Antibodies from multiple sclerosis brain identified Epstein-Barr virus nuclear antigen 1 & 2 epitopes which are recognized by oligoclonal bands

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Research

Abstract

Background Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), the etiology of which is poorly understood. The most common laboratory abnormality associated with MS is increased intrathecal IgG synthesis and the presence of oligoclonal bands (OCBs) in the brain and cerebrospinal fluid (CSF). However, the major antigenic targets of these antibody responses are unknown. The risk of MS is increased after infectious mononucleosis (IM) due to EBV infection, and MS patients have higher serum titers of anti-EBV antibodies than control populations.

Objectives To identify disease-relevant epitopes of IgG antibodies in MS.

Methods We screened phage-displayed random peptide libraries (12-mer) with total IgG antibodies purified from the brain of a patient with acute MS. We identified and characterized the phage peptides for binding specificity to intrathecal IgG from patients with MS and from controls by ELISA, phage-mediated Immuno-PCR, and isoelectric focusing.

Results Two phage peptides were identified that share sequence homologies with EBV nuclear antigens 1 and 2 (EBNA1 and EBNA2), respectively. The specificity of the EBV epitopes found by panning with MS brain IgG was confirmed by ELISA and competitive inhibition assays. Using a highly sensitive phage-mediated immuno-PCR assay, we determined specific bindings of the two EBV epitopes to IgG from CSF from 46 MS and 5 inflammatory control (IC) patients. MS CSF IgG have significantly higher bindings to EBNA1 epitope than to EBNA2 epitope, whereas EBNA1 and EBNA2 did not significantly differ in binding to IC CSF IgG. Further, the EBNA1 epitope was recognized by OCBs from multiple MS CSF as shown in blotting assays with samples separated by isoelectric focusing.

Conclusions The EBNA1 epitope is reactive to MS intrathecal antibodies corresponding to oligoclonal bands. This reinforces the potential role of EBV in the etiology of MS.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) characterized by demyelination and gliosis, with various degrees of axonal pathology and episodic or progressive neurological disability (Compston and Coles, 2008). A persistent synthesis of IgG antibodies (manifested as oligoclonal bands) in the cerebrospinal fluid (CSF) and brain is an immunological hallmark usually seen in MS (Compston and Coles, 2008). However, the major antigenic target(s) of this increased IgG is unknown (Graner et al, 2020). We have shown recently that in MS, there is a complex relationship between oligoclonal bands, and immunoglobulin G antibodies (Beseler et al, 2017). In MS patients, longitudinal analyses of serum samples collected more than 10 years before the onset of clinical symptoms consistently showed that the risk of developing the disease increased significantly with the level of EBV antibody titers, with the strongest association being found for IgG antibodies binding to an EBV latent antigen, nuclear antigen 1 (EBNA1) (Ascherio et al, 2001; DeLorenze et al, 2006; Levin et al, 2005). EBNA1, the crucial EBV antigen for persistence of the virus, constitutes a dominant

antigen for both humoral and cell-mediated immune responses to the virus, and the deregulation of EBV-specific immunity in MS has been reported primarily for this antigen (Lunemann et al, 2007).

Several lines of epidemiological and sero-epidemiological evidence also support the notion of EBV infection playing a potential role in MS. For example, there is an increased risk of developing MS among individuals with a history of infectious mononucleosis (Thacker et al, 2006); there are some similarities in the epidemiology of MS and infectious mononucleosis (Warner and Carp, 1981); there is an almost universal seropositivity for EBV in adults and children with MS (Alotaibi et al, 2004; Ascherio et al, 2001); there is a steep and monotonic increase in MS risk with increasing titers of antibodies to EBV in apparently healthy adults (Levin et al, 2005; Sundstrom et al, 2004); and elevated EBNA1 immune responses predict the conversion to multiple sclerosis (Lunemann et al, 2007).

An increased frequency of IgG reactivity to EBNA1 was found in MS patients using a cDNA protein-expression array containing 37,000 tagged proteins (Cepok et al, 2005). The findings of this study are in accordance with previous reports on a higher frequency of CSF-derived EBNA1-specific IgG antibodies in patients with MS (Bray et al, 1992). Using CSF IgG from MS patients and from controls to select peptides from a filamentous phage display library, five out of 14 MS patients and one out of 14 controls had distinctive reactivity for a 6 amino acid motif found in EBNA1 (Rand et al, 2000). These data suggest that EBV-specific antibodies not only are systemically elevated in MS but also are enriched in the CSF of affected patients.

Phage-displayed random peptide libraries provide an extremely powerful technique for the selection of ligands binding to antibodies. This technology has been used successfully to identify antigens/epitopes of disease-specific antibodies directed at either infectious agents (Ferrer and Harrison, 1999; Heiskanen et al, 1999; Owens et al, 2006), auto-antigens (Rudolf et al, 1998; Zhang and Davidson, 1999) or recombinant antibodies from MS (Yu et al, 2011a). Herein we report the identification and characterization of two EBV nuclear antigen epitopes (EBNA1 and EBNA2). These were identified by screening a phage-displayed random peptide library with IgG antibodies purified from an acute MS brain, with further analyses of the peptide binding profiles to IgG antibodies in 50 MS CSF and 5 CSF obtained from inflammatory controls (IC) using a highly sensitive phage mediated immuno-PCR assay.

Materials And Methods

MS IgG and CSF

MS brain IgG antibodies (MS 95 – 2) used for panning random peptide libraries were purified from a postmortem brain plaque-periplaque white matter of an acute MS patient (Owens et al, 1997). MS and IC CSF samples were collected with approval from the University of Colorado Institutional Review Board (COMIRB # 00–688) (Beseler et al, 2017). All MS patients were of the relapsing-remitting type (RRMS). The inflammatory central nervous system diseases (IC) controls included subacute sclerosing panencephalitis (2), Behcet's disease (1), viral meningitis (1), and cryptococcal meningitis (1).

Biopanning

The PhD-12™ Phage Display Peptide Library (New England BioLabs, Beverly, MA) was used for affinity selection of specific peptides by MS brain IgG antibodies. Detailed procedures for panning, phage amplification and DNA sequencing were carried out as previously described (Yu et al, 2006a). Among the phage clones sequenced, phage/phage peptides 3 – 2 and 3–3 were used in the rest of the analyses.

Sequence analyses

Single-stranded phage DNA was sequenced, and the deduced amino acid sequences were obtained and the peptide sequences identified. The consensus peptide sequences in the EBV virome were identified by sequence alignment using ClustalW (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) with the following options used: endgaps = 1;;gapdist = 8; gapext = 0.2; gapopen = 10.0; hgapresidues = GPSNDQERK; matrix = gonnet; maxdiv = 30; outorder = input; pwgapext = 0.1; pwgapopen = 10.0; pwmatrix = gonnet; type = PROTEIN. EBV sequences used for EBNA1 were UniProt P03211 (Epstein-Barr nuclear antigen 1, Epstein-Barr virus (strain B95-8) (HHV-4) (Human herpesvirus 4)), and for EBNA2 were UniProt P12978 (Epstein-Barr nuclear antigen 2, Epstein-Barr virus (strain B95-8) (HHV-4) (Human herpesvirus 4). Alignments were performed against the entire EBV virome/proteome (both reviewed/annotated and hypothetical) from these sources:

http://www.virosnet.cs.huji.ac.il/viralproteins/showproteins.php?var=10376&a=Human+herpesvirus+4&key=

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Peptide sequence abundance was calculated based on the number of identical clones from the total phage clones sequenced.

Peptide antigenicity/epitope characterization

Putative B cell antigens/epitopes were predicted for phage peptides 3–3 and 3 – 2, as well as for the stretches of peptide sequence from the parental EBNA1 and EBNA2 proteins that were identified in the ClustalW predictions. We used the following programs, algorithms and servers for this:

"Peptide Antigen Prediction", Universidad Complutense Madrid Immunomedicine Group Tools

(http://imed.med.ucm.es/Tools/antigenic.pl) From (Kolaskar and Tongaonkar, 1990), predictions are based on the occurrence of amino acid residues in experimentally known segmental epitopes. The average antigenic propensity for each overlapping 8-mer is determined and compared to the average for the whole protein. If the average for the whole protein is above 1.0, then all residues having above 1.0 are potentially antigenic. If the average for the whole protein is below 1.0, then all residues having above the average for the whole protein are potentially antigenic.

SVMTriP

(http://sysbio.unl.edu/SVMTriP) This assesses potential linear B cell epitopes using Support Vector Machine learning which integrates tri-peptide similarity and propensity scores (SVMTriP) (Yao et al, 2012).

BcePred Prediction Server

(http://crdd.osdd.net/raghava/bcepred/) The BcePred server predict B-cell epitopes of peptides using physico-chemical properties (hydrophilicity, flexibility/mobility, accessibility, polarity, exposed surface and turns) or their combinations and puts them in the context of an antigen propensity score. Graphically, the server plots amino acid residue properties along the protein backbone. The peak of the amino acid residue segment above the threshold value is considered a predicted B-cell epitope. The table output gives the normalized score of the selected properties with the corresponding amino acid residue of a protein along with the maximum, minimum and averages values of the combined methods selected. Threshold values are as follows: hydrophilicity = 1.9; flexibility = 2.0; accessibility = 1.9; turns = 2.4; expose surface = 2.3; polarity = 1.8; antigenic propensity = 1.9.

ABCpred Prediction Server

(https://webs.iiitd.edu.in/raghava/abcpred/index.html) The ABCpred server uses a form of artificial neural networking. In this case it is a partial recurrent neural network with a single hidden layer of 35 residues, and an optional network window length of 10. The output consists of a single binary number of 1 or 0 (epitope or non-epitope). The predicted B cell epitopes are ranked according to their score obtained by trained recurrent neural networks; a higher score for a peptide indicates a higher probability as an epitope. Peptides shown are above the threshold value chosen (0.51 in these cases) (Saha and Raghava, 2006).

BepiPred-2.0: Sequential B- Cell Epitope Predictor

(http://www.cbs.dtu.dk/services/BepiPred/) To predict B-cell epitopes from a protein sequence, the BepiPred-2.0 server uses a Random Forest algorithm trained on epitopes and non-epitope amino acids determined from crystal structures, followed by a sequential prediction smoothing. Sequences colored in the orange gradient are predicted to be above the threshold (here set at 0.51) (Jespersen et al, 2017).

Peptide structure predictions

Structures for Pep 3–3, Pep 3 – 2, and their corresponding homologous sequences in EBNA1 and EBNA2 (EBNA1$_{386-416}$ and EBNA2$_{263-286}$) were rendered using PepFold 3.5 (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/) (Lamiable et al, 2016).

ELISA

Purified MS brain 95 – 2 IgG in 0.1 M carbonate buffer (50 µl, 200 µg/ml) was coated onto wells of ELISA plates overnight at 4 °C. The wells were then blocked with 3% BSA for 2 h followed by incubation with

purified phage 3 – 2 and 3–3 (5×10^9/well) for 1 h. After washing with 0.05% Tween-20/Tris buffered saline (TBST), the wells were incubated with a 1:500 dilution of mouse anti-M13 IgG-HRP (New England BioLabs) antibody for 1 h, followed by incubation with peroxidase substrate ABTS (Vector Laboratories, Burlingame, CA) for 20 min. The optical absorbance was measured at 415 nm with a Microplate Manager (BioRad, Hercules, CA). All samples were tested in duplicate and the ELISA was repeated at least one additional time.

Phage mediated immuno-PCR for phage binding specificity. Wells of protein A coated plates were added with panning MS antibodies (5-fold serial dilutions) and incubated with equal amount of phage 3–3 and 3 – 2 (10^7/well) respectively. Bound phage were assayed by real-time PCR using lysed phage DNA directly as template (Yu et al, 2007). PCR relative cycle threshold (Ct) values (100 minus Ct.) were plotted against IgG concentration. Phage bindings are indicated by low Ct values.

Inhibition ELISA

For inhibition ELISA, MS 95 – 2 IgG at a concentration of 20 µg/ml was pre-incubated with the indicated amounts of competitive synthetic peptide 3–3 or irrelevant peptide for 30 min at room temperature and the mixture was added to wells of ELISA plates pre-coated with EBNA1 protein antigen (50 µg/ml) (DevaTal, Inc, Hamilton, NJ). Bound IgG was detected by incubation of a 1:500 dilution of anti-human IgG-HRP (Sigma) for 1 h followed by ABTS color reaction as described above. EBNA1 peptide competed with EBNA1 protein for binding to MS 95 – 2 IgG, whereas the irrelevant peptide (2-6-12, HVQLAPPIALRH) did not. The higher the concentration of Peptide 3–3 that is added, the less 95 – 2 IgG was observed binding to the EBNA-1 coated on the plate.

Phage mediated immuno-PCR for screening multiple CSF

MS and control CSF at the IgG concentration of 1 µg/ml were added to wells of Reacti-Bind™ protein A coated clear strip plate (Thermo Fisher/Pierce, Waltham, MA) and incubated at room temperature for 2 h. The wells were then blocked with 3% BSA for 2 h and incubated for 1 h with phages 3 – 2 and 3–3 (10^8/well) in 1% BSA, respectively. After washing with TBST, bound phages were collected by heating the wells with 50 µl of deionized water at 95 °C for 15 min. Phage solution (4 µl) was used as templates for real-time PCR. For phage mediated real-time PCR, specific primers for M13 phage were used (Yu et al, 2006b). All real-time PCR was performed in an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Forest City, CA). Each PCR reaction (20 µl) consisted of 1X power SYBR® Green master mix, 750 nM of each primer and 4 µl of phage template. A dissociation-curve analysis was performed at 95 °C for 15 seconds, 60 °C for 1 min and 95 °C for 15 seconds. A control reaction without template and standard curve analysis were included in each run. Bound phages were calculated based on standard curve analysis. The thermal cycle conditions included 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 45 seconds. The 100 bp amplicon was confirmed by electrophoresis.

IEF Immunoblot of MS CSF with EBNA1 phage peptide probe

CSF (200–500 µl) was centrifuged through an Amicon Ultra 0.5 ml 30 K cellulose centrifugal filter unit (Amicon, MilliporeSigma, Burlington, MA) at 14,000 × g for 30 min at room temperature before IEF using the SPIFE® IgG IEF kit (Helena Laboratories, Beaumont, TX) and a SPIFE 3000 electrophoresis analyzer. Wicks were soaked in an anode (0.3 M acetic acid) or cathode (1 M NaOH) solution and applied to the edge of a SPIFE® IgG IEF gel. Five microliters of MS CSF/sera (3–5 µg IgG for phage probe and 100 ng IgG for alkaline phosphatase (AP)-conjugated anti-human IgG probe) (Vector Lab) were loaded into wells of an SPIFE IEF gel. After electrophoresis at 700 V for 1 h at 15 °C, samples were transferred to PVDF membranes (Helena Lab) for 45 min, followed by blocking in Helena blocking agent (1 g bovine milk protein/50 ml 1X TBS) for 1 h at room temperature. Membranes were incubated with the respective phage peptides at concentrations ranging from 5.0×10^{10} to 1.5×10^{11} pfu/ml in 1:10 Helena blocking agent/TBST (blocking buffer) at room temperature for 2 h. After washing with TBST membranes were incubated with mouse anti-M13 mAb at a 1:500 dilution in blocking buffer, followed by incubation with 1:500 dilution of AP conjugated anti-mouse IgG at room temperature for 1 h. Membranes were developed with NBT/BCIP substrate (Vector Labs). For control blots, membranes were incubated for 1 h with 1:1000 dilutions of AP-anti-human IgG (H + L) in blocking buffer, followed by NBT/BCIP detection.

Peptide synthesis

EBV-related peptides (3 – 2 and 3–3) that were selected by panning with the MS 95 – 2 IgG were commercially synthesized by Sigma Genosys (The Woodlands, TX), and were more than 90% pure as determined by reverse-phase high-performance liquid chromatography. Each peptide contains 16 amino acids with the specific 12-mer peptide sequence being analyzed followed by the C terminal peptide sequence GGGC (GGGS is the spacer sequence between the peptide and the phage pIII minor protein). The serine residue was replaced with cysteine to facilitate coupling the thiol group in the resulting peptide to the maleimide-activated ELISA plate (Pierce Thermo Fisher). The C-terminus carboxyl group on the cysteine residue was amidated to block the negative charge.

Statistics

Statistics programs utilized were Microsoft Excel and Graph Pad Prism; other analyses are from specialized software packages and are cited above.

Results

Phage peptides selected by MS plaque IgG share sequence homology with EBNA1 and EBNA2

There are continually mounting epidemiological data regarding the association between EBV infection and MS (Laurence and Benito-Leon, 2017) (including the antibody recognition of EBV antigens (Almohmeed et al, 2013)). In this background, to identify antigenic targets of the antibody response of IgG in MS, we panned phage-displayed random peptide libraries on IgG that was purified from plaque-periplaque white matter of an acute MS brain using similar panning protocols (Graner et al, 2020; Yu et al,

2011b). We identified 2 phage peptides (Pep 3−3 and Pep 3 − 2), that shared conserved amino acid residues with EBV nuclear antigen 1 and 2 (EBNA1 and EBNA2), which were further confirmed by searching the peptides for homology against the EBV virome/proteome (Fig. 1A &D). Note that of the 20 phage clones sequenced, Pep 3−3 represented 2 of those, and Pep 3 − 2 represented 13 of the clones.

We analyzed the antigenic potential of Peps 3−3 and 3 − 2, along with their corresponding sequences in EBNA1 and EBNA2 proteins, using a variety of algorithms. For Pep 3−3 and EBNA1, we see that based on methods of Kolaskar and Tongaonkar (Kolaskar and Tongaonkar, 1990), (http://imed.med.ucm.es/Tools/antigenic.pl) portions of the mid- C-terminus of $EBNA1_{386-416}$ would be considered antigenic, including the determinant PFFHPVGE. The C-terminus of Pep 3−3 shows potential antigenic propensity (Fig. 1B, C) and this is a region of sequence homology between Pep 3−3 and EBNA1 (Fig. 1A). Both Pep 3 − 2 and the corresponding region of EBNA2 (EBNA $_{263-286}$) have average antigenic propensities above threshold with higher scores at the peptides' C-termini despite no noted specific antigenic determinants (Fig. 1E, F). Further, additional multiple epitope prediction programs suggest that homologous regions of $EBNA1_{386-416}$ and Pep 3−3, and of $EBNA2_{263-286}$ and Pep 3 − 2, are potential B cell epitopes (Supplemental Figs. 1 &2, Supplemental Table 1).

Predicted structural relationships between $EBNA1_{386-416}$ and Pep 3−3, and between $EBNA2_{263-286}$ and Pep 3 − 2

There is no definitive structural information available concerning the region of EBNA1 ($EBNA1_{386-416}$) that is homologous to Pep 3−3. Protein Database Entries covering a portion of the EBNA1 protein are in the context of an EBNA1 peptide in the pocket of an HLA molecule being presented to a T cell receptor. $EBNA2_{263-286}$ (homologous to Pep 3 − 2) also lacks structural data.

To compare the EBNA proteins regions with their homologous peptides, we used PepFold 3.5 to render possible structures for three-dimensional alignments of the homologous protein sequences and the phage peptides. For $EBNA1_{386-416}$, the amino acid residues in that peptide's positions 17−23 represent the longest contiguous stretch of homology with Pep 3−3 (Fig. 1A).

In Fig. 2A, these charged and aromatic amino acids protrude from the plane of the peptide toward the reader, suggesting an exposed surface, portions of which are considered potential B cell/antibody recognition epitopes. Likewise, the residues of Pep 3−3 that show a similar structural configuration (Arg5, Pro6, Phe7, Phe8, Pro10, Fig. 2B) to the homologous stretch in $EBNA1_{386-416}$ (Arg18, Pro19, Phe20, His22, Pro23, Fig. 2A) are also within portions of predicted epitopes. For $EBNA2_{263-286}$ and Pep 3 − 2, we see positional alignments of aromatic and polar amino acids (Pro16, Thr17, Pro13, Phe19 in the arrangement of $EBNA2_{263-286}$ (Fig. 2C) with Pro5, Thr6, Tyr9, and Phe8 in Pep 3 − 2 (Fig. 2D). Again, these amino acids are depicted as protruding from the plane of the image toward the reader, and they correspond to regions that may be B cell/antibody epitopes.

The specificity of phage peptides for MS plaque IgG

To validate the specificity of the selected phage peptides, we performed ELISAs and showed that these two EBNA-related peptides bound specifically to MS brain IgG antibodies used in the original panning (Fig. 3A). Furthermore, dose response phage-mediated immune-PCR demonstrated that these phage peptides bound to panning target antibodies in a dose-dependent manner (Fig. 3B).

Phage peptide 3−3 (EBNA1 $_{386-416}$ epitope) inhibits binding of EBNA1 recombinant protein to panning MS brain IgG

We showed that phage peptide 3−3 has high sequence and structural homology to EBNA1, along with predicted epitope potential. To demonstrate that Pep 3−3 indeed resembles an epitope of EBNA1, we synthesized peptide 3−3 and carried out inhibition ELISA using commercial EBNA1 protein. MS brain IgG 95 − 2 at a final concentration of 20 μg/ml was incubated with varying concentrations of peptide 3−3 or irrelevant peptide (2-6-12, HVQLAPPIALRH) and were transferred to ELISA plates pre-coated with EBNA1 protein. Bound IgG were detected with HRP-conjugated anti-human IgG. Pep 3−3 competed with EBNA1 protein for binding to MS 95 − 2 IgG, whereas the irrelevant peptide did not. The higher the concentration of peptide 3−3 that is added, the less 95 − 2 IgG was observed binding to the EBNA-1 coated on the plate (Fig. 4).

MS CSF contained significantly higher levels of antibodies bound to EBNA1 epitope (Pep 3−3) compared to EBNA2 epitope (Pep 3 − 2)

We applied highly sensitive phage-mediated IPCR to assess the binding specificity of putative EBNA1/2 epitopes (represented by phage peptides 3−3 and 3 − 2) to MS CSF IgG (Yu et al, 2011a). Phage peptides 3−3 and 3 − 2 were added to MS and IC CSF coated wells of protein A plates. Bound phage were lysed and detected by real-time PCR (Yu et al, 2007), with the number of bound phage determined. CSF IgG from 46 MS patients overall bound more EBNA1 epitope/phage peptide 3−3 and did so more often (Fig. 5A) compared to the binding of EBNA2 epitope/phage peptide 3 − 2, but with some clear exceptions (e.g., MS02-6, MS03-2, MS04-4 MS04-8, MS05-4). Of note, all 5 inflammatory control (IC) CSF IgG bound more EBNA2 epitope/phage peptide 3 − 2 than EBNA1 epitope/phage peptide 3−3, with IC004-5 binding large numbers of phage peptide 3 − 2 in particular. When accounting for all the patients' MS CSF IgG binding of phage peptide 3−3 vs peptide 3 − 2, there were significantly more phage peptide 3−3 bound than phage peptide 3 − 2 (p = 0.0220, Fig. 5B). For IC patient CSF IgG, phage peptide 3 − 2 was bound in greater numbers than phage peptide 3−3, but the differences were not significant (Fig. 5B).

EBNA1 epitope Pep 3−3 reacts with intrathecal IgG antibodies from patients with MS

To further evaluate the intrathecal IgG reactivity to the putative EBNA1 epitope Pep 3−3, we performed isoelectric focusing (IEF) immunoblotting on selected MS CSF. MS CSF samples were separated on SPIFE IEF agarose gels. Proteins were transferred to PVDF membranes, and the blots were probed with EBNA1 phage peptide 3−3 followed by anti-M13 primary antibody, then by anti-mouse secondary antibody, with colorimetric detection. EBNA1 epitope Pep 3−3 selected by panning with MS brain IgG was recognized by

multiple high-density IgG bands in both CSF and serum from representative MS patients. (Fig. 6A). These results suggest that the EBNA1 epitope is reactive to MS intrathecal antibodies corresponding to oligoclonal bands. We further performed dose-dependent phage-IPCR and demonstrated that EBNA1 epitope Pep 3–3 preferentially binds to intrathecally synthesized IgG in MS (Fig. 6B-D).

Discussion

We have shown in this study that phage peptides discovered by panning with IgG antibodies derived from a lesion in an acute MS brain (MS 95 – 2 IgG) shared high sequence and putative structural homologies with Epstein-Barr virus antigens EBNA1 and EBNA2. The phage peptides, termed Pep 3–3 and Pep 3 – 2, respectively, are homologous with the sequences $EBNA1_{386-416}$ and $EBNA2_{263-286}$. We demonstrated predicted B cell epitope potential for the peptides and for the homologous EBNA1/2 protein regions. IPCR assays showed that phage peptide/Pep 3–3 showed significantly higher binding to MS CSF IgG than phage peptide/Pep 3 – 2, while Pep 3 – 2 had higher (but statistically non-significant) to IC CSF IgG. Furthermore, we show that EBNA1 epitope/Pep 3–3 could block MS 95 – 2 IgG reactivity with EBNA1 protein, and that phage/phage peptide Pep 3–3 was recognized by MS intrathecal and serum IgG antibodies and oligoclonal bands.

EBNA1 is critical for maintaining viral replication and maintenance of the viral genome (Shannon-Lowe and Rickinson, 2019; Wu et al, 2002). EBNA1 is an important viral protein expressed in Burkitt's Lymphoma (Rowe et al, 2014) as well as during various latency phases (Latency I, I/II, II, III) in EBV-associated malignancies (Shannon-Lowe and Rickinson, 2019). EBNA1 has long been recognized as an antigen in MS in the contexts of both antibody (Comabella et al, 2010; Farrell et al, 2009; Hecker et al, 2016; Honarmand et al, 2015; Lunemann et al, 2008a; Mameli et al, 2014; Nociti et al, 2010; Sundqvist et al, 2012; Tengvall et al, 2019) and T cell targets (Lunemann et al, 2006; Ramasamy et al, 2020). Some studies include both B and T cell responses to EBNA1 (Comabella et al, 2012; Lunemann et al, 2008b; Lunemann et al, 2010; Mameli et al, 2016; Pender et al, 2017; Pender et al, 2009). In some cases where peptide antigens/targets were used in assays, the region of EBNA1 covered by Pep 3–3 is included (Lunemann et al, 2006; Lunemann et al, 2008a; Lunemann et al, 2008b; Lunemann et al, 2010; Mameli et al, 2014; Mameli et al, 2016; Sundqvist et al, 2012). Hecker et al (Hecker et al, 2016) using a high-density peptide array of overlapping EBNA1 peptides (among many others) found that antibodies from both sera and CSF of RRMS and/or PPMS patients reacted with $EBNA1_{391-410}$ or $EBNA1_{392-411}$. These amino acid ranges cover an RRPFF motif that appears central to Pep 3–3 in its homology with $EBNA1_{386-416}$ and is likely part of the epitope recognized by the various MS patient IgG used here (IgG from brain itself, and from sera and CSF).

In a study going back over two decades (Rand et al, 1998) CSF IgG from 5 of 14 MS patients selected the motif RRPFFX (where X = H, R, M, L, N or I) from a 6-mer phage library ($> 10^7$ possible sequences). Using a biotinylated peptide in the context of the phage pIII protein, the peptide could be detected bound to certain OCBs from MS CSF, indicating the presence of selective antibodies. A GenBank search at that time found the motif in the EBV protein EBNA1, and in the chaperone protein α B crystallin (CRYAB/HSPB5). Current

BLASTp searches reveal scores of matches in many proteins, but at least 10% of the hits are to EBNA1 from various EBV isolates, suggesting that EBNA1 and the motif are highly represented. In a follow-up to those studies, the same group (Rand et al, 2000) used the aforementioned peptide to probe blots of more MS patient CSF-derived OCBs for reactivity to the EBNA1 peptide motif. Five of 15 MS patients' OCBs (CSF samples from a different repository) again showed significant reactivity to the peptide, with no controls reacting (and all were serologically EBV positive).

Our studies here showed that MS brain IgG antibodies show activities to both EBNA1 and EBNA2 epitopes, while IgG in the CSF from multiple patients largely react with the EBNA1 epitope. This was verified with reactivity of the EBNA1 Pep 3–3 with OCBs in MS patient CSF and sera. This may be the first time that an EBNA1 epitope has been recognized by MS patients' antibodies culled from a brain lesion as well as recognition by OCBs from CSF and sera, and underscores the potentially significant role played by EBV in MS through EBNA1.

The roles of EBNA2 in MS etiology are less clear, with indications that its transcription factor activities may be important (Afrasiabi et al, 2019; Ricigliano et al, 2015). After phage peptide selection by MS 95 – 2 IgG, we found relatively few MS patient CSF IgG interactions with Pep 3 – 2 (EBNA2$_{263-286}$ homolog) suggesting it was not as prominent of an antigen/epitope in the broader MS patient population. While EBNA2 genetic variants may have some impact on the disease (Mechelli et al, 2015), it is unclear how this may relate to antigenicity, and should be an area of further study.

A recent cohort study consisting of more than 25,000 Scandinavian patients with infectious mononucleosis (IM) who were followed up for the occurrence of MS (Nielsen et al, 2007) and a meta-analysis of smaller previous studies on the association of IM and MS reported that individuals with a history of IM have a > 2-fold increased risk of developing MS compared to subjects who acquired the virus without symptoms (Thacker et al, 2006). Most healthy virus carriers maintain anti-EBNA-1 as well as anti-gp350 and anti-VCA (viral capsid antigen) IgG responses during persistent EBV infection (Lunemann et al, 2007), and these responses are used diagnostically to assess whether individuals have been infected with EBV (Tierney et al, 2007).

The necessity and sufficiency of EBV infection as a causative agent in MS remains a point of debate, particular given the widespread nature EBV infection in humans (Burnard et al, 2017; Hutchinson, 2013; Owens and Bennett, 2012; Pakpoor et al, 2013; Pakpoor and Ramagopalan, 2013; Salzer and Myhr, 2013). Nonetheless, large prospective sero-epidemiological studies analyzing antibody responses via standard diagnostic ELISAs to suspected antigens have consistently demonstrated that MS patients are almost universally seropositive for EBV. These data support the argument that infection with EBV is a possible requirement for the development of MS (Lunemann et al, 2007). Among more than 80 lytic and 8 latent EBV gene products, the latency-associated EBNA1 is the only protein consistently expressed in infected proliferating memory B cells in healthy virus carriers (Hochberg et al, 2004). IgG responses to the EBV latent antigen EBNA2 peak during acute infection and are directed against latent and lytic antigens (Cohen, 2000). EBNA1 is thus the only EBV antigen consistently expressed in proliferating cells with latent

EBV infection in healthy virus carriers. It represents a key target antigen for CD4$^+$ T cell-mediated immune control mechanisms of EBV infection in healthy individuals (Lunemann et al, 2007). The persistent detection of EBNA1 under these circumstances could imply a role for the antigen as a trigger for MS under the appropriate conditions (eg, involving a wide variety of environmental and/or genetic factors including HLA (human leukocyte antigen) type) (Alfredsson and Olsson, 2019).

There are multiple links between MS and EBV infection (reviewed in (Haahr and Hollsberg, 2006)): studies of MS- and EBV-epidemiology; prospective and retrospective analyses of EBV-serology; EBV DNA sequences in blood and tissues; the specificity of antibodies amongst oligoclonal bands in MS patients; and the results from antiviral chemotherapy of MS patients. Further, regarding EBNA1 and EBNA2, the geometric mean titers of serum antibodies to EBNA1 were significantly higher in MS patients when compared with matched controls. Though the geometric mean titers to EBNA2 were not significantly higher among MS cases when compared with matched controls, they were significantly elevated when compared with all controls (DeLorenze et al, 2006). These findings were consistent with earlier work showing that patients with MS have higher titers of CSF antibodies to EBNA1 than control subjects with other neurologic conditions, and with epidemiologic studies suggesting an association of EBV infection with MS (Chen et al, 1995; Tierney et al, 1994; Wu et al, 2002). However, as noted above and further here (Laurence and Benito-Leon, 2017), the connections between EBV infection and immune responses to EBV antigens and their roles in MS remain indistinct.

A possible link between EBV infection and autoimmunity may lie in the realms of molecular mimicry leading to "mistaken self" autoimmune attacks (Lucas et al, 2011). There have long been reports of the small chaperone/heat shock protein α B-crystallin (CRYAB/HSPB5) as a target for T cell responses (van Sechel et al, 1999). This protein is ordinarily not expressed in human lymphoid tissues; however, perhaps due to the stress of EBV infection, infected B cells express CRYAB, along with presentation of CRYAB peptides on HLA-DR to CD4$^+$ T cells, promoting an inflammatory response. Curiously, in a constructed system of antigen presentation on HLA-I molecules, the expression of CRYAB within the antigen-presenting cells did not result in CD8$^+$ T cell recognition or activation (van Nierop et al, 2016). This implies a more restricted T cell response to CRYAB epitopes in the form of memory CD4$^+$ T cells.

As noted above (Hecker et al, 2016), a high-density peptide array that screened MS patient sera and CSF found antibodies that reacted with EBNA1 in the domains possessing the RRPFF motif, which was also present in CRYAB. The authors suggested that antibody responses against EBV infection may lead to a breakdown of tolerance to CRYAB. As T cell help is requisite in generating effective B cell/antibody responses (Turner et al, 2017), it is conceivable that the co-recognition of the RRPFF motifs from both EBNA1 and CRYAB by B and T cells may induce anti-EBV responses that lead to autoimmune/anti-CRYAB responses, with ensuing inflammation in the brain. The roles of CRYAB in brain inflammation are complicated (van Noort et al, 2012) and perhaps contradictory, but the extracellular presence of CRYAB from stressed astrocytes and microglia is established (Zhu et al, 2015). It is possible that potential cross-reactivity of EBNA1 antibodies to CRYAB in these scenarios may generate an autoimmune response further exacerbated by CRYAB-reactive T cells. While this discussion is obviously hypothetical, our

findings over the presence of EBNA1-reactive antibodies within an MS lesion (and further validation of such epitope recognition by antibodies in sera and CSF) reaffirms the potential of EBV infection and response as a factor in MS etiology and disease progression.

The approach adopted here could conceivably facilitate the identification of amino acid sequence motifs in antigens specific for different diseases even if the etiologies of those diseases were unknown. Notwithstanding this possibility, it is clear from several lines of evidence, including these current results, that EBV likely plays some role in MS. Whether that role proves to be causal or as a co-factor will require further investigation in the different MS phenotypes likely involving the merged environments of injury, inflammation, and immunity.

Conclusion

Using a phage peptide library, we screened IgG purified from a postmortem brain plaque-periplaque from an acute MS patient. These IgG bound to phage peptides representing epitopes from Epstein-Barr virus nuclear antigens 1 and 2 (EBNA1/2), The EBNA1 epitope in particular was recognized by IgG from 46 MS patients while IgG from inflammatory control patients had significantly less binding. Further, OCBs of MS patients from both sera and CSF also recognized the phage-display EBNA1 epitope. These data reaffirm the potential of EBV infection and response as a factor in MS etiology and disease progression.

Declarations

Availability of data and materials

Data are contained within the publication; otherwise, contact the corresponding author for data requests.

Ethics declarations

Ethics approval and consent to participate

Written informed consents were obtained from all patients, and this study conformed to the principles of the Declaration of Helsinki and was approved by the MS and IC CSF samples were collected with approval from the University of Colorado Institutional Review Board (COMIRB # 00−688)

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Contributions

XY had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. ZW, PGEK, TDL, MWG, and XY contributed to the study concept and design. CD, MW, CL, TP, and XY were responsible for the acquisition of data. ZW, PGEK, TDL, MWG, and XY contributed to the analysis and interpretation of data. ZW, PGEK, MWG, and XY drafted the manuscript. CD, MW, CL, TP, and TDL provided administrative, technical, or material support. MWG and XY supervised the study. MWG and XY obtained funding. The authors read and approved the final manuscript.

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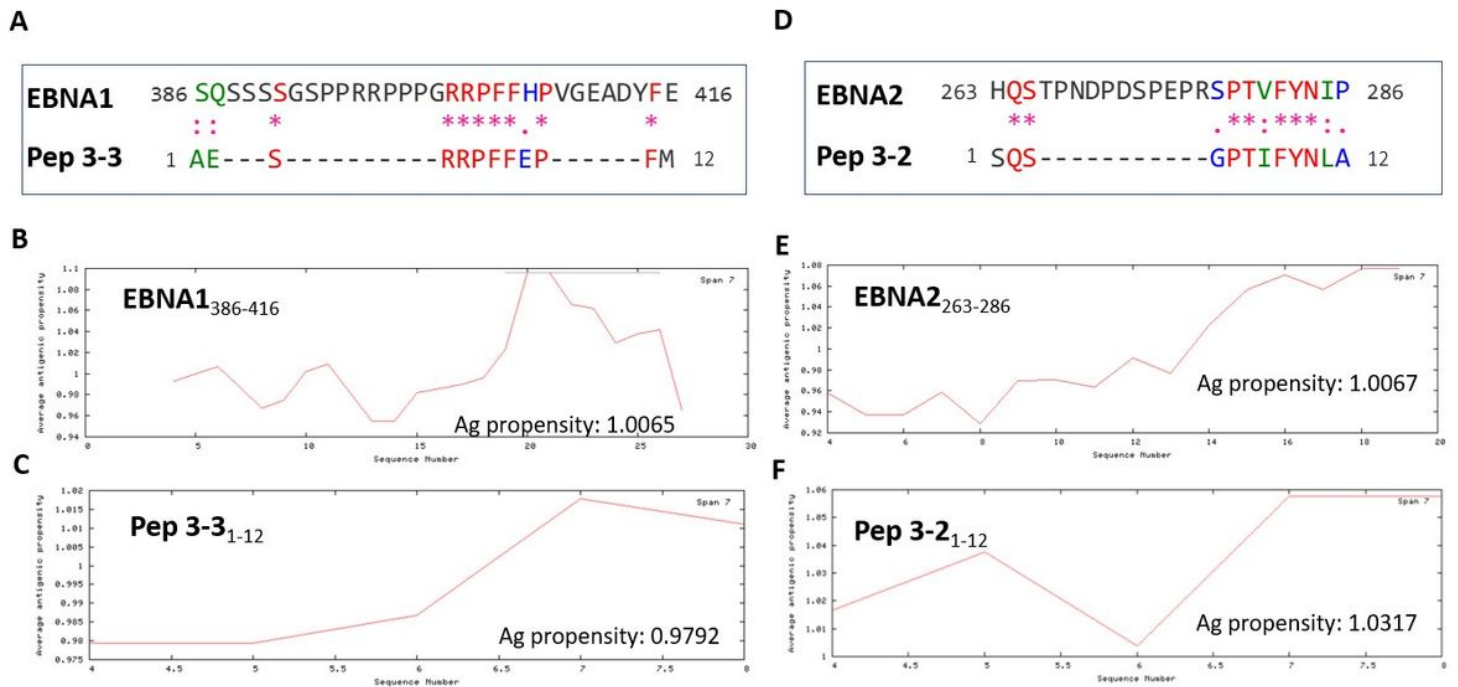
Figures

Figure 1

Phage peptides selected by MS brain plaque IgG antibodies share high sequence homologies with Epstein-Barr Virus Nuclear Antigen-1 (EBNA1) and EBNA2 and are predicted with high antigenic propensity. Two unique phage peptides (Pep 3-3 and Pep 3-2) were identified by panning phage-displayed random peptide libraries with IgG antibodies purified from plaques of a MS brain with acute disease. CLUSTAL W alignment was performed for sequence homology alignments. A. Pep 3-3 is shown with identical ("*", red lettering; 8/12), strongly similar (":", green lettering; 2/12), and weakly similar (".", blue lettering; 1/12) residues designated over the EBNA1 amino acid sequence from 386-416. This phage accounted for 2 of the 20 phage peptides sequenced. B. Graphic presentation of predicted antigenic peptide in EBNA1 386-416 (SQSSSSGSPPRRPPPGRRPFFHPVGEADYFE). Average Antigenic Propensity = 1.0065; Antigenic determinant = PFFHPVGE. C. Graphic presentation of predicted antigenic peptide for Pep 3-3 (AESRRPFFEPFM). Average Antigenic Propensity = 0.9792; No noted antigenic determinants. D. CLUSTAL W alignment of Pep 3-2 with EBNA2. Pep 3-2 is shown with identical ("*", red lettering; 7/12), strongly similar (":", green lettering; 2/12), and weakly similar (".", blue lettering; 2/12) residues designated over the EBNA2 amino acid sequence from 263-286. This phage accounted for 13 of the 20 phage peptides sequenced. E. Graphic presentation of predicted antigenic peptide in EBNA2 263-286 (HQSTPNDPDSPEPRSPTVFYNIP). Average Antigenic Propensity = 1.0067; no noted antigenic determinants. F. Graphic presentation of predicted antigenic peptide for Pep 3-2 (SQSGPTIFYNLA). Average Antigenic Propensity = 1.0317; No noted antigenic determinants.
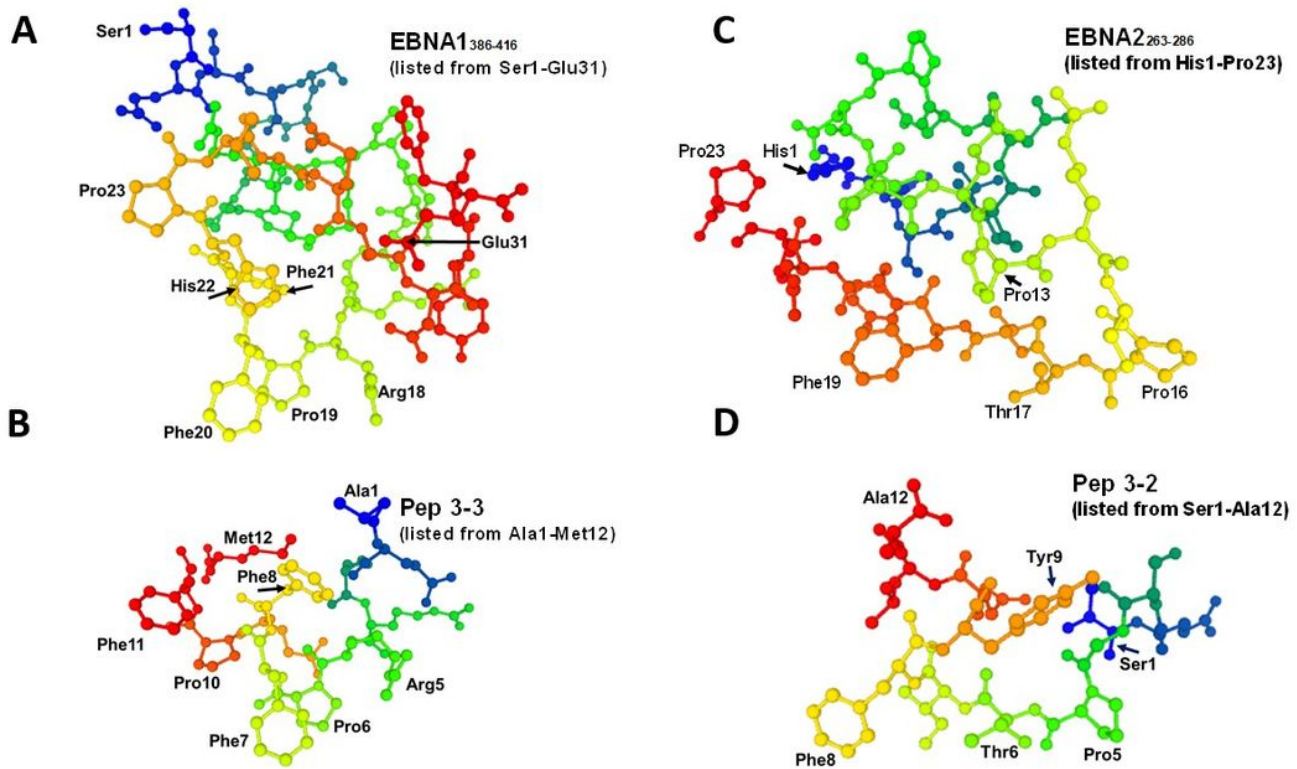
Figure 2

Predicted structural relationships between EBNA1386-416 and Pep 3-3, EBNA2 263-286 and Pep 3-2. Using PepFold 3.5 (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD3/), we rendered potential structural information comparing the peptides. A, B. Ser1 and Glu31 (first and last residues for EBNA1386-416) and Ala1 and Met12 (first and last residues for Pep 3-3) are shown for orientation. Amino acid residues of interest as potentially antigenic are shown as coming out of the plane of the figure toward the reader (18RPFFHP23 in EBNA1386-416, and 5RPFF[E]PF11 in Pep 3-3). C, D. His1 and Pro23 (first and last residues for EBNA2263-286) and Ser1 and Ala12 (first and last residues for Pep 3-2) are shown for orientation. Amino acid residues of interest as potentially antigenic are shown as coming out of the plane of the figure toward the reader (13P[RSP]T[V]F19 in EBNA2263-286 and 5PT[I]FY9 in Pep 3-2).
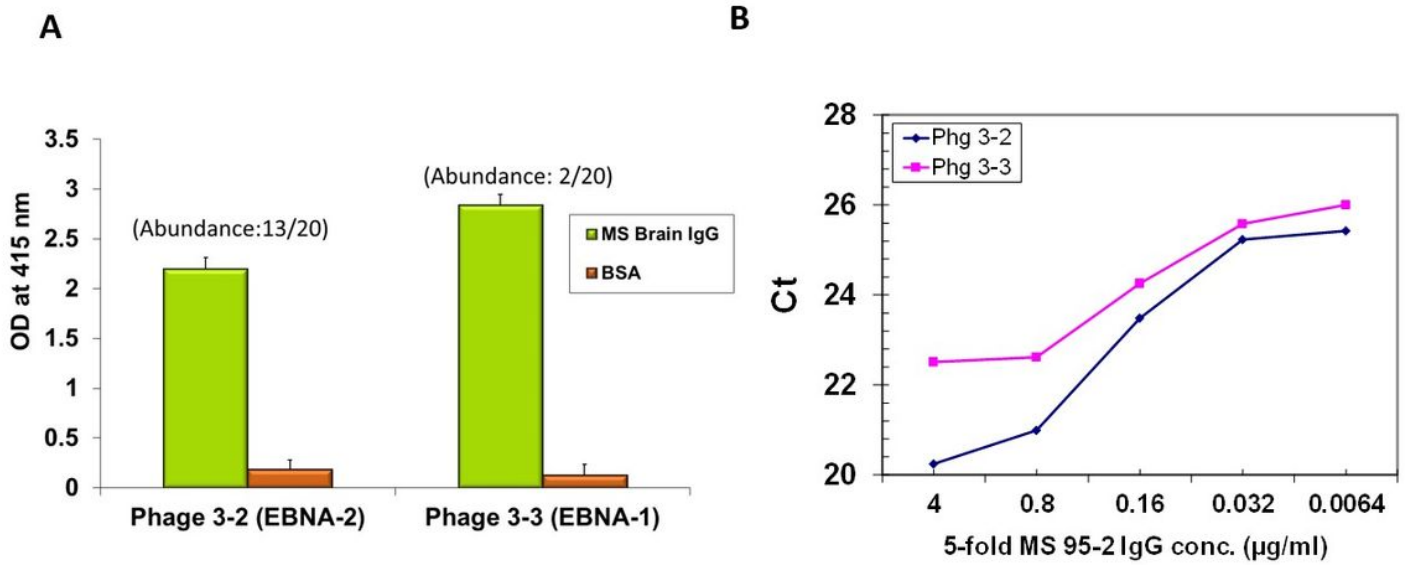
Figure 3

Phage peptides 3-3 and 3-2 show specific binding to panning MS brain antibodies. A. ELISA demonstrating specific bindings of the selected phage peptides to the MS brain antibodies used in the panning step. BSA coating serves as negative control. The abundance shown in the parenthesis indicates the frequency of the peptide out of total number of phage clones sequenced. B. Dose response phage IPCR showing specific binding of phage peptides to panning MS brain antibody 95-2 IgG. Wells of protein A coated plate were added with panning target MS antibodies (5-fold serial dilutions) and incubated with equal amount of each phage (107/well). Bound phage were assayed by real-time PCR using lysed phage DNA directly as template. PCR relative cycle threshold (Ct) values (100 minus Ct.) were plotted against IgG concentration. Avidly bound phage are indicated by low Ct values. Each data point is the average of two independent measurements.
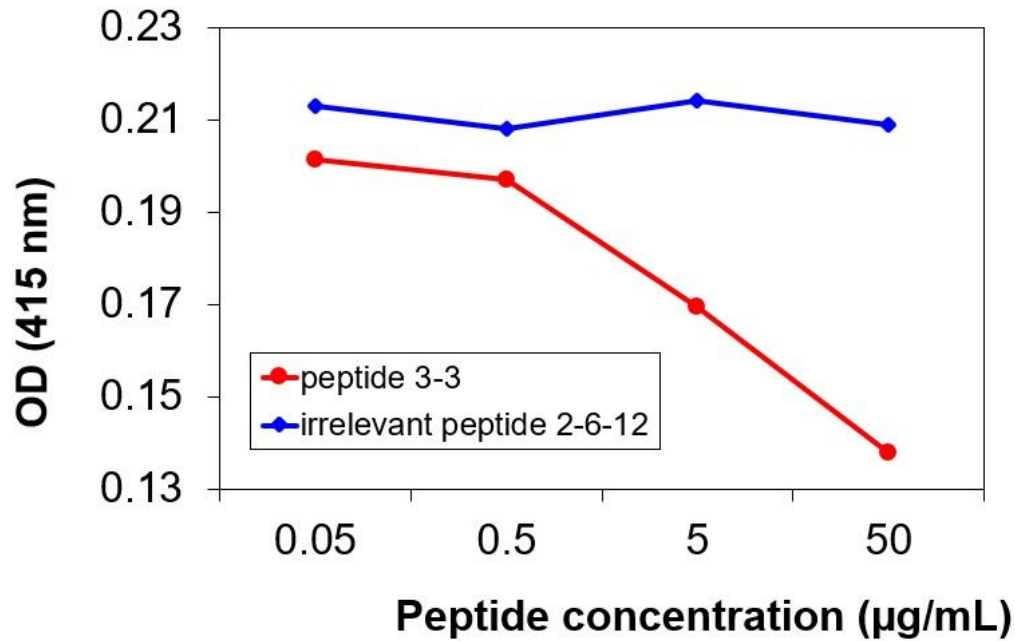
Figure 4

EBNA1 epitope (peptide 3-3) inhibits binding of EBNA1 protein to MS 95-2 IgG. ELISA wells were coated overnight with recombinant EBNA-1 protein at 50 µg/ml. MS brain IgG 95-2 at a final concentration of 20 µg/ml were incubated with varying concentrations of synthetic peptide 3-3 or irrelevant peptide (2-6-12) and were transferred to the plate pre-coated with EBNA-1 protein. Bound IgG were detected with HRP-conjugated anti-human IgG. Pep 3-3 competed with EBNA1 protein for binding to MS 95-2 IgG, whereas the irrelevant peptide did not. The higher the concentration of Peptide 3-3 that is added, the less 95-2 IgG was observed binding to the EBNA1 coated on the plate.
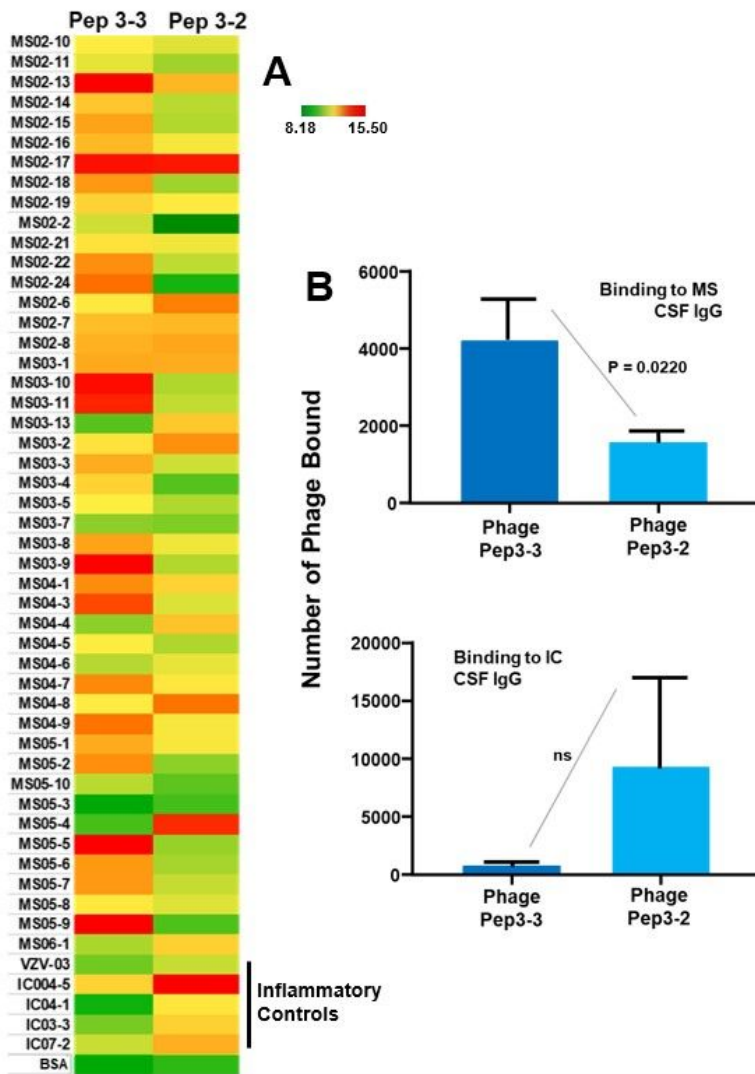
Figure 5

EBNA1 and EBNA2 epitope-specific antibodies are detected differentially in MS CSF, and EBNA1/Pep 3-3 has significantly higher bindings to MS CSF IgG compared to EBNA2/Pep 3-2. EBNA1/Phage 3-3 (Pep 3-3) and EBNA2/Phage 3-2 (Pep 3-2) at concentrations of 108/well were added to Protein A wells pre-coated respectively with 46 MS CSF and 5 IC CSF (at IgG concentration of 1µg/ml). Bound phages were detected by IPCR. A. Heat map (phage numbers converted to log2) showing differential binding of 46 MS patient CSF IgG and 5 IC patient CSF IgG to either Pep 3-3 or Pep 3-2, with low-to-high scale depicted. Inflammatory controls are noted. B (top). Quantified means of all MS CSF IgG binding to phage Pep 3-3 vs phage Pep 3-2, showing significantly more phage Pep 3-3 bound than phage Pep 3-2. B (bottom). Quantified means of all MS CSF IgG binding to phage Pep 3-3 vs phage Pep 3-2, showing no significant difference despite higher binding to phage Pep 3-2. Error bars = SEM. Phage counts (averages of duplicate values) and conversion to log2 are available in Supplemental Table 2.
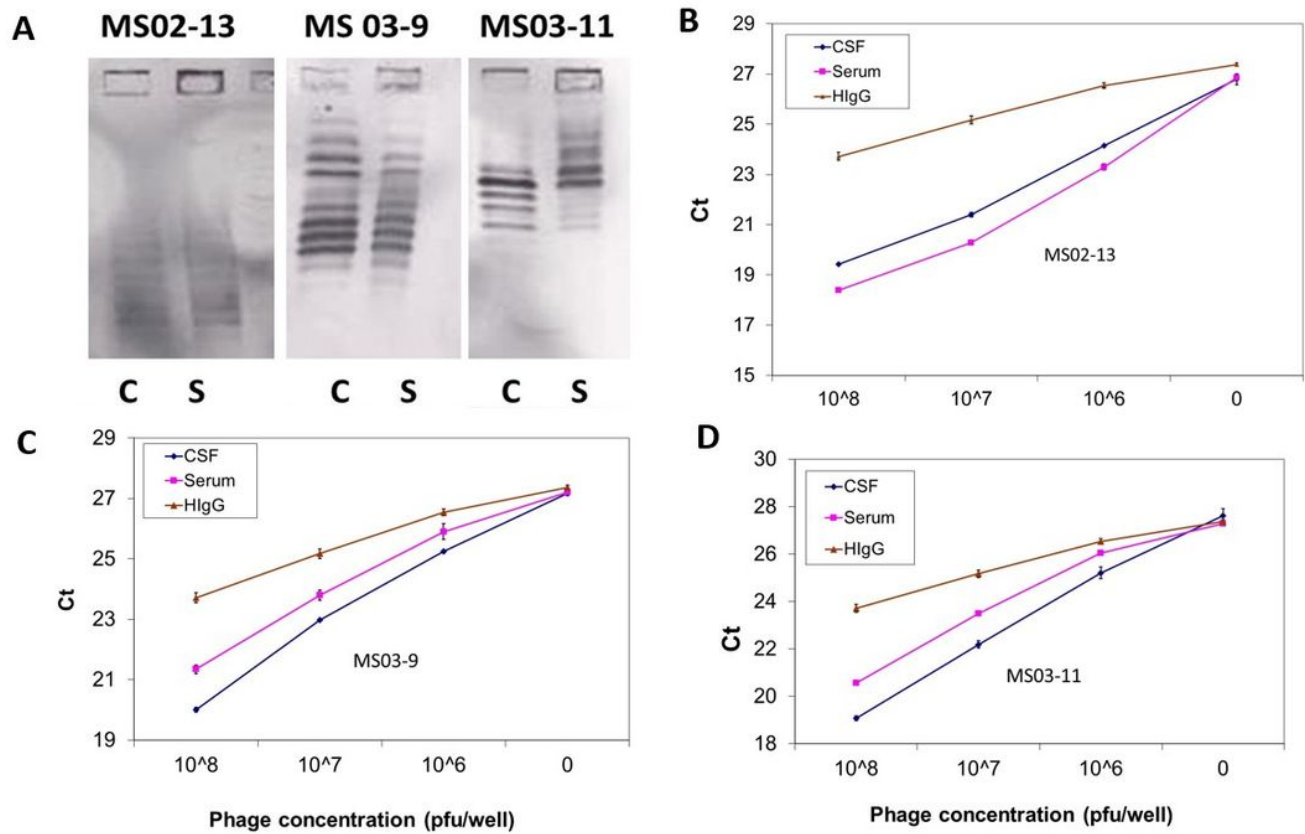
Figure 6

EBNA1 epitope (phage peptide 3-3) was recognized by intrathecally synthesized IgG in MS. A. IEF-immunoblots demonstrating EBNA1 epitope (phage peptide 3-3) reacts to oligoclonal IgG antibodies in MS. Paired MS CSF (C) and serum (S) (3–5 µg total IgG) from three patients (MS02-13, MS03-9, MS03-11) were resolved on agarose IEF gels and transferred to PVDF membranes. Blots were probed with EBNA1 phage peptide 3-3 (1010 pfu/ml) and incubated with mouse anti-M13 antibody followed by AP-anti-mouse antibody detection. Peptides selected by MS IgG recognized multiple high density IgG bands in both CSF and serum. B-D. Dose-dependent phage-IPCR demonstrates EBNA1 epitope (phage peptide 3-3) binds to intrathecally synthesized IgG in MS. Paired MS serum and CSF, as well as pre-immune human IgG control (50 µl at IgG concentration of 1 µg/ml), were coated in duplicate wells of protein A-plates before addition of the corresponding phage peptides (serial 10-fold dilutions starting with 108 pfu/well). Bound phage was determined by real-time PCR. Phage peptide bound at higher levels to IgG in CSF than in serum in a dose-dependent manner. Preimmune human IgG served as negative control. Experiments were performed at least twice. Error bars represent SD. B, patient MS02-13; C, patient MS03-9; D, patient MS03-11. Data represent at least three independent experiments. Lower Ct values indicate more phage bound.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SUPFIGS1.pptx
- SUPFIGS2.pptx
- SUPPLEMENTALTABLE1.docx
- SupplementalTable2IPCRphagecountsandlog2conversion.xlsx