

Elevated EBNA-1 IgG in MS is associated with genetic MS risk variants

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ABSTRACT

Objective: To assess whether MS genetic risk polymorphisms (single nucleotide polymorphism [SNP]) contribute to the enhanced humoral immune response against Epstein-Barr virus (EBV) infection in patients with MS.

Methods: Serum anti-EBV nuclear antigen 1 (EBNA-1) and early antigen D (EA-D) immunoglobulin γ (IgG) levels were quantitatively determined in 668 genotyped patients with MS and 147 healthy controls. Anti-varicella-zoster virus (VZV) IgG levels were used as a highly prevalent, non-MS-associated control herpesvirus. Associations between virus-specific IgG levels and MS risk SNPs were analyzed.

Results: IgG levels of EBNA-1, but not EA-D and VZV, were increased in patients with MS compared with healthy controls. Increased EBNA-1 IgG levels were significantly associated with risk alleles of SNP rs2744148 (SOX8), rs11154801 (MYB), rs1843938 (CARD11), and rs7200786 (CLEC16A/CIITA) in an interaction model and a trend toward significance for rs3135388 (HLA-DRB1*1501). In addition, risk alleles of rs694739 (PRDX5/BAD) and rs11581062 (VCAM1) were independently associated and interacted with normal EBNA-1 IgG levels. None of these interactions were associated with EA-D and VZV IgG titers.

Conclusions: Several MS-associated SNPs significantly correlated with differential IgG levels directed to a latent, but not a lytic EBV protein. The data suggest that the aforementioned immune-related genes orchestrate the aberrant EBNA-1 IgG levels. *Neurol Neuroimmunol Neuroinflamm* 2017;4:e406; doi: 10.1212/NXI.000000000000406

GLOSSARY

CI = confidence interval; **EA-D** = early antigen D; **EBNA-1** = Epstein-Barr nuclear antigen 1; **EBV** = Epstein-Barr virus; **HC** = healthy control; **HLA** = human leukocyte antigen; **IgG** = immunoglobulin γ ; **IM** = infectious mononucleosis; **miR** = micro RNA; **NF- κ B** = nuclear factor kappa B; **OR** = odds ratio; **SNP** = single nucleotide polymorphism; **VCA** = viral capsid antigen; **VLA-4** = very late antigen 4; **VZV** = varicella-zoster virus.

The etiology of MS involves genetic and exposure to environmental factors, including Epstein-Barr virus (EBV) infection.^{1–3} Genetic risk factors for MS include specific human leukocyte antigen (HLA) alleles and currently approximately 100 mainly adaptive immune-related single nucleotide polymorphisms (SNPs) with modest odds ratios (ORs) compared with the major HLA-DRB1*1501 association^{4–6} and EBV exposure.⁷

Almost all patients with MS are infected with EBV, compared with 90%–95% in healthy controls (HCs).^{7,8} A history of EBV-related infectious mononucleosis (IM) and elevated EBV nuclear antigen 1 immunoglobulin γ (IgG) levels increases the risk to develop MS later in life.^{9–11} Recently, a meta-analysis showed that antibodies against the latency-associated Epstein-Barr nuclear antigen 1 (EBNA-1) are consistently increased in patients with MS compared

Supplemental data
at Neurology.org/nn

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Funding information and disclosures are provided at the end of the article. Go to Neurology.org/nn for full disclosure forms. The Article Processing Charge was funded by *Neurology*[®], *Neuroimmunology* & *Neuroinflammation*.

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with healthy EBV carriers, whereas results for the lytic viral capsid antigen (VCA) and early antigen D (EA-D) are more heterogeneous.⁷

Epidemiologic and genetic studies support interactions between EBV and HLA-DRB1*15 on an additive scale.^{12–14} Whether the MS risk SNPs are associated with enhanced IgG levels against EBV is currently unclear.^{13,14}

We hypothesized that certain MS risk SNPs are involved in the increased humoral immune response against EBV. Serum IgG levels against the EBV antigen EBNA-1 were measured and associations with MS risk SNPs were assessed.

METHODS Patients and controls. Consecutive patients with MS (n = 668) seen at the MS center ErasMS between 2003 and 2013 were included. A diagnosis of MS was made based on the 2005 McDonald criteria for MS¹⁵ and subclassified as clinically isolated syndrome, primary progressive, relapsing-remitting, or secondary progressive MS based on the clinical course of disease. Unrelated HCs (n = 147) were persons accompanying patients with MS to our outpatient clinic. HCs were age matched to the patients with MS. Exclusion criteria for HCs to participate in this study were prior neurologic symptoms suggestive of MS or the use of immunomodulatory drugs for other autoimmune diseases.

Standard protocol approvals, registrations, and patient consents. This study was performed according to the guidelines specified in the Declaration of Helsinki and approved by the Medical Ethical Committee of the Erasmus MC, and all participants provided written informed consent.

Determination of IgG levels against viral proteins. Plasma from blood collection tubes containing EDTA (BD) was used to measure IgG levels against EBNA-1, EA-D, and varicella-zoster virus (VZV) as a control. Subsequently in samples negative for

EBNA-1 and EA-D, we measured anti-VCA IgG to ascertain anti-EBV serostatus. All samples were determined using well-validated chemiluminescent assays and IgG levels measured on a Liaison XL (all DiaSorin, Saluggia, Italy) according to the manufacturers' guidelines at the National Referral Center for Virus Diagnostics at the Erasmus MC. All samples were automatically diluted by a factor 20 by the Liaison XL. If antibody levels were below or above the threshold, these samples were reanalyzed undiluted or manually prediluted by factor 10 and subsequently diluted by the Liaison XL, respectively. Using this protocol, all samples were in the linear range of the assays. Patients negative for EBNA-1, EA-D, and VCA IgG were omitted from further study to prevent bias between patients with MS and HCs due to differences in EBV seroprevalence.

DNA isolation and genotyping. Genomic DNA was isolated from peripheral blood cell pellets using standard laboratory techniques. Isolated DNA was resuspended in Tris-EDTA (pH = 7.5) buffer and stored at -20°C. Genotyping was performed using the Illumina 610K Quad array (Illumina, San Diego, CA) or Sequenom platform (on both platforms, genotyping was performed in 1 run according to good laboratory practice following the manufacturers' protocol) for 78 MS-associated risk SNPs at the Erasmus MC^{4,5} (table e-1 at Neurology.org/nn). All SNPs were in the Hardy-Weinberg equilibrium (all *p* values >0.30).

Statistical analysis. IgG levels for EBNA-1, EA-D, and VZV were 10 log transformed to normalize titers. T-tests were used to compare groups, and analysis of variance was used to compare more than 2 groups (clinical parameters: use of immunomodulatory drugs, age, sex, and disease form). For logistic regression, IgG levels were dichotomized as above or below the 75th percentile, and the ORs for MS risk SNPs were determined. In the first phase of this study, 330 patients with MS were included, and all MS risk SNPs included in this study (table e-1) were assessed. All SNPs (in interaction) associated with EBNA-1 IgG with a *p* value <0.10 (i.e., VCAM-1, PRDX5, SOX8, MYB, CARD11, CLEC16A, and HLA-DRB1*1501), EBNA-1, EA-D, VZV, and optionally VCA IgG levels were subsequently determined in a validation cohort of 336 patients with MS and 147 HCs. All associations were confirmed in the validation cohort. Subsequently, both data sets were pooled for further analysis. Lastly, interaction models between MS risk SNPs associated with IgG levels were constructed using forward conditional logistic regression. Initially, in a univariate model, SNPs associated with increased EBV IgG were identified. Subsequently, the most significant SNP was assessed in an interaction term with all remaining SNPs. Significant interactions were subsequently taken into the next model with an additional SNP in the interaction term, until no more significant interactions were found. Lastly, using this interaction term, remaining interactions between the other SNPs were assessed using the same method. Logistic regression models were determined both uncorrected and corrected for aforementioned clinical parameters. If the number of homozygous SNP carriers was smaller than 40 persons, this group was pooled with heterozygous allele carriers.

Using bootstrapping analysis, 95% confidence intervals (CIs) were calculated, with 10,000 random samples from the assessed population with a bias-corrected accelerated method (unless otherwise indicated). All analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY).

p Values <0.05 were considered statistically significant, and significance is indicated in the figures as **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. Graphs were constructed using GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA).

Table 1 Clinical and demographic characteristics of patients and controls

	HC (n = 147)	MS (n = 668)	<i>p</i> Value
Age at sampling (SD) in years	41 (12)	42 (13)	0.46
Age at onset (SD) in years	NA	34 (11)	NA
No. of female:male	78:60	477:190	5 × 10 ⁻⁴
Disease duration in months (IQR)	NA	98 (13-152)	NA
CSF abnormal, %		82	
IgG index raised, %		72	
Oligoclonal bands	NA		NA
Multiple, %		61	
1 additional, %		12	
Treated patients, %	NA	17	NA

Abbreviations: HC = healthy control; IgG = immunoglobulin γ ; IQR = interquartile range; NA = not applicable. CSF abnormal defined as either oligoclonal bands specific in CSF or a raised IgG index >0.67 or both.

RESULTS Patients and controls. Patients with MS and HCs were age matched; however, the sex distribution was significantly different. Demographic characteristics and clinical information are shown in table 1.

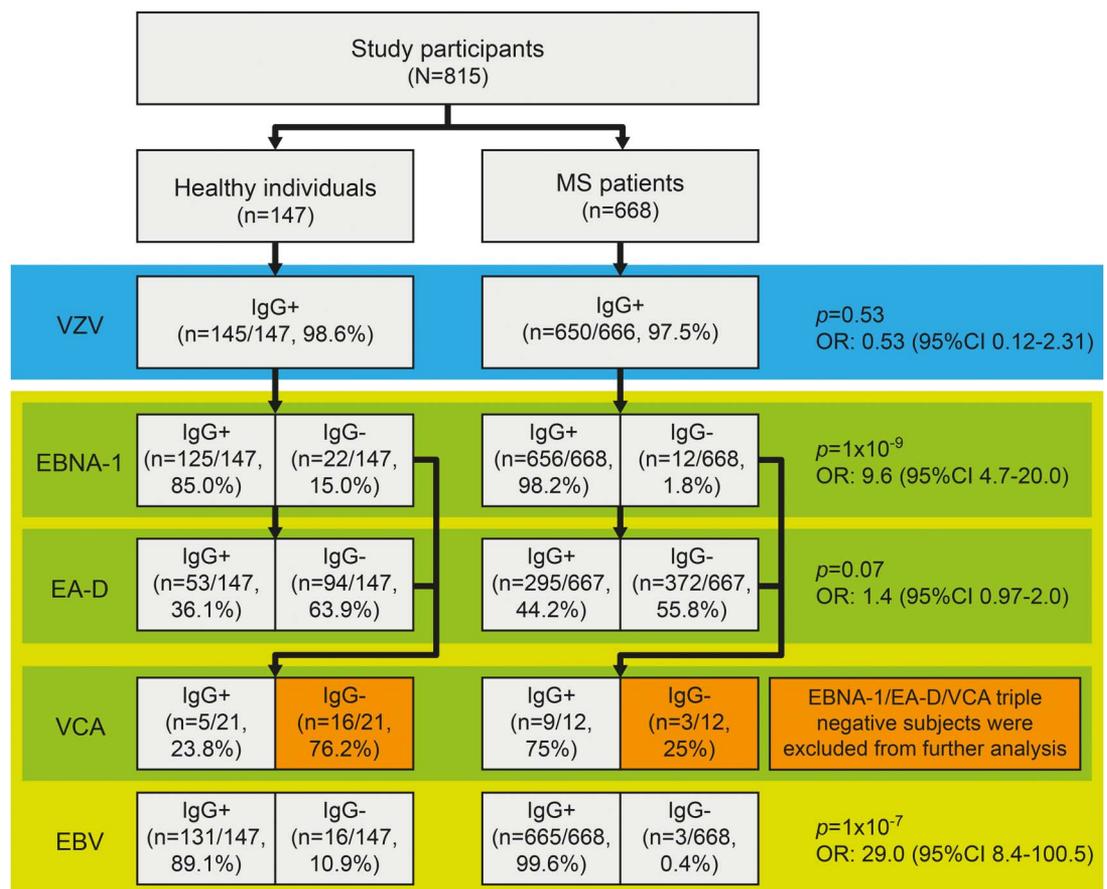
EBV seroprevalence and EBNA-1 IgG levels are increased in patients with MS compared with controls. More patients with MS (665 of 668; 99.6%) compared with HCs (131 of 147; 89.1%) were EBV seropositive based on EBNA-1, EA-D, and VCA IgG levels (OR 29.0, 95% CI 8.4–100.5; $p = 1 \times 10^{-7}$, figure 1). This difference in EBV seroprevalence was irrespective of HLA-DRB1*1501 carriership (HLA-DRB1*1501 adjusted OR 43.6, 95% CI 9.7–196.7; $p = 9 \times 10^{-7}$). No difference in VZV seropositivity between MS and HCs was observed ($p = 0.40$, figure 1). In addition, EBNA-1 IgG levels were higher in patients with MS compared with healthy EBV carriers (HEC) ($p = 9 \times 10^{-7}$, figure 2A). Differences in EBNA-1 IgG levels were not related to sex differences ($p = 0.36$, figure 2D), clinical disease course ($p = 0.16$, figure 2E), or immunomodulatory drug treatment ($p = 0.62$,

figure 2F). By contrast, no differences were observed for EA-D ($p = 0.55$, figure 2B) and VZV IgG levels between MS and HCs ($p = 0.08$, figure 2C).

HLA-DRB1*1501 is associated with enhanced serum EA-D IgG levels. EBNA-1, EA-D, and VZV IgG levels were dichotomized as above or below the 75th percentile of patients with MS. Subsequently, the OR of HLA-DRB1*1501 carriers for high IgG levels was determined. Previous associations of HLA-DRB1*1501 with elevated EBNA-1 IgG titers¹² showed a trend for significance (OR 1.43, 95% CI 0.96–2.13; $p = 0.08$) using a multivariate logistic regression model (figure 3A). Moreover, we observed an association of HLA-DRB1*1501 with EA-D IgG levels (OR 2.08, 95% CI 1.19–3.64; $p = 0.01$), but not VZV (data not shown).

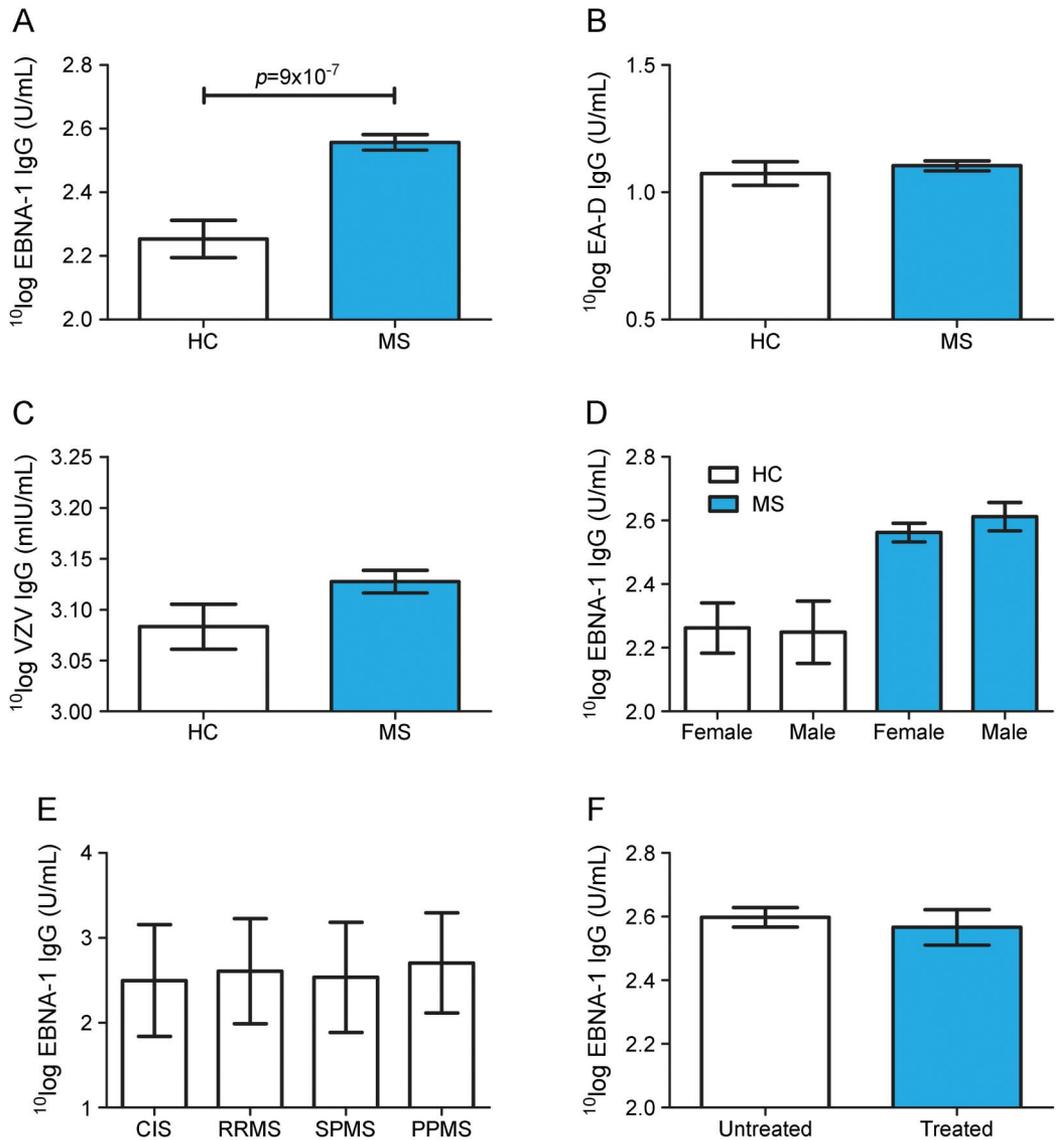
PRDX5 and VCAM1 protective alleles are associated with elevated EBNA-1 IgG levels. Next, we assessed the OR of 78 MS-associated risk SNPs (table e-1) for high serum EBNA-1, EA-D, and VZV levels. The non-MS

Figure 1 Flowchart of the study and virus seroprevalence



Immunoglobulin γ (IgG) levels against varicella-zoster virus (VZV) and the Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) and early antigen D (EA-D) peptides were determined. For EBNA-1 and EA-D IgG double-negative patients, viral capsid antigen (VCA) IgG was assessed to ensure EBV serostatus. IgG levels against VZV was used as a highly prevalent herpes control virus to assure the specificity of the outcomes. EBNA-1, EA-D, and VCA triple negative patients were excluded from further study. CI = confidence interval; OR = odds ratio.

Figure 2 EBNA-1, but not EA-D nor VZV, increased in patients with MS



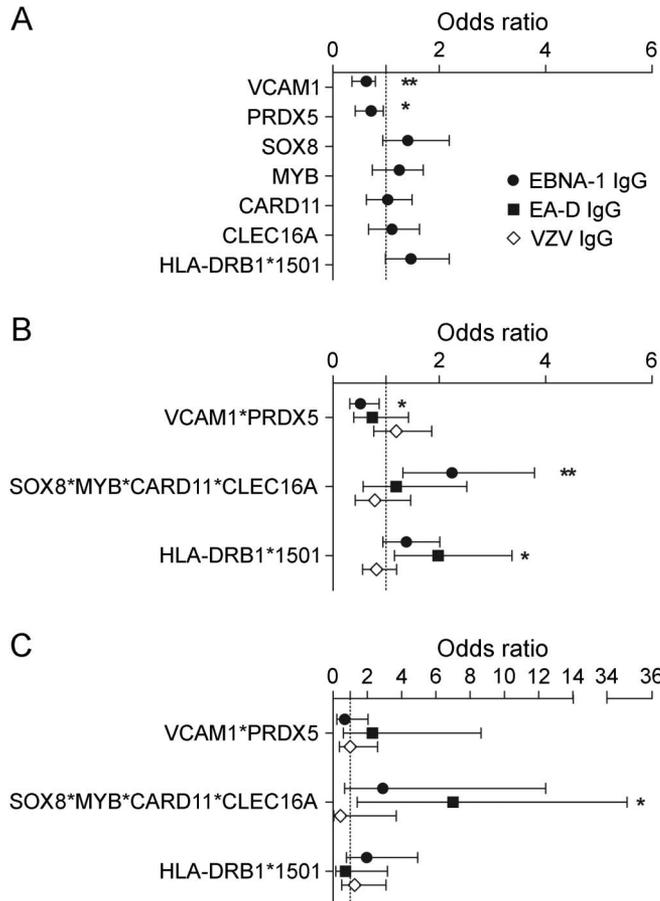
(A) Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA-1) immunoglobulin γ (IgG) titers are significantly increased in patients with MS compared with healthy EBV carriers (HEC). No significant differences in IgG levels between MS and healthy controls (HCs) were observed for (B) early antigen D (EA-D) and (C) varicella-zoster virus (VZV). (D) IgG levels against EBV are similar in (D) women and men, (E) relapsing-remitting (RR) MS, secondary progressive (SP) MS, primary progressive (PP) MS, or clinically isolated syndrome (CIS) patients, and (F) patients treated with immunomodulatory drugs or untreated.

risk alleles of PRDX5 (OR 0.63, 95% CI 0.42–0.95; $p = 0.028$) and VCAM1 (OR 0.54, 95% CI 0.36–0.81; $p = 0.003$) were correlated with high EBNA-1 IgG levels. Conversely, MS risk alleles of MYB, SOX8, CARD11, CLEC16A, and HLA-DR showed a trend toward significance for high EBNA-1 IgG levels ($p < 0.10$, figure 3A). No significant differences were found for the remaining 71 MS risk SNPs. No association between these MS risk SNPs and EA-D IgG and VZV IgG was found (all $p > 0.52$).

Complex interaction between VCAM1-PRDX5 and SOX8-MYB-CARD11-CLEC16A associated with EBNA-1 IgG. Next, we assessed whether interactions between

MS risk SNPs are associated with the enhanced anti-EBNA-1 humoral response observed in patients with MS. All associated SNPs with $p < 0.10$ and HLA-DRB1*1501 (figure 3A) were included in logistic regression models with interaction terms. Interaction was observed between the non-MS risk polymorphisms in VCAM1 and PRDX5 ($p = 0.01$, figure 3B) in association with high EBNA-1 IgG levels. A second interaction term between the risk alleles of SOX8-MYB-CARD11-CLEC16A was associated with high EBNA-1 IgG ($p = 0.004$). In a combined model, HLA-DRB1*1501 was not associated with increased EBNA-1 IgG titers ($p = 0.1$, figure 3B). Adjustment for sex, clinical disease

Figure 3 VCAM1-PRDX5 and SOX8-MYB-CARD11-CLEC16A interactions are associated with EBNA-1 and EA-D IgG levels



Epstein-Barr nuclear antigen 1 (EBNA-1) (circle), early antigen D (EA-D) (filled square), and varicella-zoster virus (VZV) immunoglobulin γ (IgG) levels were dichotomized, and logistic regression was performed to assess which MS risk SNPs are associated with EBNA-1, EA-D, and VZV IgG levels. (A) Univariate logistic regression in patients with MS showed significance for EBNA-1 IgG above the 75th percentile for VCAM1 (odds ratio [OR] 0.54, 95% confidence interval [CI] 0.36–0.81; $p = 0.003$) and PRDX5 (OR 0.63, 95% CI 0.42–0.95; $p = 0.028$). Multivariate logistic regression with interaction terms between MS risk SNPs showed significant interactions of VCAM1*PRDX5 (OR 0.54, 95% CI 0.32–0.89; $p = 0.01$) and SOX8*MYB*CARD11*CLEC16A (OR 2.1, 95% CI 1.23–3.66; $p = 0.004$) for EBNA-1 IgG, but not HLA-DRB1*1501 ($p = 0.1$), in patients with MS (B) and in healthy Epstein-Barr virus carriers (C). HLA = human leukocyte antigen. * $p < 0.05$, ** $p < 0.005$.

course, and immunomodulatory treatment did not affect the results ($p > 0.20$, data not shown). Age at sampling was significantly associated with EBNA-1 IgG, adjusting our model did not alter the conclusions (data not shown). No associations between these interacting genes and EA-D or VZV IgG were found (figure 3B). Similar, though less robust, trends in HEC were observed (figure 3C). Lastly, the 95% CIs of these interactions were assessed using a bootstrapping approach with 10,000 random samples to nonparametrically fit the model. Only very minor differences after bootstrapping were observed (approximate bias between the actual data and the bootstrapped data set was 0.019 or less), indicating that the OR on EBNA1 IgG levels are accurate. In conclusion, gene-gene interaction between VCAM1-PRDX5 and

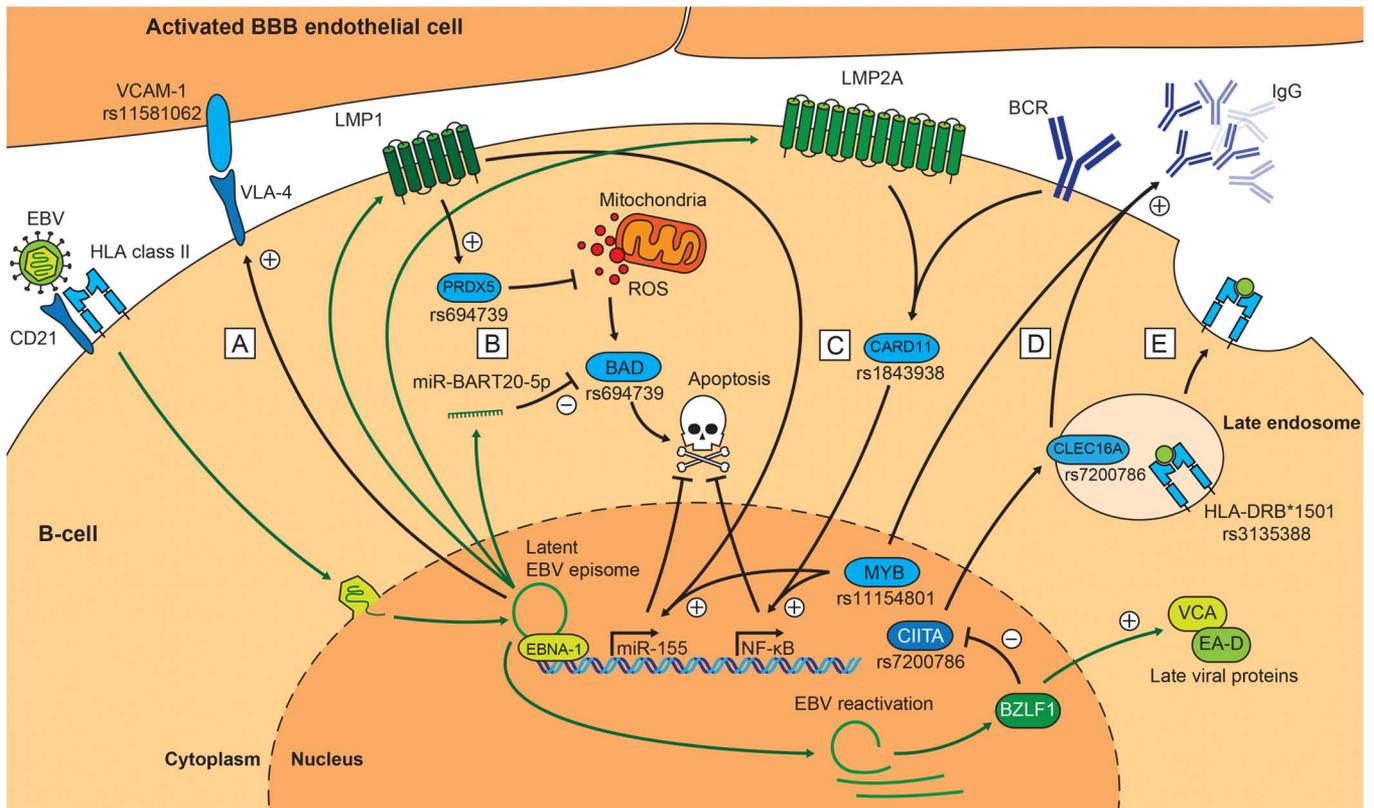
SOX8-MYB-CARD11-CLEC16A is specifically associated with EBNA-1 IgG and not with EA-D and VZV.

Gene-gene interaction associated with EBNA-1 IgG is not secondary to MS. Next, we assessed whether the different gene-gene interactions are related to the MS risk SNPs or secondary to MS disease activity. Therefore, we used logistic regression with abundant EBNA-1 IgG as the dependent variable and patients with MS as a covariate. Correcting for MS as a covariate did not alter the previously described interactions, indicating that the gene-gene interactions itself are important for the increased EBNA-1 IgG and that the associations are not secondary to MS alone. In addition, correcting for HLA-DR did not affect our results (table e-2).

DISCUSSION Increased EBV seroprevalence and, more specifically, enhanced IgG specific for EBNA-1 immune response in patients with MS have been widely reported^{2,8,10,11} and confirmed in this study. Longitudinal studies demonstrating EBV infection in the years preceding the onset of MS^{2,8,10,11} are often used as (circumstantial) evidence for a causal role of EBV in MS. Alternatively, this might be an epiphenomena as a result of a dysregulated immune system observed in patients with MS, leading to an altered handling of the virus. In addition, the virus might be more active in patients with MS, although the antibody titers against EA-D, a marker for active viral replication, are not increased in patients with MS in the majority of published studies⁷ as well as in our study. Lastly, it might be explained that patients with MS develop IM at a later age than HCs, especially because EBV infection at later age is more likely to cause IM.¹⁶ The enhanced EBNA-1 IgG titers in MS might at least partially be explained by both HLA and non-HLA MS-associated risk genes.^{13,14} Recently, a study with 1,367 Mexican Americans assessed association between genome wide over 900,000 SNPs and EBNA-1 seropositivity, irrespective of antibody titers. They found that only HLA-DRB1 and HLA-DQB1 were associated with EBNA-1 positivity. This is slightly in contrast to the current study, which might be explained by the fact that we took the EBNA-1 IgG titers into account in our models. In addition, their study might have suffered from a power problem with multiple testing issues, whereas we only assessed MS-associated SNPs. Lastly, differences in study population (patients with cardiovascular disease and diabetes mellitus) might explain the different results.¹³

MS-associated risk SNPs are shown to affect the Th- and B-cell transcriptomes.^{17,18} This may, on the one hand, influence humoral immune responses against EBV and on the other hand influence regulation of EBV latency in B cells. To assess whether MS

Figure 4 Model proposing interaction between EBV latency proteins with here described MS risk genes



(A) Epstein-Barr virus (EBV) infection of B cells induces very late antigen 4 (VLA-4) expression, the ligand of VCAM-1 expressed by activated brain epithelial cells. (B) Latency-associated membrane protein 1 (LMP1) of EBV induces expression of the peroxiredoxin family of antioxidant enzymes (PRDX5), thereby reducing reactive oxygen species (ROS) and preventing caspase-mediated apoptosis. EBV-expressed micro RNA (miR)-BART20-5p targets B-cell chronic lymphocytic leukemia/BAD transcripts, reduces BAD protein levels, and inhibits apoptosis. (C) Analogous to B-cell receptor signaling, EBV LMP2A activates miR-155 and NF- κ B expression via CARD11 and thereby inhibits apoptosis and promotes proliferation. (D) Immediate early lytic EBV protein BZLF1 blocks loading of peptides to HLA class II in late endosomes by inhibiting CIITA and induced expression of early antigen D (EA-D) and viral capsid antigen (VCA). (E) CLEC16A is involved in HLA-II antigen presentation via regulation of the endosomal compartment and is important for the production of Ig. Plus and minus indicate increased and reduced expressions, respectively. BBB = blood brain barrier; BCR = B-cell receptor; EBNA-1 = Epstein-Barr nuclear antigen 1; HLA = human leukocyte antigen; IgG = immunoglobulin γ ; NF- κ B = nuclear factor kappa B.

risk genes are associated with EBNA-1 IgG, we stratified quantitative EBNA-1, EA-D, and VZV IgG titers as above the 75th percentile to MS-associated SNPs in healthy EBV carriers and EBV-positive MS patients and determined the OR of these polymorphisms.

In this study, we observed a similar trend between EBNA-1 IgG and of HLA-DRB1*1501 in line with previously reported associations.¹² Moreover, here, HLA-DRB1*1501 was also associated with EA-D IgG levels, but not VZV IgG. The HLA-DRB1*1501 influences the repertoire of T cells recognizing peptide antigens.¹⁹ It can therefore be suggested that this HLA-DRB1*1501 association with EBNA-1 and EA-D IgG levels reflects Th-/B-cell interactions, having an effect on the EBV-specific humoral immune response of patients with MS.

By contrast, we found that the HLA-independent MS risk SNPs in VCAM-1 and PRDX5 inversely correlated with EBNA-1 IgG levels. Of interest, in

a multivariate logistic regression model, interactions between VCAM1-PRDX5 and SOX8-MYB-CARD11-CLEC16A were associated with EBNA-1 IgG levels in a contrasting manner. Strikingly, the HLA-DRB1*1501 effect on EBNA-1 IgG titer is overshadowed by these 2 genetic interactions associated with altered EBNA-1 humoral responses. The increased EBNA-1 IgG observed in patients with MS is partially explained by these genetic interactions.

A similar genetic association was found in HCs previously exposed to EBV, although not statistically significant presumably due to the lack of statistical power. The in part common genetic background of HCs and patients with MS (e.g., the quite high proportion of HCs carrying MS risk SNPs) might explain the common effects observed in the immune response against EBV observed in this study. The identified MS risk genes associated with EBNA-1 IgG are likely candidates to be functionally involved

in the enhanced humoral immune response, as discussed here below.

EBNA-1 is expressed in all phases of the EBV lifecycle except latency 0, where only noncoding EBV transcripts are expressed.²⁰ EA-D, an EBV polymerase processivity protein encoded by BamHI fragment M rightward open reading frame 1, is restricted to lytic-phase EBV.²⁰ The observed association with EBNA-1 IgG levels therefore suggests that the genes associated with MS risk SNPs found in this study interact with latency-associated transcripts of EBV in infected B cells. Strikingly, most genes associated with the SNPs that correlate with EBNA-1 IgG levels identified in this study have been shown to interact with EBV-associated proteins and transcripts.

VCAM1 is expressed by cytokine-activated blood-brain barrier endothelial cells in MS lesions.²¹ Memory B cells constitutionally express the ligand of VCAM1, integrin $\alpha 4\beta 1$ (very late antigen 4, VLA-4).¹⁷ EBV infection considerably increases VLA-4 expression in B cells,²² suggesting increased migration of EBV B cells to the CNS (figure 4A).

PRDX5 is a member of the peroxiredoxin family of antioxidant enzymes. PRDX5 expression prevents reactive oxygen species-mediated damage in mitochondria, thereby inhibiting caspase-induced apoptosis. The EBV latent membrane protein 2A induces PRDX5 expression in B cells.²³ Alternatively, the PRDX5-associated SNP, rs694739, is related to B-cell chronic lymphocytic leukemia/BAD in a type I diabetes study.²⁴ Expression of BAD is induced during caspase-mediated apoptosis. The constitutionally EBV-expressed micro RNA (miR)-BART20-5p targets the 3'-untranslated region of BAD, resulting in decreased BAD levels and reducing apoptosis in infected B cells.²⁵ EBV inhibits caspase-mediated apoptosis via PRDX5 and BAD during latent- and lytic-phase infections (figure 4B), resulting in reduced immune exposure of EBNA-1, which may partly explain the PRDX5 association with lowered EBNA-1 IgG levels in patients with MS (figure 4B).

The rs11154801 SNP is linked to Abelson helper integration site 1 or MYB.²⁶ MYB is a transcription factor required for B-cell development, and expression is reduced during the process. Aberrant expression of MYB leads to lymphoproliferative disorders by inducing nuclear factor kappa B (NF- κ B) and miR-155 expression.²⁷ Direct interaction of EBV transcripts or proteins with MYB are not shown. However, latency-associated membrane protein 1 induces miR-155 and LMP2A/B potently induces NF- κ B expression during latencies I and II, preventing apoptosis and promoting proliferation.^{27–29} CARD11 is a scaffolding protein required for NF- κ B activation.

Of interest, gain-of-function mutations in CARD11 lead to B-cell malignancies and anergic T cells by continuous NF- κ B activation, which is severely exacerbated by EBV infection³⁰ (figure 4C).

The rs7200786 SNP is in close proximity to CLEC16A, DEXI, and CIITA and alters expression of DEXI.³¹ CIITA and CLEC16A are involved in the regulation of HLA class II via the endosomal system.³² In addition, CLEC16A knockout in mice leads to reduced numbers of B cells and an altered immunoglobulin spectrum.³³ The immediate early lytic cycle-associated EBV transcription factor BZLF1 downregulates MHC class II antigen presentation through inhibition of CIITA during EBV reactivation (figure 4, D and E).

rs2744148 is located 36.6 kb downstream of the nearest gene SOX8, sex-determining region Y-box 8. To date, no interactions of rs2744148 with SOX8 or EBV transcripts are known.

As we here followed a candidate gene approach using a set of predefined recently discovered MS risk genes, we did not correct for multiple testing. This can be seen as a limitation of this study, with a risk of being underpowered. To attenuate this issue, we used first and second cohorts to validate the findings. Moreover, higher numbers obtained by pooled analysis of patients and controls provided results in the same direction with lower *p* values. The results are plausible, as the SNPs associated with elevated EBNA-1 IgG are all, except SOX8, highly expressed in B lymphocytes (data not shown, based on biogps.org) and already shown to be involved in EBV latency machinery. In the current study, we were able to assess 78 of the 100 MS risk SNPs currently published, thereby possibly missing some genetic associations with EBNA-1 IgG. It is unlikely that this has affected the underlying results. It might be an underestimation of the real genetic contribution to EBNA-1 IgG levels in MS, as there are even more MS risk genes to be identified. Lastly, although we assessed whether or not our results are secondary to MS disease itself, and we found that correcting for MS in our regression models did not alter the results, we cannot fully rule out that MS disease itself is at least partially associated with increased EBNA-1 IgG. To fully assess this, we would need a larger cohort of HCs to draw reliable conclusions for this association.

Taken together, the genes associated with the identified genomic MS risk SNPs have previously been shown to be involved in migration (VCAM1), anti-apoptosis (PRDX5/BAD, MYB/NHI1, and CARD11), and MHC class II antigen presentation (HLA-DRB1*1501 and CLEC16A/CIITA) in latently EBV-infected B cells presumably resulting in altered EBNA-1 IgG levels. Functional differences

in these pathways may lead to distinct viral inhibition and appear at the same time in MS-related autoimmunity. This deserves to be further explored at a functional level.

AUTHOR CONTRIBUTIONS

Study design: K.L.K., G.P.V.N., S.M.J.S., G.M.G.M.V., and R.Q.H. Data acquisition: K.L.K., G.P.V.N., S.M.J.S., M.J., G.M.G.M.V., and R.Q.H. Statistical analysis: K.L.K., G.P.V.N., and R.Q.H. Writing the manuscript: K.L.K., G.P.V.N., M.J., G.M.G.M.V., and R.Q.H.

STUDY FUNDING

The MS center ErasMS is financially supported by the MS Research Foundation, Voorschoten, The Netherlands.

DISCLOSURE

K.L. Krefl reports no disclosures. G.P. Van Nierop received research support from the Dutch MS Research Foundation. S.M.J. Scherbeijn reports no disclosures. M. Janssen reports on disclosures. G. M.G.M. Verjans received research support from the Dutch MS Research Foundation, Niedersachsen-Research Network on Neuroinfectiology of the Ministry of Science and Culture of Lower Saxony. R.Q. Hintzen participated in trials with Biogen Idec, Merck Serono, Roche, and Novartis; he is on the editorial board of Multiple Sclerosis and Related Disorders. Go to Neurology.org/nn for full disclosure forms.

Received December 20, 2016. Accepted in final form July 31, 2017.

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