologic, and CSF characteristics of PwMS, together with EBV transcript expression and EBV DNA quantification in PBMCs and CSF cells. The expression levels of EBV latent (EBER1, LMP1, LMP2A, EBNA1, EBNA3A) and lytic (BZLF1, gp350/220) transcripts were evaluated by preamplified real-time PCR. EBV DNA levels were quantified using droplet digital PCR. Data are expressed as relative EBV RNA levels normalized to GAPDH, or as the ratio between EBV BamHI-W repeats and the human EIF2C2 reference gene. Samples from HD were included as controls for PBMC analyses and therefore lack clinical, radiologic, and CSF data. Empty cells indicate either undetectable EBV targets (below the assay limit of detection) or data not applicable/not available for HD samples. The table includes only PwMS (n = 30) and HD (n = 8) samples in which at least one EBV RNA transcript was detected in PBMCs and/or CSF cells.

EBV transcripts were detected in 5 of 69 CSF cell samples (7.3%), a relatively low frequency that likely reflects several intrinsic limitations of CSF analysis. These include the low total cellular content of CSF and the rarity of B cells (the primary EBV reservoir). Importantly, as presented in Table 1, all EBV RNA-positive CSF samples displayed a transcriptional profile indicative of viral activity, with expression of latency II/III genes (LMP1, LMP2A, EBNA3A) and/or lytic transcripts (BZLF1, gp350/220). These findings closely mirror the dysregulated EBV gene expression patterns observed in PBMCs, reinforcing the idea that EBV latency disruption is not limited to the periphery and supporting a potential role for intrathecal EBV activity in MS immunopathogenesis. Although EBV DNA could not be systematically evaluated due to the limited cellular yield of most CSF samples, in 3 patients with high CSF cellularity (>100,000 cells; MS08, MS39, MS70), we could analyze EBV DNA. Two of the 3 samples were EBV DNA-positive, both showing viral loads approximately tenfold higher in the CSF than in their paired PBMCs (MS08: 0.0011 vs 0.00012; MS39: 0.00346 vs 0.00015), whereas in MS70, EBV DNA was undetectable in both compartments. Despite the small subset, these findings further support compartmentalized EBV replication.

Figure 4 EBV DNA Load in PwMS Stratified by EBV RNA Detectability



EBV DNA levels in PBMCs from PwMS were compared based on whether EBV RNA was detected or not. Samples with detectable EBV RNA exhibited significantly higher EBV DNA load (p = 0.002, Mann-Whitney U test), suggesting a correlation between viral transcriptional activity and DNA burden. The red lines inside the boxes represent the median value. Notably, the median EBV DNA load in RNA-negative PwMS was zero, indicating that in most cases both RNA and DNA were undetectable. Boxes extend from the 25th to the 75th percentile, covering the interquartile range (IQR), and whiskers extend from the 25th percentile−1.5 IQR to the 75th percentile +1.5 IQR. Maximum outliers outside the whiskers are represented by individual marks. EBV = Epstein-Barr virus; HD = healthy donors; PBMC = peripheral blood mononuclear cell; PwMS = persons with multiple sclerosis

Immune-Related Gene Expression

To evaluate systemic immune dysregulation in MS, we analyzed the expression of 47 immune-related genes in PBMCs from PwMS and HD. The gene panel was designed to capture key elements of inflammation, immune regulation, and antiviral defense, including markers of immune cell subsets, cytotoxic and activation molecules, cytokines, chemokines, type I IFN signaling components, pathogen-recognition receptors, and Ki67.

Table 2 summarizes the median expression levels and ranges of the selected immune-related genes in PBMC samples from PwMS and HD, along with the p values indicating the statistical significance of the differences between the 2 groups. We found a higher expression of several immune-related genes in PwMS compared with HD. In particular, the strongest differences ($p \leq 0.01$) were observed in genes associated with plasma cells (CD138), B-cell survival (BAFF), T cells (CD4, FoxP3, T-bet, Eomes) and cytotoxic T-cell response (CD8, Granzyme-B, LT-α), innate immunity (NKp46, CD68, MHCII), costimulation (CD86), type-I IFN signaling pathway (IRF3, IRF8, IFNβ, OAS1, CXCL10), T-cell recruitment and migration (CCR5, CCL5, CXCR3), and inflammation (NAMPT, IL35). Except for CD4, these differences remained significant when applying post hoc Bonferroni correction based on the number of EFA-derived factors (n = 6, see below, i.e., corrected p value = 0.05/6 = 0.008). These findings reveal broad immune activation in therapy-naïve PwMS, involving cytotoxic, regulatory, and antiviral responses. The resulting gene expression profile reflects systemic immune dysregulation and may contribute to MS pathogenesis, possibly in association with the EBV transcriptional activity.

Multivariate Analysis Integrating Immune and Viral Parameters

To characterize immune alterations in PwMS and their relationship with EBV, we performed an EFA integrating immune gene expression, viral RNA/DNA levels, and anti-EBV antibody titers. This multivariate approach allowed us to capture key patterns within the data set and to identify clusters of coexpressed genes that differentiate PwMS from HD with enhanced statistical power. EFA grouped the variables into 6 factors, each representing a set of highly correlated genes or markers with minimal overlap between factors. These 6 components explained a cumulative 63.7% of the total variance (17.1%, 11.2%, 10.5%, 9.6%, 9%, and 6.4%, respectively). Table 3 presents the genes with the strongest correlation (factor loadings >0.5) with each factor. Factor 1 comprises genes related to general immune activation, including T and NK cell cytotoxic responses (CD4, CD8, Eomes, IFN-γ, Granzymes) as well as pattern recognition and inflammation (CD68, TLR3, TLR9). Factor 2 consists of viral genes associated with EBV latency (EBER1, LMP2A, EBNA1, EBNA3A) and lytic reactivation (BZLF1, gp350/2200), along with CD69, marker of T-cell activation. Factor 3 is associated with inflammation (IL-1β, IL-23 subunit p19, NAMPT), type I IFN antiviral response (IFN-β), and chemokines involved in

Table 2 Comparison of Immune Gene Expression Between PwMS and HD, With Associated *p* Values

		MS			HD			
Category	**Target**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	***p* Value**
B-lymphocyte markers	CD20	0.212	0.006	1.656	0.113	0.017	0.871	0.131
	CD138	0.000	0.000	0.002	0.000	0.000	0.001	0.007
	BCMA	0.002	0.000	0.019	0.002	0.001	0.032	0.124
T-lymphocyte and NK cell markers	CD4	0.254	0.031	0.924	0.174	0.032	0.830	0.01
	CD8	0.483	0.039	3.487	0.150	0.025	2.046	0.001
	CD56	0.004	0.000	0.248	0.006	0.001	0.045	0.019
	CD57	0.014	0.000	1.125	0.013	0.001	0.167	0.904
	NKp46	0.015	0.000	0.156	0.006	0.000	0.077	0.005
	FoxP3	0.018	0.002	0.089	0.003	0.001	0.029	<0.001
	Eomes	0.032	0.001	0.240	0.010	0.000	0.149	<0.001
	T-bet	0.123	0.008	0.921	0.049	0.011	5.239	0.001
Cytotoxic/activation markers	CD69	0.359	0.013	7.749	0.207	0.035	1.818	0.034
	Perforin	0.218	0.022	1.249	0.106	0.008	1.083	0.030
	Granzyme-A	0.130	0.000	0.768	0.086	0.013	1.218	0.787
	Granzyme-B	0.078	0.005	1.354	0.019	0.001	0.206	<0.001
	Granzyme-K	0.085	0.009	0.663	0.050	0.005	0.835	0.040
	LT-α	0.018	0.000	0.660	0.006	0.002	0.037	<0.001
	LT-β	0.087	0.005	0.557	0.086	0.014	0.442	0.850
	CD86	0.025	0.002	0.102	0.006	0.001	0.087	<0.001
Monocyte/macrophage markers	CD68	0.539	0.168	1.416	0.158	0.071	3.198	<0.001
	MHCII	1.416	0.094	4.909	0.182	0.009	1.142	<0.001
Cytokines/inflammatory mediators	IFN-γ	0.004	0.000	0.053	0.002	0.000	0.071	0.069
	TNF	0.023	0.000	0.170	0.016	0.005	0.090	0.029
	BAFF	0.070	0.012	0.444	0.030	0.011	0.275	<0.001
	IL-1 β	0.275	0.020	10.487	0.182	0.010	2.277	0.283
	1L-10	0.001	0.000	0.013	0.000	0.000	0.010	0.011
	IL-23 p19	0.006	0.000	0.895	0.004	0.001	0.016	0.043
	IL-27 p28	0.002	0.000	0.068	0.003	0.000	0.055	0.096
	IL-12/IL-35 p35	0.005	0.000	0.137	0.003	0.000	0.019	0.007
	IL-12/IL-23 p40	0.000	0.000	0.001	0.000	0.000	0.000	0.051
	EBI3	0.000	0.000	0.013	0.000	0.000	0.001	<0.001
	NAMPT	0.177	0.023	4.654	0.039	0.010	1.104	<0.001
Pathogen recognition receptors	TLR3	0.001	0.000	0.003	0.001	0.000	0.004	0.798
	TLR9	0.002	0.000	0.008	0.002	0.000	0.020	0.024

Continued

Table 2 Comparison of Immune Gene Expression Between PwMS and HD, With Associated *p* Values *(continued)*

		MS			HD			
Category	**Target**	**Median**	**Min**	**Max**	**Median**	**Min**	**Max**	***p* Value**
Type-I IFN signaling	IRF3	0.172	0.008	1.374	0.071	0.018	0.290	<0.001
	IRF7	0.010	0.000	0.257	0.010	0.002	0.076	0.722
	IRF8	0.270	0.024	2.565	0.092	0.029	0.685	<0.001
	IFNβ1	0.001	0.000	14.634	0.000	0.000	0.002	<0.001
	MXA	0.111	0.007	3.209	0.071	0.012	3.095	0.122
	OAS1	0.079	0.002	1.435	0.016	0.004	0.245	<0.001
Chemokines	CXCL10	0.001	0.000	0.186	0.000	0.000	0.081	<0.001
	CCR5	0.031	0.004	18.362	0.003	0.000	0.030	<0.001
	CCL2	0.000	0.000	0.039	0.000	0.000	0.122	0.230
	CCL5	5.816	0.323	54.996	2.396	0.341	11.968	<0.001
	CXCR3	0.004	0.000	4.391	0.002	0.000	0.008	<0.001
	CXCL13	0.000	0.000	0.000	0.000	0.000	0.000	0.032
Proliferation marker	KI67	0.013	0.001	0.077	0.009	0.008	0.019	0.063

Abbreviations: BAFF = B-cell activating factor; BCMA = B-cell maturation antigen; CCL = C-C motif chemokine ligand; CCR = C- chemokine receptor; CXCL = C-X-C motif chemokine ligand; CXCR = C-X-C chemokine receptor; EBI3 = Epstein-Barr virus induced gene 3; Eomes = eomesodermin; FoxP3 = forkhead box P3; IFN = interferon; IL = interleukin; IRF = interferon regulatory factor; LT = lymphotoxin; M x A = myxovirus resistance protein A; NAMPT = nicotinamide phosphoribosyltransferase; NCD = cluster of differentiation; OAS1 = 2′-5′-oligoadenylate synthetase 1; T-bet = T-box expressed in T cells; TLR = Toll-like receptor. Post hoc Bonferroni correction was applied based on the number of EFA-derived factors (n = 6), resulting in a corrected significance threshold of $p < 0.008$.

immune cell migration (CCR5, CXCR3), together with the lytic EBV transcript BZLF1. Factor 4 correlates with NK cell activation markers (CD56, CD57) and cytokines regulating NK and cytotoxic T-cell responses, including IL-12 and IL-27 components (IL-27 subunit p28, IL-12/IL-35 subunit p35, IL-12/IL-23 subunit p40, EBI3). Factor 5 is linked to immune regulation, featuring Treg-associated genes (FoxP3), antigen presentation (MHCII), costimulation (CD86), and key components of the IFN-driven antiviral response (IRF3, IRF7, IRF8, OAS1). Finally, Factor 6 comprises markers of Th1-driven inflammation and chemokine signaling (CCL5, CXCL10, IL-27 subunit p28, T-bet). Among the 6 factors detected, Factors 1 and 3 exhibited high internal consistency, with Cronbach alpha values exceeding 0.8. Notably, scores of Factor 3 significantly differed between PwMS and HD ($p = 0.002$), suggesting enhanced inflammation, T-cell recruitment and migration, and a stronger type I IFN-mediated antiviral response in PwMS (this difference is shown in eFigure 1). The association with BZLF1, marker of early EBV reactivation, highlights a potential link between EBV reactivation and immune dysregulation in MS and underscores the value of integrative molecular approaches in identifying disease-relevant immune signatures.

Discussion

Extending the solid epidemiologic association between EBV infection and MS risk,[2] our findings demonstrate that EBV may act not only as an initial trigger but also as a persistent immunologic contributor of disease activity.[33] A key original contribution of this work is the integration of virologic data and host transcriptional profiles which reveals a clear association between EBV dysregulation and altered immune gene expression in the peripheral blood of PwMS compared with healthy donors. This combined virus-host perspective positions EBV as a key orchestrator of disease-associated immune dysfunction and highlights novel mechanistic pathways with therapeutic potential.

By applying sensitive ddPCR, we observed a higher prevalence of EBV DNA and increased viral load in PBMCs from PwMS, particularly in patients with quantifiable viral copies. While previous studies reported variable results depending on cohort and technical approaches,[19,26-28] our data provide molecular confirmation and reinforce the notion that EBV persistence is a consistent feature of MS. Furthermore, in the few cases with sufficient CSF cellularity, EBV DNA levels were markedly higher than in matched blood samples, reinforcing the concept of compartmentalized viral activity associated with intrathecal inflammation.

Importantly, we extended our analysis beyond DNA to investigate EBV transcriptional activity. We detected a significantly higher proportion of EBV RNA-positive PBMC samples in PwMS. Our data show that transcripts linked to viral latency II/III and lytic reactivation are far more frequent

Table 3 Factor Loadings on EBV (RNA Expression, Viral Load, and Serology) and Immune-Related Gene Expression in PBMCs of HD and PwMS

Variable	Factor1	Factor2	Factor3	Factor4	Factor5	Factor6
CD20						
CD138						
BCMA						
CD4	0.6307					
CD8	0.8192					
CD56				0.7714		
CD57				0.8845		
NKP46	0.6486					
FoxP3					0.5992	
CD69		0.7221				
Eomes	0.8011					
T-bet						0.7404
Perforin	0.8319					
Granzyme-A	0.8750					
Granzyme-B						
Granzyme-K	0.8110					
LT-α						
LT-β	0.7051					
IFNγ	0.7468					
CD68	0.6874					
MHCII					0.6779	
CD86					0.5533	
TNF						
IL-1 β			0.6021			
IL-10						
IL-23 p19			0.7573			
IL-27 p28				0.6530		0.5479
IL-12/IL-35 p35				0.8322		
IL-12/IL-23 p40				0.5890		
EBI3				0.7485		
NAMPT			0.5762			
TLR3	0.7152					
TLR9	0.6866					
BAFF						
IRF3					0.5175	
IRF7					0.6345	
IRF8					0.5305	

Continued

Table 3 Factor Loadings on EBV (RNA Expression, Viral Load, and Serology) and Immune-Related Gene Expression in PBMCs of HD and PwMS *(continued)*

Variable	Factor1	Factor2	Factor3	Factor4	Factor5	Factor6
IFNβ1			0.9427			
MXA						
OAS1					0.7038	
CXCL10						0.6271
Ki67						
CCR5			0.9471			
CCL2						0.8809
CCL5						
CXCR3			0.9196			
CXCL13						
EBER1		0.9020				
LMP1						
LMP2A		0.8046				
EBNA1		0.8885				
EBNA3A		0.7330				
BZLF1		0.6477	0.5750			
gp350/220		0.9240				
EBV DNA load						
Anti-EBNA1 IgG						
Anti-VCA IgG						

Factor loadings >0.5 in absolute value are shown.

in PwMS than in healthy donors. In the control HD group, RNA positivity was largely restricted to EBER1 and, rarely, LMP1. Detection of EBV RNA in a subset of CSF samples expressing latency disruption and/or lytic genes further supports the concept of compartmentalized reactivation, consistent with the presence of EBV-infected B cells in the MS brain.[12-15] Interestingly, most patients with detectable EBV transcripts in CSF were clinically stable at the time of sampling, suggesting that intrathecal EBV dysregulation may occur independently of overt disease activity. Persistence and intermittent reactivation of the virus within immune-privileged CNS niches, such as meningeal B-cell aggregates, may sustain low-grade smoldering inflammation and chronic compartmentalized immune activation.[20,34]

Crucially, our targeted approach overcomes the limitations of unbiased transcriptomics, which have largely failed to detect EBV transcripts due to their low abundance in samples dominated by human RNA.[28,35,36] Moreover, combining RNA and DNA data we found that RNA-positive PwMS displayed markedly higher viral DNA loads, whereas in RNA-negative individuals, viral DNA levels were lower or largely undetectable. This tight association between the 2 viral readouts underscores the biological consistency of our findings and strengthens the evidence that EBV transcriptional activity reflects a meaningful increase in viral burden.

Our transcriptional profiling revealed widespread upregulation of immune-related genes in PwMS. Notably, Exploratory Factor Analysis identified a molecular signature (Factor 3) combining the EBV lytic transcript BZLF1 and host genes linked to inflammation (IL-1β, IL-23 subunit p19, NAMPT), type-I IFN antiviral response (IFN-β), and T-cell trafficking (CCR5, CXCR3). This virus-host transcriptional axis provides new evidence that EBV reactivation can directly shape the immune landscape in MS. In line with this, Soldan and colleagues[29] reported lytic gene expression in spontaneous EBV-positive LCLs derived from PwMS, particularly during active disease and in association with a proinflammatory phenotype, further supporting a mechanistic link between EBV reactivation and inflammatory programs. In addition, the convergence of BZLF1 with CXCR3 is particularly compelling,

as it suggests a role for EBV in shaping chemokine-guided immune cell recruitment in MS. Supporting this interpretation, experimental studies in humanized mice demonstrated that EBV infection can drive the expansion of T-bet$^+$CXCR3$^+$ B cells infiltrating the CNS and recruiting inflammatory T cells.[37]

A recent paper reported serologic and molecular evidence of EBV reactivation in PwMS, together with altered immune profiles in blood and CSF.[38] Using flow cytometric and plasma marker approaches, the authors reached conclusions which are consistent with our data. We extend these findings and provide direct molecular evidence of EBV transcriptional activity in paired PBMCs and CSF, and integrate viral and host immune signatures in peripheral blood. Collectively, these results further support a role for EBV dysregulation in immune compartmentalization and inflammatory imbalance in MS.

Our study also provides molecular support for the systemic failure to control EBV in PwMS. The presence of transcripts reflecting EBV dysregulation, coupled with immune activation, suggests persistent low-grade viral activity that continuously perturbs immune balance. This is consistent with previous evidence of ineffective T-cell–mediated EBV control in MS.[34,39] Within the CNS, additional mechanisms of immune evasion, such as exploitation of the PD-L1/PD-1 axis by infected B cells,[40] may further sustain viral persistence. Genetic susceptibility also contributes to this scenario, with genome-wide analyses showing enrichment of MS risk loci in genes interacting with EBV proteins. Recent evidence indicates that the EBNA2 1.2 risk variant converges on immune pathways such as CD40 signaling, linking host and viral genetic variability to immunopathogenic mechanisms specific for MS.[41]

Altogether, our findings support the notion that EBV may act as a chronic immunologic driver in MS, maintaining a state of persistent immune activation through transcriptional activity that reawakens antiviral pathways, promotes proinflammatory responses, and alters T-cell migration. This integrative model reinforces the positioning of EBV as a central contributor to MS pathogenesis.

From a translational perspective, our data underscore the potential of targeting EBV and its immunologic consequences to alter disease progression in MS. The proven efficacy of B-cell–depleting therapies in MS[42] supports this rationale and our identification of specific virus-host transcriptional signatures opens the way for more selective strategies. While conventional antivirals have limited impact on latency,[43] early clinical evidence suggests that agents targeting the EBV DNA polymerase, such as tenofovir, may help control disease activity.[44,45] Additional approaches include EBNA1 inhibitors[46] and EBV-specific adoptive T-cell therapies,[47] which have shown promise in early-phase studies. Although autologous T-cell therapies are encouraging, allogeneic approaches have so far failed to show benefit in controlled trials.[48] An evolving view of MS immunology underscores that relapses are sustained by peripheral cellular immune interactions, particularly the dialogue between B and T cells. The efficacy of anti-CD20 therapies illustrates the therapeutic benefit of disrupting this pathogenic crosstalk.[49,50] Future strategies may further optimize approaches that selectively target B/T-cell interactions while simultaneously controlling viral persistence, thereby integrating immune modulation with EBV-targeted interventions. Such approaches will require reliable molecular biomarkers to stratify patients and guide precision interventions.[9,23] In this context, our work contributes directly to this effort by providing evidence of EBV transcriptional activity and its association with specific immune signatures.

This study has some limitations. The use of total PBMCs may underestimate EBV abundance, although it enabled assessment of virus-host interactions in a physiologically relevant immune context. Future studies focusing on EBV detection and quantification should use B-cell enrichment strategies to increase sensitivity. CSF was analyzed only in PwMS and not in healthy donors; while CSF collection from healthy individuals is rarely feasible, comparison with other neurologic disease controls could provide additional insights. Longitudinal analyses linking EBV activity with clinical and radiologic outcomes, including treatment response, will be important. Finally, innovative CNS profiling approaches, such as spatial transcriptomics, may help clarify the local role of EBV in MS pathology.

In conclusion, our study provides molecular evidence that EBV latency disruption and lytic reactivation contribute to immune dysregulation in MS. These findings support a model in which persistent EBV activity sustains inflammation beyond the initial trigger phase. Integrated virus-host transcriptional profiling may identify novel biomarkers to stratify patients most likely to benefit from EBV-targeted therapies and pave the way for future personalized treatment strategies.

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Author Contributions

C. Meloni: major role in the acquisition of data; analysis or interpretation of data. F. Marnetto: major role in the acquisition of data. C. Fagnani: analysis or interpretation of data. L Benincasa: major role in the acquisition of data. D. Galano: major role in the acquisition of data. P. Trivedi: drafting/revision of the manuscript for content, including medical writing for content. P. Valentino: major role in the acquisition of data. S. Martire: major role in the acquisition of data. A. Di Sapio: major role in the acquisition of data. A. Bertolotto:

major role in the acquisition of data. A.M. Repice: major role in the acquisition of data. C. Ballerini: major role in the acquisition of data. C. Mancosu: major role in the acquisition of data J. Frau: major role in the acquisition of data. E. Cocco: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data. C. Veroni: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data.

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