Peer Review File

Manuscript Title: Clonally Expanded B Cells in Multiple Sclerosis Bind EBV EBNA1 and GlialCAM

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

The auto-immune mechanisms involved in multiple sclerosis are thought to involve both T and B cell components; oligoclonal immunoglobulins in CSF suggest a limited number of antigens may be targeted by the B cells that are involved. This paper provides an impressive analysis of the antigen specificity of B cells present in CSF of MS patients and identifies a relatively frequent and novel cross reaction between antibodies specific for a defined epitope in the EBV EBNA1 protein and the cell GlialCAM protein (which is present in some of the cells in MS lesions).

EBV infection is a risk factor for MS and this work provides very detailed evidence for part of a mechanism by which EBV could play a role. Although some parts of the EBNA1 protein sequence are known to vary between EBV isolates, the epitope of interest here is conserved. The proposed target is novel and potentially important.

Specific points

1. The initial analysis of antibody specificity used CSF samples from 9 patients, of which 6 were found to have antibodies that bind EBNA1. The further validation of the EBNA1 385-405 epitope involved blood plasma samples from 36 MS patients and 20 healthy controls. In Figure 3p, 10 of the 36 MS patients (27%) have the raised level of specific antibodies and 2 of the controls (10%). A similar enhancement of GlialCAM reactivity was found (Fig 3p) but only 3 these were in the same patients. This seems to indicate that the novel mechanism proposed might only be significant in a small minority of MS cases and probably needs validation in a larger MS/control panel.

2. The EBNA1 antibody cross reaction with GlialCAM recognises the intracellular domain of GlialCAM. How could the antibody gain access to the intracellular part of GlialCAM in Glial cells for the proposed mechanism to be correct? Manuscript p16, line 440 seems to be a tacit recognition of this and the need to investigate whether the same EBNA1/GlialCAM peptide might also be a T cell epitope (perhaps restricted through HLA-DRB1, a risk allele for MS?).

Referee #2 (Remarks to the Author):

This work is innovative. It bundles an impressive technological armamentarium to detail a pathogenic component contributing to the CNS changes underlying early Multiple Sclerosis. Specifically, the observations shed light on several long-standing conundrums. First, they help us better understand the well-known but elusive role of EBV infection in the pathogenesis, and, second, they identify a "new" humoral autoantigen, at least in a subgroup of patients. Third, they reveal an IgG affinity maturation process putatively happening within the CNS, the target organ of the autoimmune disease.
Here, Robinson, Steinman and colleagues have screened the CSF of people with MS (CIS and early RR) for IgG producing plasmablasts (PB). MS derived CSF PBs differed from blood counterparts by increased HLA-DR and IgG, and low a4 integrin and IgA expression. Single-cell sequencing revealed increased clonality of PB along with extended CDR3 lengths, which is in stark contrast to

polyclonal B cells. Among 148 OCB-derived recombinant MAbs 1/3 bound EBV related proteins (shown by EBV protein microarrays), including the EBNA1p394-399 sequence. Another 20% of this set react to VZV or CMV. Structural features of the AB/Ag interaction were derived from crystallography. A screen on HuProt human microarray gave two major hits, a cytoplasmic GlialCAM determinant (10/148), and the actin filament associated protein AFAP. Binding affinity to unmodified GlialCAM was low, but was substantially increased by phosphorylation. Interestingly, cross-reactivity was noted in CSF IgG, but not in non-mutated germline progenitor antibodies. GlialCAM binding was verified in situ by immunohistochemistry on mouse brain sections. Pathogenic relevance was confirmed in actively induced EAE, where pre-immunization of mice with EBNA1 peptide/CpG exacerbated subsequently induced EAE in the presence of anti-GlialCAM activity. Finally, GlialCAM binding antibodies were demonstrated in the plasma of a subgroup of MS donors. This ligation could be quenched by soluble EBNA1 antigens.
This elegant work is technically impressive. It raises very few questions.
The pleocytosis in the CSF of most donors appears exceptionally high, up to 57/ul. Here, please indicate the proportions of B cells and PB, respectively. Were the cohorts selected for high pleocytosis values?
GlialCAM expression largely recapitulates the one of aquaporin-4. Patients MS30, MS16 and MS49 (Fig.3p) had top levels of GlialCAM reactivity – did they present an atypical clinical picture, perhaps one reminiscent of NMOSD?
Amazingly, anti-GlialCAM IgGs bind to intracellular protein determinants, would they bind to cultured glia cells?
Other groups (e.g. Obermeier et al.) have produced recombinant OCB antibodies and found binding to several intracellular epitopes, but not to EBV. Did the investigators test the effect of cross-reactive IgG on SJL/J EAE?

Referee #3 (Remarks to the Author):

In this very thorough paper by Lanz et al., WH Robinson and an international team of stellar investigators used high throughput single B cell cloning to analyze the antibody repertoire in plasma blasts from CSF and PB. Characterization of representatives of a large number of clonal expanded antibodies from CSF led to the discovery of a sizable subset of antibodies that bound the EBV and to a lesser extent other viral antigens, with a prominent antigen being the EBNA1 TF. The authors show convincingly that some EBNA1 specific antibodies from MS bind to GlialCAM and with substantially higher affinity to phosphorylated GlialCAM peptides, a finding that was nicely explained by the structure of the antibody:antigen complex. Finally the authors present mouse experiments showing that co-administration of EBNA1 antigen peptide together with M- inducing PLP peptide exacerbates the disease.
Overall the authors present an impressive amount of carefully conducted work and the paper is of a caliber suitable for Nature. However there are some lingering major question and several secondary points that need to be addressed:
Major: 1) While the data is convincing in establishing that anti-EBNA1 antibodies with cross-reactivity to GlialCAM can be found in MS patients and could have a role in pathogenicity, it is not clear how prevalent such antibodies are in MS patients. In Fig 3o and p analysis of patient samples seems to suggest an elevation of GlialCAM cross-reactive antibodies however this effect appears to be driven largely by three patients with high GlialCAM titers. Based on Fig 3o the majority of MS patients tested seem to have no GlialCAM reactivity at least in serum. For the three high GlialCAM reactivity patients is there anything else known e.g. could it be that they have higher cross reactivity to auto antigens more broadly, did they have more or less severe disease etc. In short, the authors need to clarify whether the the EBNA1 386-405 epitope is of likely clinical significance and if that is not the case, the paper needs to be very clear about it (esp in light of several instances of proposed MS auto antigens e.g. Kir4.1 whose clinical relevance ended up being questionable). Of note I do not believe lack of generality or clinical significance should preclude publication in a high impact journal such as Nature but should be addressed.

2) Does MS39p2w174 and other EBNA1 antibodies isolated by the authors show polyreactivity? This could contribute to their putative pathologic role apart from cross reactivity to GliaCAM. Reactivity to common polyreactivity antigens such as ssDNA, RNPs, cardiolipin, etc should be examined and if polyreactivity is seen then its role should be properly addressed.

Less important:
1)Fig 1: Some of the data in Fig 1 is well established from earlier reports e.g. Fig 1a 1e,g is well known from the literature and hence should be moved to the SI. Also the data in 1b and 1c is not really relevant to the main story. Ref 8 by some of the cii-authors ha already established that there are transcriptional differences between CSF and peripheral blood plasma blasts!
2) Fig 1g: The clonality comparison of PBs in peripheral blood and CSF does not seem valid. Sampling of peripheral blood PBs is much more sparse than for CSF (because of the relative numbers of cells). It would take the analysis of many 10 of thousands peripheral blood PCs to determine whether clonal expansions are comparable to CSF. In general it is not appropriate to compare clonal expansions between different compartments, only within the same compartment in different populations/patients.
3) What was the fraction of clones found in both CSF and in peripheral blood? Any features of note for shared clones? Fig 3i shows V gene distribution but not whether clones are found in both compartments.
4) Fig 1j,k; Were all the PSMs for peptides from CDR3? If not then how do we know that they match "sequences unique to the patient" and not matches that arose because of the relatively small number of BCRs for each patient which could make framework peptides appear "unique". Also were there any peptides from EBNA1 antibodies detected?
5) The authors show convincingly that reactivity to GlialCAM by MS39p2w174 is acquired by SHM. Was that the case for the two other antibodies with similar reactivity?

Referee #4 (Remarks to the Author):

The manuscript by Lanz et al. shows a mechanistic link between multiple sclerosis and Epstein-Barr virus, whereby a mature antibody cross-reacts with an EBV transcription factor (EBNA1), and glia-specific type-1 membrane protein glialCAM. The authors provide convincing structural, biophysical and in vivo results to demonstrate the relevance of such mechanism.
These findings provide novel insights into the molecular mechanisms underlying an important and complex human pathology, and could serve to develop therapeutic tools. Hence, I find the manuscript as potentially suitable for publication, but I have concerns that should be address before considering publication:
1-The manuscript lacks necessary background on the subject that will help readers to better follow the flow of the work. Instead of a proper introduction providing such background and exposing clearly the problem to be addressed, the manuscript presents an extended summary of results (lines 88-107) that overlaps with both the abstract and discussion sections. The introduction should be extensively edited, stating for instance current knowledge on EBV (and other viruses) and MS relationship, relevant EBV antigens particularly regarding EBNA-1, etc.
2-The abstract states (line 76) “crystal structure of EBNA1-antibody complex…”, I think this is misleading, as the structure solved is that of a Fab with an antigenic-EBNA1 peptide. Also in line 214, it should be stated that the complex is with monovalent Fab, and not divalent mAb. Although the methods section describes clearly that is a Fab-peptide complex, I think it is important to clarify this in the text, as both native EBNA1 and glialCAM form oligomers potentially affecting binding of divalent mAbs, compared to monovalent-Fab/peptide binding (see also comment #3).
3-mAb MS39p2w174 binds native glialCAM with an order of magnitude higher affinity than the phosphorylated glialCAM derived peptides. What do the authors think is the reason for this? Could it be oligomerization of the native protein? It is worth including some discussion about this after that related to the effect of phosphorylation (after line 433)
4-Regarding interferometry experiments. The authors only present dissociation constants (KD),

but it would be of interest to show "on and off" kinetic constants, since they already have these data, to get insight on binding differences. Also, I couldn't find anywhere the number of biological binding experiments performed. Finally, please include in the text errors associated to KD determination.
5-Regarding anisotropy correction of diffraction data, it would be informative to include in the crystallographic table the resolution limits along the three axes. Also data completeness before staraniso (I assume the reported completeness -96.1/78.0%- is after) to show that incompleteness does not come from insufficient sampling of reciprocal space.
6-The authors mention in the abstract that results in the manuscript could guide developments of MS-related therapies. It is a bit disappointing that there is no discussion about this in the text. Is it possible that EBNA1 and/or glialCAM derived peptides could serve to neutralize auto-antibodies? Please, include some hypothesis about the therapeutic implications of the work in the discussion, or remove the sentence in the abstract.
7-Line 294, reference 24 does not correspond to the PDB ID mentioned (J Mol Biol 1998)

Author Rebuttals to Initial Comments:

Point-by-Point Response to Reviewers' Comments **November 23, 2021**

Manuscript: Clonally Expanded B Cells in Multiple Sclerosis Bind EBV and GlialCAM

Nature manuscript 2021-06-09836A

Authors: Tobias V. Lanz, R. Camille Brewer, Peggy P. Ho, Jae-Seung Moon, Kevin M. Jude, Daniel Fernandez, Ricardo A. Fernandes, Alejandro M. Gomez, Gabriel-Stefan Nadj, Christopher M. Bartley, Ryan D. Schubert, Isobel A. Hawes, Sara E. Vazquez, Manasi Iyer, J Bradley Zuchero, Bianca Teegen, Jeffrey E. Dunn, Christopher B. Lock, Lucas B. Kipp, Victoria C. Cotham, Beatrix M. Ueberheide, Blake T. Aftab, Mark S. Anderson, Joseph L. DeRisi, Michael R. Wilson, Rachael J.M. Bashford-Rogers, Michael Platten, K. Christopher Garcia, Lawrence Steinman, William H. Robinson

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Specific points

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Response 1.1 We thank the reviewer for the suggestion regarding the need to validate our findings of EBNA1 – GlialCAM reactivity. We now add new data from 2 additional cohorts of MS patients, and in both cohorts demonstrated replication of our original results showing EBNA1 – GlialCAM reactivity (Revision Fig. 1a,b). To further address the question if MS plasma antibodies cross-react between the $EBNA1_{AA386-405}$ epitope and phosphorylated $GlialCAM_{AA370-389\ pSer376}$, we now present the results of blocking experiments in which we showed $GlialCAM_{AA370-389\ pSer376}$ blocks binding of MS plasma antibodies to $EBNA1_{AA386-405}$ (Revision Fig. 1c). Our new data are described in detail below:

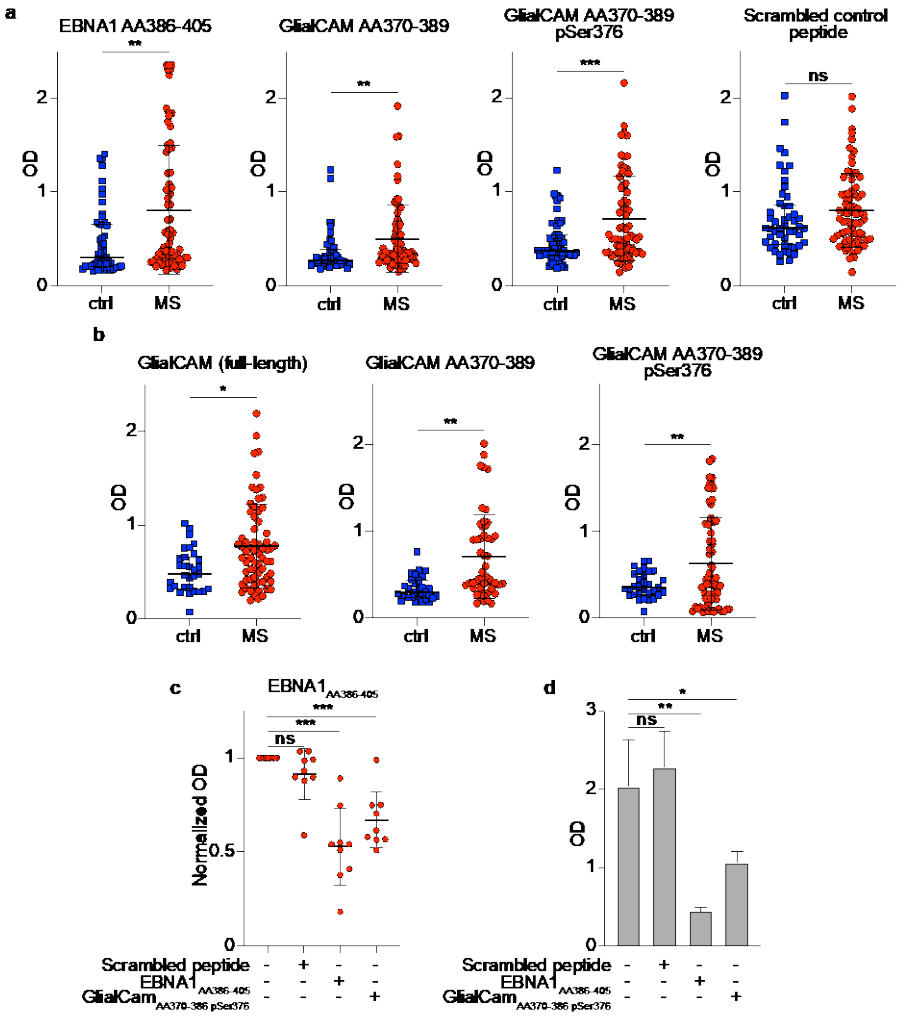
In the first new cohort of 71 MS patients and 50 healthy control individuals, we measured plasma IgG reactivity against the peptides $EBNA1_{AA386-405}$, $GlialCAM_{AA370-389}$, and phosphorylated $GlialCAM_{AA370-389\ pSer376}$ (Revision Fig. 1a). The new results are concordant with our results in the original Manuscript Fig. 3n-p. IgG reactivity against the peptides $EBNA1_{AA386-405}$, $GlialCAM_{AA370-389}$, and phosphorylated $GlialCAM_{AA370-389\ pSer376}$ was significantly higher in MS patients than in healthy individuals, with differences being more

pronounced for phosphorylated GlialCAM$_{AA370\text{-}389\ pSer376}$. No significant differences in reactivities were detected against scrambled control peptide. The data builds on several prior publications cited in our manuscript, which described elevated antibody reactivity against the broader EBNA1 region AA365-425 in MS patients over healthy individuals in larger cohorts[1–5].

In a second new patient cohort of 67 MS patients and 31 healthy control individuals, we measured reactivity to GlialCAM protein and peptides to further validate our findings (Revision Fig. 1b). Corroborating the data in the original manuscript as well as the new data above, anti-GlialCAM IgG levels in this cohort were again significantly higher in MS patients than in healthy control individuals, with approximately 20 - 25% of MS patients harboring increased plasma IgG antibodies binding the key EBNA1 and GlialCAM epitopes GlialCAM$_{AA370\text{-}389}$ and phosphorylated GlialCAM$_{AA370\text{-}389\ pSer376}$ (Revision Fig. 1b).

To address the question of cross-reactivity, we selected 9 MS plasma samples from the first new MS patient cohort, with higher than average reactivities against EBNA1$_{AA386\text{-}405}$ and GlialCAM$_{AA370\text{-}389\ pSer376}$. Using ELISA, we tested the ability of EBNA1$_{AA386\text{-}405}$, GlialCAM$_{AA370\text{-}389\ pSer376}$, and scrambled control peptide to block the binding of human MS plasma IgG to EBNA1$_{AA386\text{-}405}$. We showed that blocking with GlialCAM$_{AA370\text{-}389}$ pSer376 significantly reduced anti-EBNA1AA386-405 reactivity in most samples (Revision Fig. 1c), thereby corroborating our data in the original Manuscript Fig. 3q and further validating the observed cross-reactivity in MS plasma samples. We included the new data in the updated Manuscript Fig. 3q and the Extended Data Fig. 9a-c.

Together, we found elevated IgG levels against EBNA1$_{AA386\text{-}405}$ and GlialCAM (protein and peptide) in MS patients over healthy individuals, which we now confirmed in two additional independent cohorts. As the reviewer pointed out, anti-EBNA1/GlialCAM cross-reactivity is not universally elevated in all MS patients. Rather, high reactivity seems to be present in a subset of MS patients, which amounts to 20 - 25% of MS patients. MS is a heterogenous disease, and we believe that our findings reveal molecular mimicry between EBNA1$_{AA386\text{-}405}$ and GlialCAM$_{AA370\text{-}389\ pSer376}$ as the pathogenic driver in one quarter of human MS patients. Moreover, the data from studies on patients' plasma represents a "snapshot in time". The duration of antibodies that can be measured outside the CSF in patients with MS to elevated IgG levels against EBNA1$_{AA386\text{-}405}$ is a matter that will be the subject of future studies. Whether levels of antibodies correlate with duration of disease, subtype of disease, or other factors will be of great interest, and will inform us regarding the potential utility of such antibodies as a clinical biomarker.



Revision Fig. 1. Increased plasma reactivity against EBNA1 and GlialCAM proteins and peptides in healthy control individuals and MS patients. a, ELISA measurement of antigen-specific IgG reactivity against peptides EBNA1$_{AA386-405}$, GlialCAM$_{AA370-389}$, phosphorylated GlialCAM$_{AA370-389\ pSer376}$, and scrambled peptide control in plasma samples of healthy control individuals (*n=50*) and MS patients (*n=71*). Mean values for each patient and means ± SD across patient groups are shown. Representative OD (450 nm) measurements of two independent experiments, each carried out in duplicates. **b**, ELISA measurements of antigen-specific IgG reactivity against GlialCAM full-length protein, GlialCAM$_{AA370-389}$, and phosphorylated GlialCAM$_{AA370-389\ pSer376}$ in plasma samples of a separate cohort of healthy control individuals (*n=31*) and MS patients (*n=67*). Mean values for each patient and means ± SD across patient groups are shown. Representative OD (450 nm) measurements of two independent experiments, each carried out in duplicates. **c**, ELISA measurements of MS plasma antibody reactivity against EBNA1$_{AA386-405}$, without interference as well as blocked with scrambled peptide control, EBNA1$_{AA386-405}$, or GlialCAM$_{AA370-389\ pSer376}$. Mean OD (450 nm), normalized to unblocked sample, of quadruplicate measurements ± SD from one experiment are shown. **d**, Positive control (mAb MS39p2w174) for the data shown in (**c**). *P* values according to **a**,**b**, Mann-Whitney U test, **c**,**d**, one-way two-tailed ANOVA, corrected for multiple comparisons using the Dunnett method. * P < 0.05, ** P < 0.01, *** P < 0.001

Comment 1.2 The EBNA1 antibody cross reaction with GlialCAM recognises the intracellular domain of GlialCAM. How could the antibody gain access to the intracellular part of GlialCAM in Glial cells for the proposed mechanism to be correct? Manuscript p16, line 440 seems to be a tacit recognition of this and the need to investigate whether the same EBNA1/GlialCAM peptide might also be a T cell epitope (perhaps restricted through HLA-DRB1, a risk allele for MS?).

Response 1.2 As the reviewer points out and as mentioned in the manuscript, the identified epitope GlialCAM$_{AA370-389}$ is indeed localized on the intracellular domain. There is ample precedent for intracellular B cell antigens in autoimmune diseases. These include most prominently anti-nuclear antibodies, anti-dsDNA antibodies, and anti-Sm antibodies in systemic lupus erythematosus[6,7], anti-citrullinated antibodies in rheumatoid arthritis[8], anti-tRNA synthetase antibodies in myositis, and anti-nuclear cytoplasmic antibodies in ANCA vasculitis. We now include these references in the updated manuscript in the paragraph in which we describe the epitope's intracellular location and linear structure. A common interpretation of the pathogenicity of intracellular B cell antigens is their exposure during cell death, followed by accumulation of inflammatory autoantibodies, aided by mechanisms such as immune complex formation. Another potential role of autoantibodies targeting intracellular antigens, is binding and thereby capturing the intracellular antigen to load dendritic cells and thereby activate a coordinated T cell response. In support of such a mechanism, we now provide data showing anti-GlialCAM CD8 T cells in MS blood (Revision Fig. 4c; described in detail below). Thus, there are multiple mechanisms by which autoantibodies against intracellular antigens could contribute to glial and neuronal injury in MS.

In addition, there might be a molecular mechanism that is distinct for GlialCAM in MS patients, which could expose the intracellular epitope to systemic antibodies. GlialCAM has a diverse set of functions as a cellular adhesion molecule, a chaperone, and an ion channel subunit[9–11]. It multimerizes and interacts with itself and several partners, including CLC2, MLC1, and aquaporin 4[12]. During cell death, GlialCAM could become part of inflammatory multi-protein complexes or it could stay attached to its binding partners on neighboring cell membranes, thereby exposing the intracellular domain to antibodies and directing complement to glial cells. We believe the full exploration of the exact mechanisms is beyond the scope of the current manuscript, and they are part of an upcoming grant proposal and our follow-on studies.

We agree with the Reviewer that the T cell response to EBNA1$_{AA386-405}$ and GlialCAM$_{AA370-389}$ might be an additional important factor contributing to inflammation. To investigate the T cell response, we measured T cell activation and phenotypes in the EAE-model presented in our original Manuscript Figure 4. Immunization with EBNA1$_{AA386-405}$ induced a strong CD4+ T cell response while the T cell response against PLP$_{AA139-151}$ remained comparable in both groups (Revision Fig. 2a). EBNA1$_{AA386-405}$ stimulated the secretion of B cell stimulatory Th1 cytokines IFN-γ, TNF, and IL-12, as well as IL-6 and IL-10 (Revision Fig. 2b-f), but suppressed the key Th17 cytokine IL-17 (Revision Fig. 2g). In this mouse model, we could not detect a robust CD4 T cell response against GlialCAM$_{AA370-389\ pSer376}$ (Revision Fig. 2a-g). However, EBNA1-reactive T cells that secrete B cell stimulatory cytokines could promote maturation and activation of cross-reactive B cells and contribute to the anti-GlialCAM antibody titers observed in the same mice (Manuscript Fig. 4b). We included our data on mouse CD4+ T cells in the updated Extended Data Fig. 10b-h.

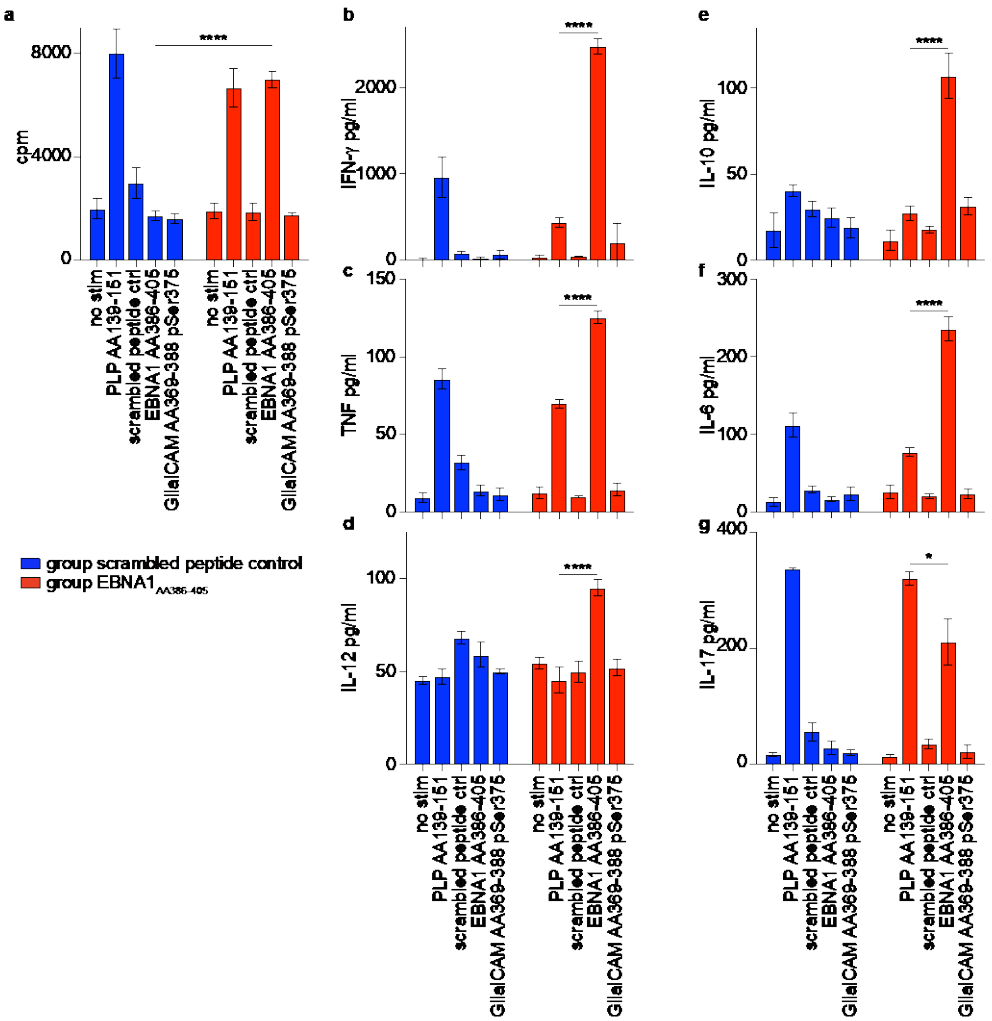
As the reviewer pointed out, the patient's HLA genotype could profoundly influence T cell reactivity to either antigen. As the EAE experiment above was carried out in wildtype mice, no relevant HLA effects would have been reflected in the EAE model. We should point out that patient MS39, from whom antibody MS39p2w174 originated, is not a carrier of the most significant MS risk allele HLA-DRB1*15:01 (Extended Data Table 2). The patient carries HLA-DRB1*03:01, a second HLA class II risk allele for MS with lower significance. The patient did not carry any of the protective HLA class I alleles (HLA-A*02:01, HLA-B*44:02,

HLA-B*38:01 and HLA-B*55:01)[13,14]. We used the prediction tools SYFPEITHI, NetMHC, and IEDB to predict binding of EBNA1 and GlialCAM peptides to the patient's molecular HLA setup, and included
the main MS risk allele HLA-DRB1*15:01 (Revision Fig. 3). We listed the 10 highest binding peptides of EBNA1 (Revision Fig. 3a) and GlialCAM (Revision Fig. 3b) for each HLA allele as well as the highest prediction for a peptide containing the central binding motifs of antibody MS39p2w174 ($EBNA1_{AA394-400}$ and $GlialCAM_{AA377-383}$). These motifs were not predicted to present particularly well on either HLA class I or HLA class II and were never amongst the top 10 predicted binders. However, multiple other regions of both proteins are predicted to be presented well on several alleles, with GlialCAM peptides binding slightly better to HLA class I alleles (Revision Fig. 3a) and EBNA1 peptides binding slightly better to HLA class II alleles (Revision Fig. 3b). Peptides of both proteins can be presented well on HLA-DRB1*15:01, but only EBNA1 binds tightly to HLA-DRB1*03:01. Presentation of EBNA1 on HLA-DRB1*15:01 and HLA-DRB1*03:01 might be part of the explanation how these alleles contribute to MS risk.

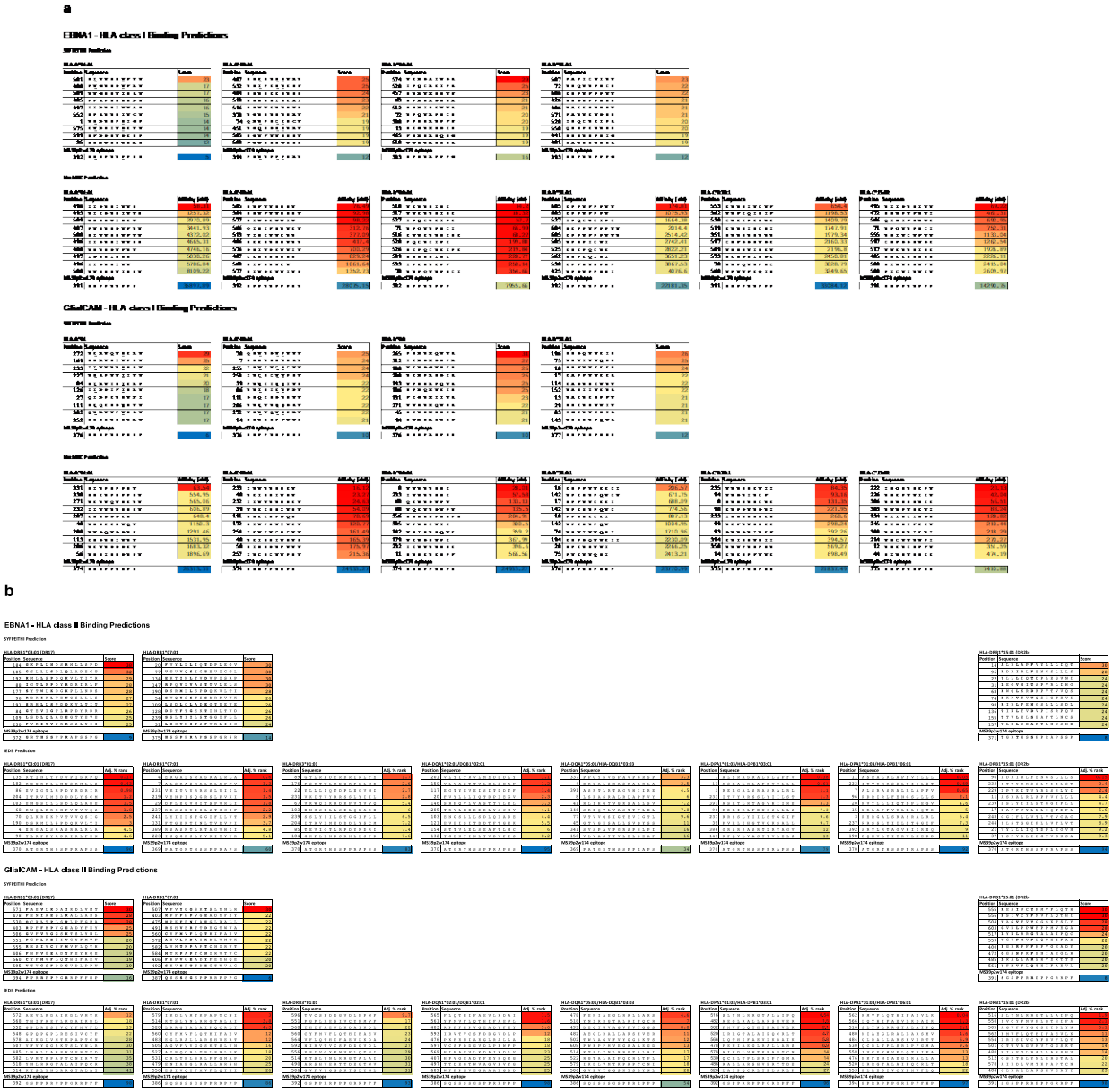
To further assess T cell reactivity in humans, we stimulated PBMCs of MS patients (*n=7*) and healthy control individuals (*n=6*) with EBNA1 and GlialCAM proteins and peptides. MS patients from our original cohort that exhibited increased plasma antibody titers against EBNA1 and GlialCAM were selected for T cell analyses (original Manuscript Fig. 3n-p). Human MS blood CD8+ T cells exhibited a robust response against EBNA1 in both groups, while only CD8+ T cells from MS patients responded to GlialCAM ICD and GlialCAM ECD, as measured by IFN-γ and granzyme-B expression (Revision Fig. 4c). One patient (MS16) showed extraordinarily high counts of IFN-γ+ granzyme-B+ CD8+ T cells upon stimulation with EBNA1, GlialCAM ICD, and GlialCAM peptide (Revision Fig. 4c,d). The same patient also responded to GlialCAM ECD, suggesting that the T cell response is raised against multiple regions throughout the whole protein. Low reactivity was detected against phosphorylated $GlialCAM_{AA370-389\ pSer376}$, suggesting that $GlialCAM_{AA370-389}$ phosphorylated at Ser376 may not be the epitope targeted by anti-GlialCAM CD8+ T cells in MS.

In CD4+ T cells, EBNA1 protein induced IFN-γ and IL-17 expression in MS patients and to a lesser degree in healthy control individuals (Revision Fig. 4a,b). We observed a trend towards more IFN-γ expression in CD4+ T cells in response to $EBNA1_{AA386-405}$, GlialCAM ECD and ICD proteins. This trend was less pronounced for IL-17. Skewing towards Th1 cytokines is in line with our observations in mouse CD4 T cells. We included the data on human CD8+ and CD4+ T cells in our updated Manuscript Fig. 4f and Extended Data Fig. 10k-m.

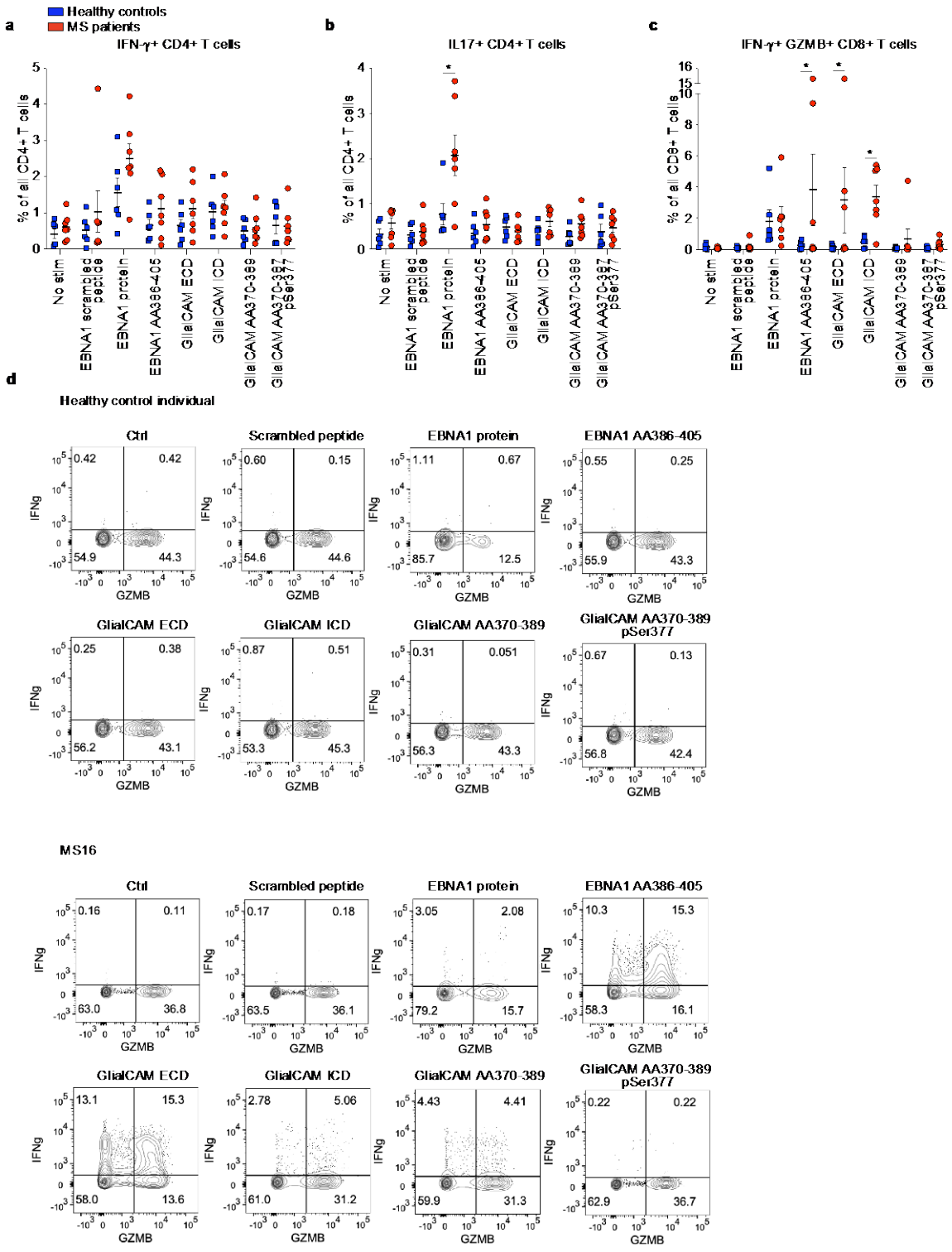
In summary, we found a robust anti-EBNA1 CD4+ T cell response in mice, which is skewed towards the secretion of Th1 cytokines and B cell stimulating cytokines IL-6 and IL-10. In human MS patients, our T cell assays demonstrate that MS patients exhibited significantly increased CD8 T cell responses to $EBNA1_{AA386-405}$ and GlialCAM ECD and ICD proteins, as compared to healthy controls. Our human CD4+ T cell data also shows a trend towards increased IFN-γ expression upon stimulation with $EBNA1_{AA386-405}$ and GlialCAM ECD and ICD proteins. EBNA1 protein robustly induces IFN-γ and IL-17 in CD4+ T cells of MS patients. T cell responses against GlialCAM could be an important pathogenic mediator in EBNA1-induced MS. Proposed longitudinal studies on CD4+ and CD8+ T cell responses to $EBNA1_{AA386-405}$ and GlialCAM ECD and ICD proteins will be illuminating in regards to duration and frequency, in both the peripheral and CSF compartments.



Revision Fig. 2. T cell response in mice immunized with EBNA1$_{AA386-405}$. a, Measurement of of ^{3}H-thymidine incorporation to assess T cell proliferation from mice immunized with scrambled peptide control (blue) and EBNA1$_{AA386-405}$ (red). **b-g**, ELISA cytokine measurements of T cell culture supernatants of **b**, IFN-γ, **c**, TNF, **d**, IL-12, **e**, IL-10, **f**, IL-6, **g**, IL-17. * $P < 0.05$, **** $P < 0.0001$ according to two-sided two-way ANOVA using the Tukey method for multiple comparisons.



Revision Fig. 3. HLA binding prediction. a, HLA class 1 prediction and **b**, HLA class 2 prediction, each for EBNA1 peptides (top) and GlialCAM peptides (bottom). Algorithms SYFPEITHI, NetMHC, and IEDB were used. HLA genetic setup of patient MS39 was investigated as well as HLA-DRB1*15:01. Top 10 binding peptides for each protein on each allele are shown as well as the respective top peptide containing the MS39p2w174-binding epitope, together with the respective score of each prediction algorithm.



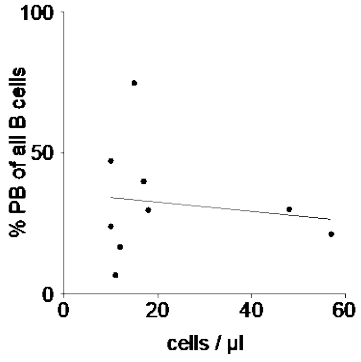
Revision Fig. 4. T cell reactivity in human CD4+ and CD8+ T cells upon stimulation with EBNA1 and GlialCAM proteins and peptides. a-d, Flow cytometry data of PBMCs from healthy control individuals and from MS patients stimulated for 16h with IL2, IL7, and the indicated proteins and peptides. **a**, % of IFN-γ and **b**, % of IL-17 expressing CD4+ T cells in all CD4+ T cells. **c**, % of IFN-γ+ GZMB+ CD8+ T cells in all CD8+ T cells. **d**, Dot plots showing flow cytometry data of live/dead- CD3+ CD8+ T cells from one representative healthy control individual and MS patient MS16. Significance levels were assessed by two-way ANOVA, followed by FDR calculation using the two-stage step-up method of Benjamini, Krieger and Yekutieli, * FDR < 0.1.

Referee #2 (Remarks to the Author):

This work is innovative. It bundles an impressive technological armamentarium to detail a pathogenic component contributing to the CNS changes underlying early Multiple Sclerosis. Specifically, the observations shed light on several long-standing conundrums. First, they help us better understand the well-known but elusive role of EBV infection in the pathogenesis, and, second, they identify a "new" humoral autoantigen, at least in a subgroup of patients. Third, they reveal an IgG affinity maturation process putatively happening within the CNS, the target organ of the autoimmune disease.
Here, Robinson, Steinman and colleagues have screened the CSF of people with MS (CIS and early RR) for IgG producing plasmablasts (PB). MS derived CSF PBs differed from blood counterparts by increased HLA-DR and IgG, and low a4 integrin and IgA expression. Single-cell sequencing revealed increased clonality of PB along with extended CDR3 lengths, which is in stark contrast to polyclonal B cells. Among 148 OCB-derived recombinant MAbs 1/3 bound EBV related proteins (shown by EBV protein microarrays), including the EBNA1p394-399 sequence. Another 20% of this set react to VZV or CMV. Structural features of the AB/Ag interaction were derived from crystallography. A screen on HuProt human microarray gave two major hits, a cytoplasmic GlialCAM determinant (10/148), and the actin filament associated protein AFAP. Binding affinity to unmodified GlialCAM was low, but was substantially increased by phosphorylation. Interestingly, cross-reactivity was noted in CSF IgG, but not in non-mutated germline progenitor antibodies. GlialCAM binding was verified in situ by immunohistochemistry on mouse brain sections. Pathogenic relevance was confirmed in actively induced EAE, where pre-immunization of mice with EBNA1 peptide/CpG exacerbated subsequently induced EAE in the presence of anti-GlialCAM activity. Finally, GlialCAM binding antibodies were demonstrated in the plasma of a subgroup of MS donors. This ligation could be quenched by soluble EBNA1 antigens.
This elegant work is technically impressive. It raises very few questions.

Comment 2.1 The pleocytosis in the CSF of most donors appears exceptionally high, up to 57/ul. Here, please indicate the proportions of B cells and PB, respectively. Were the cohorts selected for high pleocytosis values?

Response 2.1 The patients included in this study cohort were indeed selected for MS patients with CSF pleocytoses above 10 cells / µl. We had mentioned this in our manuscript (page 4, line 112: "All patients had a pleocytosis of >10 cells / µl in CSF (Extended Data Table 1)". However, we understand that this point should be emphasized more extensively and therefore we have revised this sentence to "Patients with a CSF pleocytosis of >10 cells / µl were selected (Extended Data Table 1)". This selection was primarily necessary due to technical considerations, as in our experience plate-bound single-cell B cell sorting and sequencing became increasingly challenging below a threshold of 5 cells / µl, and yielded significantly more BCR sequences when above 10 cells / µl. Naturally, the volume of CSF per patient we obtained for research purposes was limited. While our IRB allowed for 15 mL per patient, more commonly we received ~5-10 mL. While high white blood cell counts aided single-cell sorting and sequencing, high CSF white blood cell counts did not correlate with the plasmablast proportions in the CSF.



Revision Fig. 5. CSF white blood cell counts vs. plasmablast frequencies in CSF. Graph correlating white blood cell counts in the CSF of each patient with the patient's CSF plasmablast levels, represented as percent of all CSF B cells, as detected by flow cytometry.

When selecting patients with high pleocytosis, we were aware of the possible misdiagnosis of neuromyelitis optica spectrum disorders (NMOSD). Special care was taken to follow the current McDonald criteria for the diagnosis of MS[15,16]. None of the patients met the diagnostic criteria for NMOSD, in particular spinal lesions spanning ≥3 segments[17]. Patients were tested for antibodies against aquaporin-4 and MOG and showed negative results.

Patient MS37, who showed the highest pleocytosis of 57 cells / µl, had a spinal lesion spanning one segment in addition to several small subventricular contrast enhancing lesions. In order to avoid a misdiagnosis, her serum (and CSF) were tested repeatedly and in different laboratories for anti-aquaporin-4 and anti-MOG antibodies and were always negative. Inquiry with her current treating neurologist four years after the initial lumbar puncture confirmed that no new symptoms had occurred that would suggest a revision of her initial MS diagnosis.

Of note, patient MS39, from whom antibody MS39p2w174 was selected, possessed 18 cells / µl, which was in the middle of the range of CSF cell counts (Extended Data Table 1).

Comment 2.2 GlialCAM expression largely recapitulates the one of aquaporin-4. Patients MS30, MS16 and MS49 (Fig.3p) had top levels of GlialCAM reactivity – did they present an atypical clinical picture, perhaps one reminiscent of NMOSD?

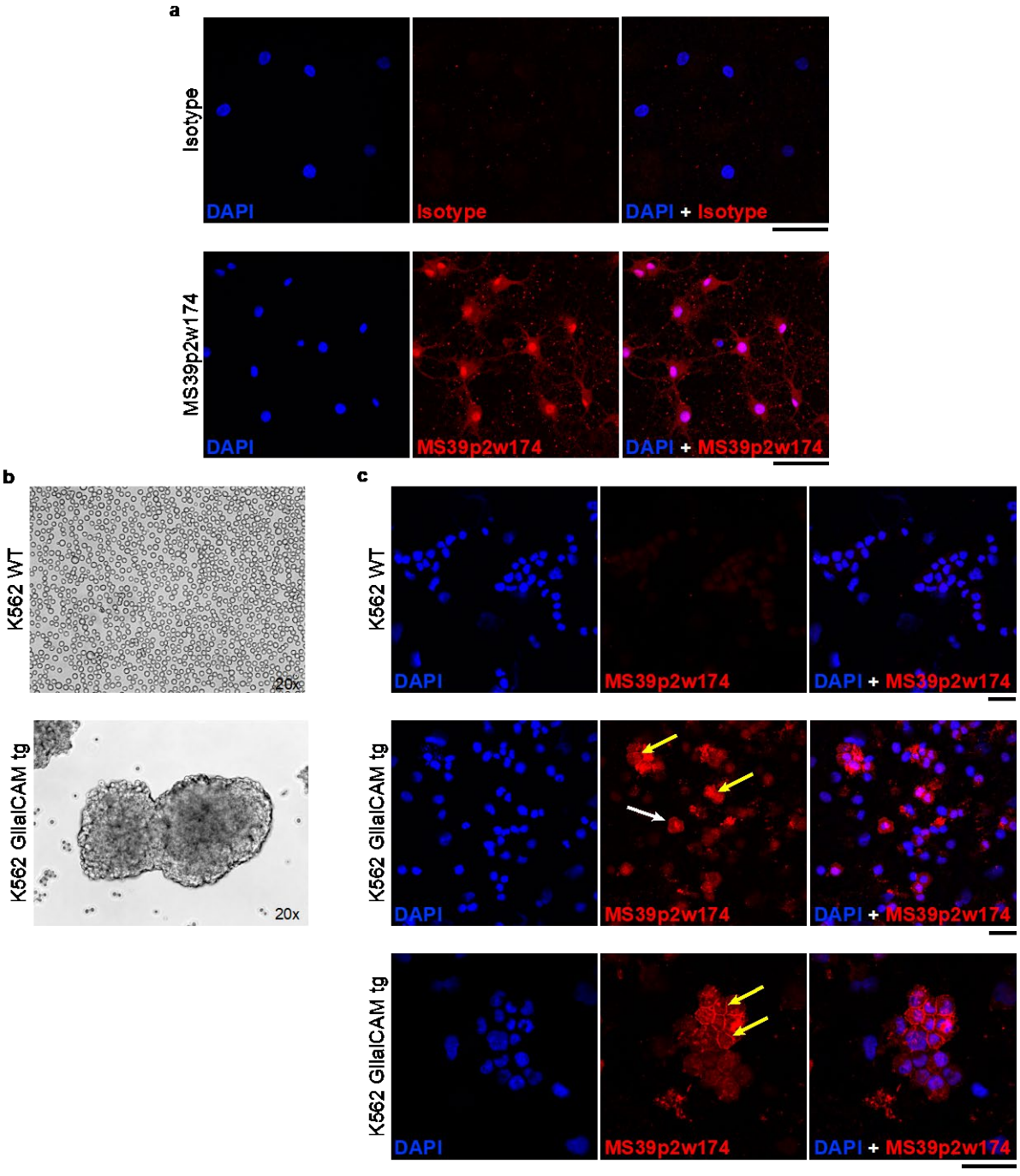
Response 2.2 Patient MS30 was part of our initial patient cohort that was included in the repertoire sequencing analysis and of which we have very detailed patient histories. The patient does not present any atypical clinical features that would suggest a diagnosis reminiscent of NMOSD. We have limited patient data from patients MS16 and MS49, but inquiries with the patients' treating physicians were made and the treating physicians relayed to us that there was no evidence of NMOSD and no reason to deviate from the initial MS diagnosis.

As mentioned above in our response to the first Reviewer, we have now added two additional cohorts of MS patients in which we replicated our findings and demonstrated elevated plasma reactivity against GlialCAM and cross-reactivity between $EBNA1_{AA389-405}$ and $GlialCAM_{AA370-389}$ in a subset of MS patients (Revision Fig. 1). Currently we do not have data on patient histories for these patients that would be detailed enough to draw conclusions on particular disease presentations in the subgroup with high anti-GlialCAM reactivity. We are planning a larger follow-up study powered with enough well-characterized patient samples to answer the important question if antibody reactivity to GlialCAM correlates with particular disease characteristics or with response to certain therapeutics.

Comment 2.3 Amazingly, anti-GlialCAM IgGs bind to intracellular protein determinants, would they bind to cultured glia cells?

Response 2.3 Primary rat oligodendrocytes, differentiated from rat embryo-derived oligodendrocyte precursor cells[18], were stained with antibody MS39p2w174. A relatively uniform staining pattern was observed in oligodendrocytes, resembling the GlialCAM expression pattern described in cultured cells at low density[9,19] (Revision Fig. 6a). To determine if antibody MS39p2w174 could stain GlialCAM in tight junctions at the cell-cell interface, we generated lentivirally transduced stable GlialCAM over-expressing K562 cell lines (GlialCAM-tg). In culture GlialCAM-tg cells clump together in bulks of several hundred cells (Revision Fig. 6b). Staining of GlialCAM-tg cells showed a similar uniform staining pattern in single cells (Revision Fig. 6c, white arrow). However, cells located in bulks

express GlialCAM at the cell border where it likely trans-dimerizes with neighbouring cells building tight junctions[12]. Immunofluorescence staining with antibody MS39p2w174 shows tight junction staining, resembling GlialCAM expression of cell cultures in high density[9,19,20] (Revision Fig. 6c, yellow arrows). We have included these images in our updated Extended Data Fig. 8d-f.



Revision Fig. 6. Immunofluorescence on primary rat oligodendrocytes and transgenic GlialCAM overexpressing K562 cells. a, Immunofluorescence staining of primary rat oligodendrocytes with isotype control antibody (top panel) and MS39p2w174 (bottom panel). **b**, K562 cells in culture, wildtype (WT) (top) and transduced with full-length GlialCAM (GlialCAM-tg, bottom). **c**, Immunofluorescence with MS39p2w174 on WT K562 cells (top) and GlialCAM-tg K562 cells (center and bottom). White arrow: single K562 cell, orange arrow: high intensity MS39p2w174 staining on the cell boarder between transgenic K562 cells in bulks. All scale bars: 40 µm.

Comment 2.4 Other groups (e.g. Obermeier et al.) have produced recombinant OCB antibodies and found binding to several intracellular epitopes, but not to EBV. Did the investigators test the effect of cross-reactive IgG on SJL/J EAE?

Response 2.4 In the publication by Brändle and Obermeier *et al.*[21], the authors characterize six monoclonal antibodies derived from the oligoclonal immunoglobulin bands from the CSF in MS patients. Interestingly, three antibodies bind three different intracellular antigens (FAM84A, MKNK1, AKAP17A), which are all ubiquitously expressed proteins. Our study initially follows a similar approach, as we also selected antibodies from the CSF of MS patients. But it expands upon their prior antibody characterizations by testing the reactivity of 148 monoclonal antibodies derived from plasmablasts in the CSF of MS patients. As noted by the Reviewer, none of the six antibodies in the publication by Brändle and Obermeier *et al*. bind cells expressing 5 different EBV antigens. In addition to the small number of antibodies tested, issues with presentation and fixation could inhibit antibody binding to the antigen in this particular assay. We believe that via our focused effort, exposing our antibodies to a set of 50 EBV protein preparations and 240 peptides spanning four EBV proteins, we substantially increased our ability to identify a significant antigenic target. Of note, MS39p2w174 has a high affinity for EBNA1 protein and $EBNA1_{AA389-405}$, but does not bind whole EBV lysate preparations (Original Manuscript Fig. 2a). Although antibody MS39p2w174 binds a linear peptide, we agree with Brändle and Obermeier *et al.* that the majority of antibodies depend on an intact protein confirmation for binding. In our experience, multiple different assays are needed in campaigns to successfully identify antibody targets.

In addition to immunizing SJL/J mice with the EBNA1 peptide, we treated SJL/J mice with antibody MS39p2w174 at a dose of 500µg/mouse on days 3, 6, and 9 post immunization. We did not observe a significant difference in disease scores between treated and untreated mice. This result indicates that a cellular B cell component as well as a T cell response might be necessary for the full inflammatory mechanism. As we pointed out under 1.2, immunization with $EBNA1_{AA386-405}$ induced a strong T cell response against $EBNA1_{AA386-405}$, while the T cell and antibody response against $PLP_{AA139-151}$ remained comparable in both groups (Revision Fig. 2a). $EBNA1_{AA386-405}$ stimulated the secretion of B cell stimulatory Th1 cytokines IFN-γ, TNF, and IL-12, as well as IL-6 and IL-10 (Revision Fig. 2b-f), but suppressed the key Th17 cytokine IL-17 (Revision Fig. 2g). In this mouse model, we could not detect a robust CD4+ T cell response against $GlialCAM_{AA370-389\ pSer376}$ (Revision Fig. 2a-g). However, EBNA1-reactive T cell that secrete B cell stimulatory cytokines could promote maturation and activation of cross-reactive B cells and contribute to the anti-GlialCAM antibody titers observed in the same mice (Manuscript Fig. 4b).

Referee #3 (Remarks to the Author):

In this very thorough paper by Lanz et al., WH Robinson and an international team of stellar investigators used high throughput single B cell cloning to analyze the antibody repertoire in plasma blasts from CSF and PB. Characterization of representatives of a large number of clonal expanded antibodies from CSF led to the discovery of a sizable subset of antibodies that bound the EBV and to a lesser extent other viral antigens, with a prominent antigen being the EBNA1 TF. The authors show convincingly that some EBNA1 specific antibodies from MS bind to GlialCAM and with substantially higher affinity to phosphorylated GlialCAM peptides, a finding that was nicely explained by the structure of the antibody:antigen complex. Finally the authors present mouse experiments showing that co-administration of EBNA1 antigen peptide together with M- inducing PLP peptide exacerbates the disease.
Overall the authors present an impressive amount of carefully conducted work and the paper is of a caliber suitable for Nature. However there are some lingering major question and several secondary points that need to be addressed:

Major:

Comment 3.1 While the data is convincing in establishing that anti-EBNA1 antibodies with cross-reactivity to GlialCAM can be found in MS patients and could have a role in pathogenicity, it is not clear how prevalent such antibodies are in MS patients. In Fig 3o and p analysis of patient samples seems to suggest an elevation of GlialCAM cross-reactive antibodies however this effect appears to be driven largely by three patients with high GlialCAM titers. Based on Fig 3o the majority of MS patients tested seem to have no GlialCAM reactivity at least in serum. For the three high GlialCAM reactivity patients is there anything else known e.g. could it be that they have higher cross reactivity to auto antigens more broadly, did they have more or less severe disease etc. In short, the authors need to clarify whether the the EBNA1 386-405 epitope is of likely clinical significance and if that is not the case, the paper needs to be very clear about it (esp in light of several instances of proposed MS auto antigens e.g. Kir4.1 whose clinical relevance ended up being questionable). Of note I do not believe lack of generality or clinical significance should preclude publication in a high impact journal such as Nature but should be addressed.

Response 3.1 We thank the Reviewer for this comment and suggestion. We addressed this question in detail in our response to Reviewer #1 (see response 1.1 and Revision Fig. 1a-c). As described above, we now include additional ELISA measurements to demonstrate plasma IgG reactivity against EBNA1$_{AA389-405}$ and unphosphorylated and phosphorylated GlialCAM$_{AA370-389}$ using plasma samples from a new patient cohort of 71 MS patients and 50 healthy control individuals (Revision Fig. 1a). We demonstrate significantly higher plasma IgG reactivities to the three peptides in MS patients as compared to healthy control individuals. We corroborated elevated reactivity to GlialCAM protein as well as unphosphorylated GlialCAM$_{AA370-389}$ and phosphorylated GlialCAM$_{AA370-389\ pSer367}$ in a third patient cohort with 67 MS patients and 31 healthy control individuals (Revision Fig. 1b).

Using nine MS plasma samples with elevated anti-EBNA1$_{AA389-405}$ and anti-GlialCAM$_{AA370-389\ pSer376}$ levels, we now demonstrate cross-blocking of IgG binding to EBNA1$_{AA389-405}$ by GlialCAM$_{AA370-389\ pSer376}$ (Revision Fig. 1c). Anti-EBNA1$_{AA389-405}$ reactivity in 5 of 9 MS plasma samples was partially blocked by GlialCAM$_{AA370-389\ pSer376}$, thereby corroborating our data in the original Manuscript Fig. 3q, and demonstrating that the observed cross-reactivity is a broader phenomenon in a subset of MS patients.

Our data suggest that there is a subset of MS patients with elevated IgG reactivity to GlialCAM and cross-reactive antibodies to EBNA1 and GlialCAM. Our current patient cohort lacks granular data on the severity of disease and parameters such as lesion location, development of disability over time, and treatment success. In the future we are planning to perform follow-on studies that will utilize a larger MS patient cohort and will allow us to identify the disease characteristics of MS patients with high GlialCAM antibody levels. We shall also perform longitudinal studies to test the duration of these antibodies, and the proximity of their appearance to onset of disease, among other characteristics of interest. Anti-GlialCAM reactivity would be most valuable if it could ultimately guide decisions on treatment and perhaps even on prognosis.

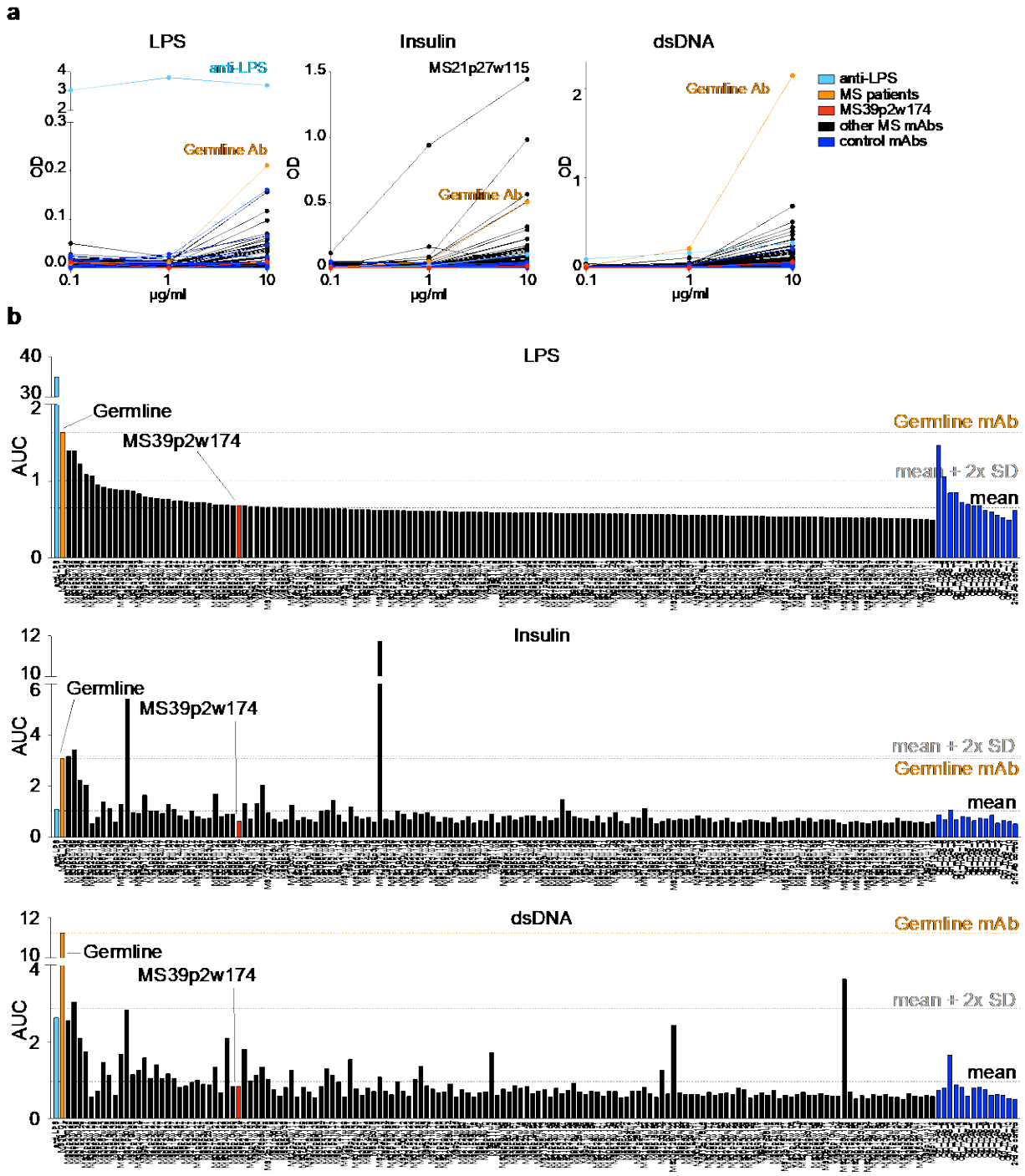
Comment 3.2 Does MS39p2w174 and other EBNA1 antibodies isolated by the authors show polyreactivity? This could contribute to their putative pathologic role apart from cross reactivity to GliaCAM. Reactivity to common polyreactivity antigens such as ssDNA, RNPs, cardiolipin, etc should be examined and if polyreactivity is seen then its role should be properly addressed.

Response 3.2 We appreciate the comment and share the concern for polyreactivity of antibody MS39p2w174. We tested all 148 monoclonal antibodies and 13 control antibodies by ELISA for reactivity against LPS, insulin, and dsDNA[22–24] (Revision Fig. 7). As expected, germline mAb showed significant levels of polyreactivity against all three antigens and generated the highest OD signals against LPS and dsDNA of all tested antibodies of

interest. Reactivity of MS antibodies was comparable to the 13 control antibodies generated in prior studies from other autoimmune and infectious diseases.

Antibody reactivity was measured at concentrations of 0.1, 1, and 10 µg/ml (Revision Fig. 7a) and the area under the curve (AUC) was compared across all antibodies (Revision Fig. 7b).
A commercial anti-LPS antibody was included, which showed an OD signal that was by orders of magnitude higher than any other expressed antibody (Revision Fig. 7a,b, LPS-panels). Antibodies were determined to be polyreactive if their reactivity was higher than the reactivity of the polyreactive Germline mAb, or above mean + 2x standard deviation of all antibodies for at least one antigen (excluding anti-LPS and 2nd AB control from the calculation of the standard deviation). Using these two criteria as thresholds, 8 out of 148 antibodies were polyreactive (5.4%) and 2 of 13 control antibodies were polyreactive (15.4%). In contrast to germline mAb, MS39p2w174 did not show elevated polyreactivity but was close to mean OD reactivity levels for all three antigens (Revision Fig. 7a,b). We have added the polyreactivity data in our new Extended Data Fig. 4.

In summary, our results show that (i) in general, antibodies in the CSF of MS patients do not show increased polyreactivity over antibodies isolated from other diseases. This is in line with a prior study in which six MS-CSF-derived antibodies were tested for polyreactivity on the same antigens[21]. (ii) The germline antibody is polyreactive and while antibody MS39p2w174 gains affinity to GlialCAM during somatic hypermutation, it concomitantly loses a significant part of its initial polyreactive binding properties.



Revision Fig. 7. Polyreactivity of recombinantly expressed monoclonal antibodies. a-b, ELISA data showing reactivity of recombinant mAbs against LPS, human insulin, and dsDNA. **a**, Reactivity represented as OD450 for select mAbs at the three indicated dilutions. **b**, Reactivity of all mAbs represented as area under the curve (AUC) of serial dilutions.

Less important:

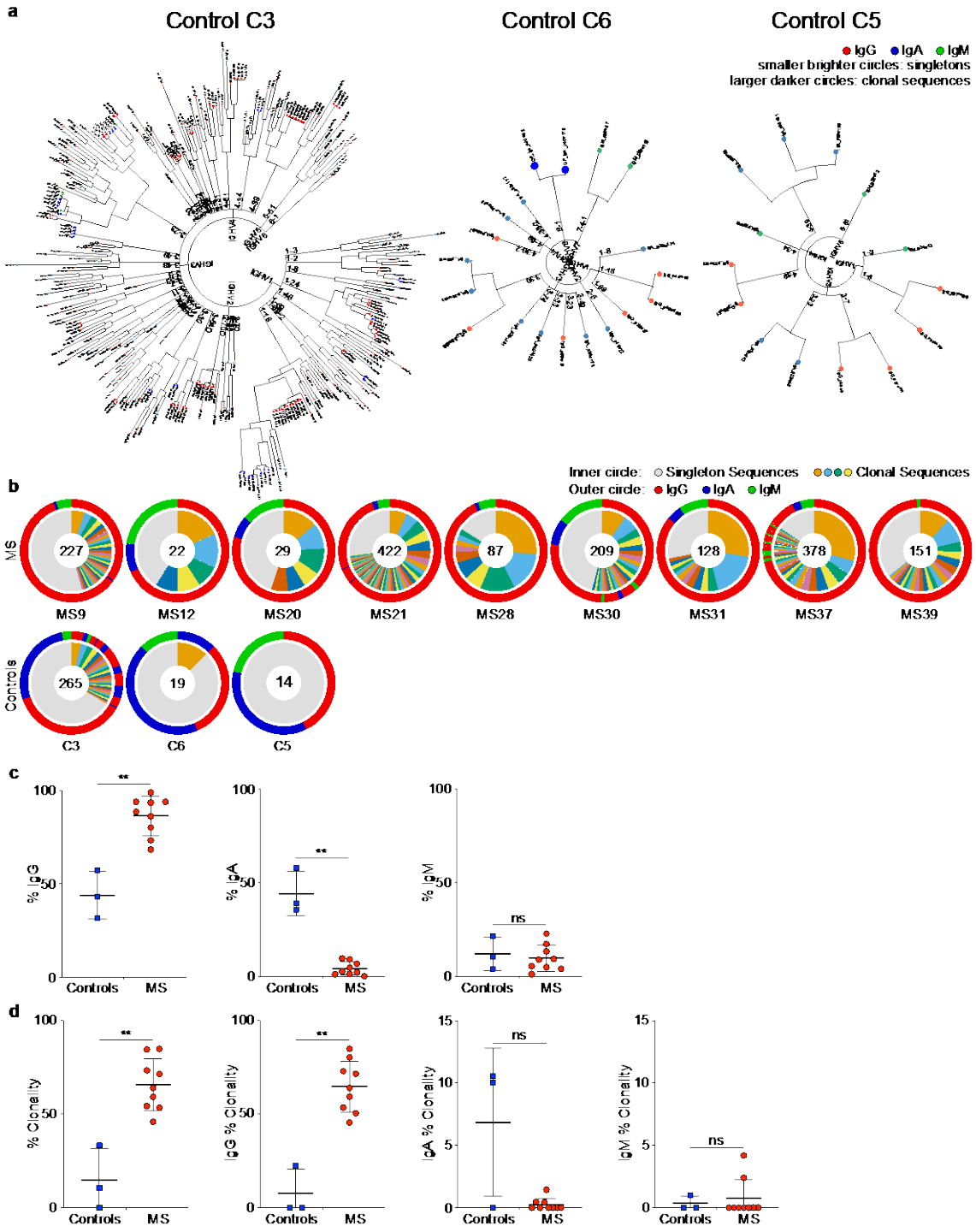
Comment 3.4 Fig 1: Some of the data in Fig 1 is well established from earlier reports e.g. Fig 1a 1e,g is well known from the literature and hence should be moved to the SI. Also the data in 1b and 1c is not really relevant to the main story. Ref 8 by some of the cii-authors ha already established that there are transcriptional differences between CSF and peripheral blood plasma blasts!

Response 3.4 We agree that some panels in Figure 1 contained data that were previously established, and we have now moved these panels to the updated Extended Data Fig. 1.

Comment 3.5 Fig 1g: The clonality comparison of PBs in peripheral blood and CSF does not seem valid. Sampling of peripheral blood PBs is much more sparse than for CSF (because of the relative numbers of cells). It would take the analysis of many 10 of thousands peripheral blood PCs to determine whether clonal expansions are comparable to CSF. In general it is not appropriate to compare clonal expansions between different compartments, only within the same compartment in different populations/patients.

Response 3.5 In order to account for the divergent cell numbers included in our single-cell repertoire analysis, we down-sampled the number of peripheral blood plasmablasts to the number of plasmablasts sequenced in the CSF. We recognize, however, that due to the low coverage of our plate-bound single-cell sequencing method, it is difficult to draw valid conclusions on the clonality of peripheral blood plasmablasts. We therefore deleted the original Fig. 1g (comparison of clonality between blood and CSF), and changed the main text to: *"The CSF repertoire is highly clonal (Fig. 1a), suggesting antigen-specific proliferation of a select set of clones within the CSF."*

In addition, we have now included three non-MS control CSF B cell repertoires — one from a patient with neuroborreliosis (C3, *n=265 sequences*), one with viral encephalitis (C6, *n=19 sequences*), and one from a patient with neuro-Behçet's disease (C5, *n=14 sequences*). We compared immunoglobulin class distributions and clonality in these control samples with the B cell repertoire data from MS patients (Revision Fig. 8a-d). The comparison highlights two features that stand out in the MS B cell repertoire data sets – (i) The IgG isotype is highly abundant and is used significantly more than any other immunoglobulin isotype in MS patients, whereas IgG and IgA are used to a similar degree in control patients, and (ii) Clonality of the CSF B cell repertoire is higher in MS patients than in control patients (Revision Fig. 8d). Higher clonality is particularly pronounced for IgG in MS patients, as IgG was by far more abundant and hardly any clonal CSF B cell used any other immunoglobulin isotype. In contrast, we did not detect elevated numbers of clonal IgG B cells in control patients as clonal IgG and IgA were present in comparable numbers. We are aware that the number of sequences in controls C5 and C6 are quite low. However, we believe this comparison adds to our repertoire analysis and emphasizes that in MS the CSF B cell repertoires are uniquely IgG-dominated and uniquely clonal, suggesting chronic intrathecal B cell stimulation with self-antigens that is not present in infectious settings or neuro-Behçet's disease. We now include the control CSF B cell repertoire data in our updated Manuscript Fig. 1a,b, and Extended Data Fig. 2b-d and 3.



Revision Fig. 8. CSF B cell repertoire data in MS and control patients. a-d, CSF B cell repertoire analysis, **a**, phylogenetic trees of CSF B cell repertoires of three control patients C3, C6, and C5. IgG (red), IgA (blue), IgM (green), smaller brighter circles depict singleton B cells, larger darker circles depict clonal B cells. **b**, Overview of 9 MS CSF B cell repertoires and 3 control repertoires. Inner circle represents clonality with singletons (grey) and clonal expansions (colors), outer circle represents immunoglobulin classes IgG (red), IgA (blue), IgM (green). **c**,**d**, Statistical analysis of B cell repertoire data shown in (**b**), **c**, immunoglobulin usage shown as percent IgG (left), IgA (center), and IgM (right) of all CSF B cell sequences in the respective patient. **d**, clonality analysis shown as percent clonal sequences of all CSF sequences in the respective patient for all immunoglobulin classes (left), IgG (center left), IgA (center right), and IgM (right). **c**,**d**, Means ± standard deviation are shown for each patient (MS, *n=9*; controls, *n=3*). ** P < 0.01 according to Mann-Whitney U test.

Comment 3.6 What was the fraction of clones found in both CSF and in peripheral blood? Any features of note for shared clones? Fig 3i shows V gene distribution but not whether clones are found in both compartments.

Response 3.6 In eight out of nine patients, we detected only marginal amounts of sequence overlap (zero or one clonal expansion) between peripheral blood and CSF in the same patient (Revision Table 1). Interestingly, patient MS21 differed substantially from the other eight patients, in that she had 31 shared clonal expansions, accounting for 73.8% of all CSF clonal expansions and 10.3% of all peripheral blood clonal expansions. While five of the other eight patients received the lumbar puncture to establish the diagnosis at the first onset of MS symptoms (Table 1, clinically isolated syndrome, CIS), patient MS21 had a years-long history of RRMS and CSF was drawn during a fulminant relapse with several new contrast-enhancing lesions after abrupt withdrawal of Fingolimod treatment. We believe that this condition caused a substantial amount of blood brain barrier leakiness, which explains the high number of shared clonal sequences. We did not identify any overlapping clones across patients.

We are aware that our coverage of the repertoire in CSF (22 - 425 paired-chain sequences per patient) and in peripheral blood (639 - 2342 paired-chain sequences per patient) is limited due to the plate-based single-cell sequencing method used. Higher coverage would likely result in the identification of additional shared clones between peripheral blood and CSF. However, the case of patient MS21 demonstrates that increased permeability of the blood brain barrier and concomitant B cell trafficking between the periphery and CNS was detected by our method. We conclude that the other eight patients have a higher degree of separation of B cell repertoires between peripheral blood and CSF.

Separation of intrathecal B cells from the B cells in the peripheral circulation is a hallmark of MS diagnostics, regularly measured by the presence of oligoclonal bands in the CSF, which differ by separation pattern from the peripheral blood. Studies that investigated the bulk B cell repertoire in both compartments with higher cell coverage showed bi-directional exchange between peripheral blood and the CSF, and more directional exchange between cervical lymph nodes and the CNS, where immature B cells were detected in the lymph nodes and more mature B cells in the CNS [25–27]. We believe that these studies, due to their methodologies, are better suited to answer questions of B cell trafficking across the blood brain barrier, whereas the strength of our study is the focus on antigen-specificity of intrathecal B cells.

	MS12	MS20	MS21	MS28	MS30	MS31	MS37	MS39	MS9
Clonal expansions in blood	219	86	302	242	100	147	54	261	132
Clonal expansions in CSF	5	6	42	10	25	13	37	23	35
Shared clonal expansions	0	0	31	0	1	1	1	0	0

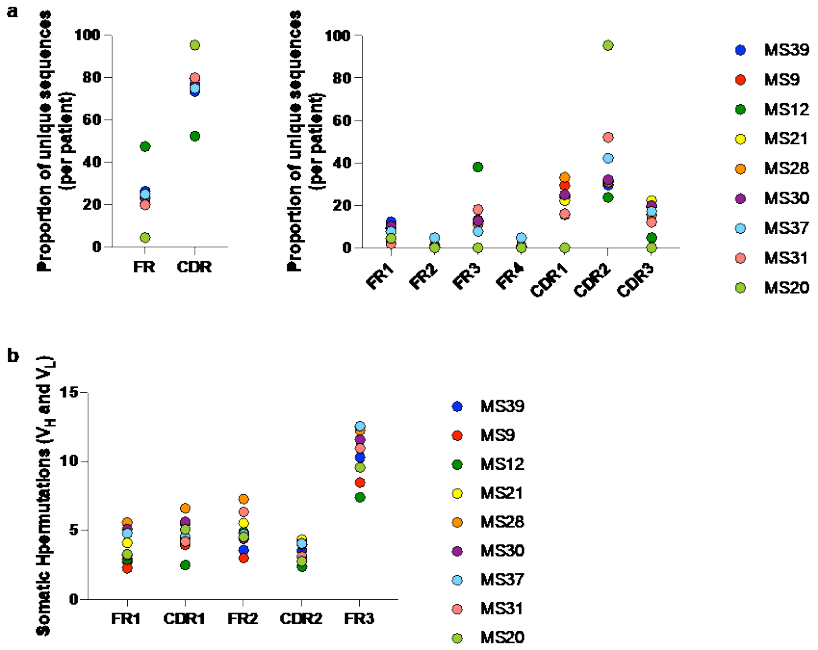
Revision Table. 1. Clonal expansions shared between blood and CSF in the same patient. Table showing number of clonal expansions of B cells in blood and CSF as well as number of clonal expansions shared between the two compartments. Members of a clonal expansion were defined by usage of the same heavy and light chain V and J genes and >70% sequence overlap between heavy and light chain CDR3 regions.

Comment 3.7 Fig 1j,k; Were all the PSMs for peptides from CDR3? If not then how do we know that they match "sequences unique to the patient" and not matches that arose because of the relatively small number of BCRs for each patient which could make framework peptides appear "unique". Also were there any peptides from EBNA1 antibodies detected?

Response 3.7 We agree with the reviewer that due to the higher variability of the hypervariable regions (CDR1, CDR2, and CDR3), they are more patient-specific, as compared to the framework regions (FR1, FR2, FR3, and FR4), with CDR3 encompassing the most variability[28]. As described in our methods, we eliminated mass spectrometry peptides that matched antibody sequences in more than one patient. Of the remaining

unique peptide sequences assigned to each patient, the majority (~80%) map to heavy chain or light chain CDR regions with most mapping to the CDR2 region (Revision Fig. 9a). This over-representation of CDR regions is likely a result of our filtering based on alignment with all other patients' repertoires, adding to stringency of our sequence selection. While framework regions are more conserved, each patient still has on average more than two mutations from germline in each framework region (Revision Fig. 9b). Additionally, as the Reviewer pointed out in comment 3.5, we cover a broader proportion of the B cell repertoire in the CSF than in the peripheral blood. Because the majority of the peptides map to hypervariable regions, and due to the broader sampling of the CSF coupled with regular occurrence of mutations in the framework region, we are confident that a high proportion of the reported sequences are unique to each patient.

We identified a total of 6 PSMs that correspond to MS39p2w174, providing direct evidence that the MS39p2w174 is secreted in the CSF (Revision Table 2).



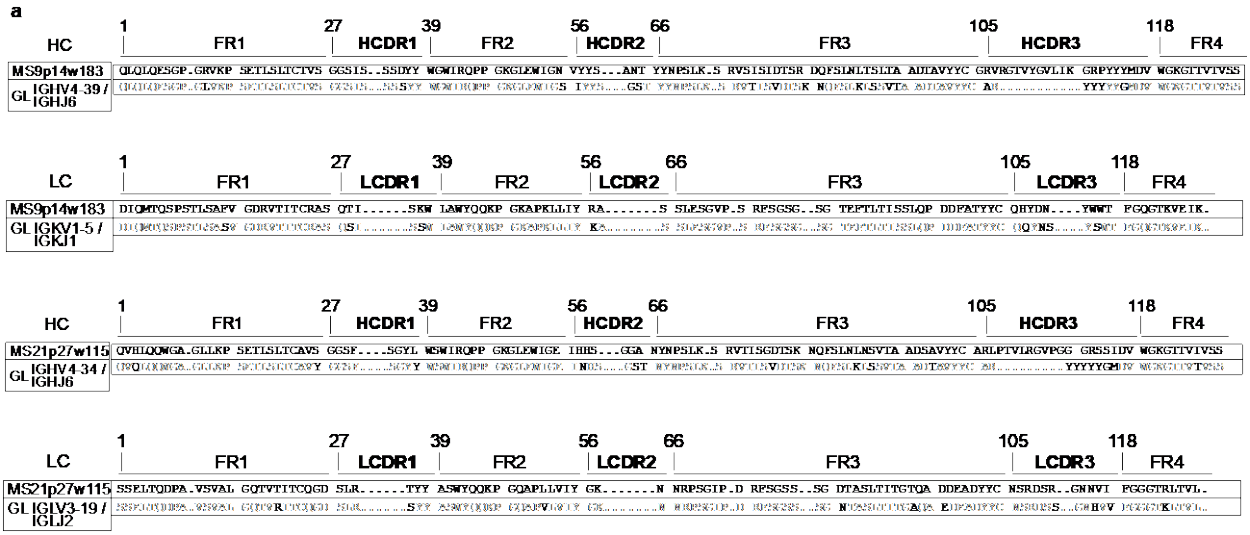
Revision Fig. 9. Mapping of unique peptides from purified CSF antibodies to CSF B cell sequences. a, Analysis of mass spectrometry data showing frequency of alignments of peptide sequences to framework and CDR regions in general (left) and separated by heavy and light chain individual framework regions and CDR regions. **b**, Repertoire analysis showing average number of somatic hypermutations (SHM) for the framework and hypervariable CDR regions in the V-region of the heavy and light chain. Means for each patient (n=9) are shown. Framework region (FR), complementarity determining region (CDR).

Annotated Sequence	Modifications	# Proteins	# PSMs	|Log Prob| A2	Byonic Score A2	Region mapped
[R].AEDTGIYYCTR.[D]	1xCarbamidomethyl [C9]	1	3	6.35	531.70	FR3, CDR3
[-].DVVLTQSPLSLPVILGQPASISCR.[S]	1xCarbamidomethyl [C23]	1	1	10.56	728.67	FR1
[R].SSQSLVYSDGR.[T]		1	1	8.36	601.33	FR1, CDR1
[R].QAPGGGLEWVANINQDGSEK.[Y]		1	1	6.66	484.58	FR2, CDR2

Revision Table. 2. Peptide-spectrum matches to MS39p2w174. Table showing all peptide-spectrum (PSM) that match the light chain or heavy chain sequence of MS39p2w174.

Comment 3.8 The authors show convincingly that reactivity to GlialCAM by MS39p2w174 is acquired by SHM. Was that the case for the two other antibodies with similar reactivity?

Response 3.8 The two antibodies MS9p14w183 and MS21p27w115 bind EBNA1 as well as non-phosphorylated GlialCAM$_{AA370-389}$ (original Manuscript Fig. 3m). We showed for antibody MS39p2w174 that its germline binds with high affinity to EBNA1 and is polyreactive, while it loses polyreactivity and gains specificity for GlialCAM during affinity maturation towards MS39p2w174. Most of our investigated CSF antibodies are not polyreactive (94.6%, Revision Fig. 7). However, MS21p27w115 stands out as a polyreactive antibody as it binds strongly to insulin (but not dsDNA or LPS). Antibody MS9p14w183 does not show increased polyreactivity in our ELISAs. The two antibodies MS9p14w183 and MS21p27w115 have a moderate level of somatic hypermutation with 33 and 30 total heavy and light chain V gene mutations, respectively (Revision Table. 3). In contrast, MS9p2w174 is more highly mutated, with a total of 50 nucleotide mutations in the V-regions of the heavy and light chain. The lower rate of somatic hypermutation suggests that both antibodies are less affinity matured than MS39p2w174, and at least in the case of MS21p27w115, reactivity to EBNA1 and GlialCAM$_{AA370-389}$ might be a function of a certain degree of polyreactivity due to a relatively early state of affinity maturation. As at least one of these two antibodies is polyreactive, we deleted their explicit mentioning from our manuscript in order to de-emphasize their importance.



Revision Fig. 10. Alignment of cross-reactive mAbs to germline sequences. a, Amino acid sequences of variable regions of mAb MS9p14w183 heavy and light chain (top) and mAb MS21p27w115 heavy and light chain (bottom).

	Heavy (V-region)		Light (V-region)	
	Total mutations	Non-silent mutations	Total mutations	Non-silent mutations
MS39p2w174	32	20	18	10
MS914w183	19	16	14	8
MS21p27w115	15	11	15	9

Revision Table. 3. Number of somatic hypermutations of cross-binding antibodies. Table showing total mutations and non-silent mutation in the V-region of the heavy and light chain of antibodies that bind EBNA1 and GlialCAM.

Referee #4 (Remarks to the Author):

The manuscript by Lanz et al. shows a mechanistic link between multiple sclerosis and Epstein-Barr virus, whereby a mature antibody cross-reacts with an EBV transcription factor (EBNA1), and glia-specific type-1 membrane protein glialCAM. The authors provide convincing structural, biophysical and in vivo results to demonstrate the relevance of such mechanism.
These findings provide novel insights into the molecular mechanisms underlying an important and complex human pathology, and could serve to develop therapeutic tools. Hence, I find the manuscript as potentially suitable for publication, but I have concerns that should be address before considering publication:

Comment 4.1 The manuscript lacks necessary background on the subject that will help readers to better follow the flow of the work. Instead of a proper introduction providing such background and exposing clearly the problem to be addressed, the manuscript presents an extended summary of results (lines 88-107) that overlaps with both the abstract and discussion sections. The introduction should be extensively edited, stating for instance current knowledge on EBV (and other viruses) and MS relationship, relevant EBV antigens particularly regarding EBNA-1, etc.

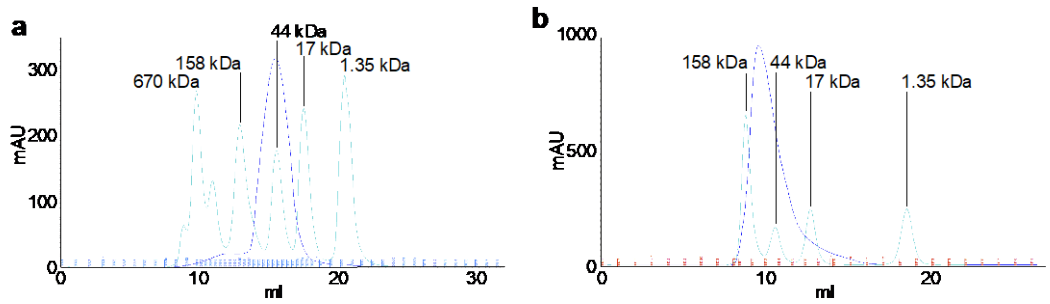
Response 4.1 We thank the reviewer for the valuable comments. We have significantly shortened the manuscript to accommodate Nature's publishing guidelines. In doing so, we revised the introduction to a short paragraph that exposes the reader to background information on the relationship between EBV and MS, and references known examples of molecular mimicry.

Comment 4.2 The abstract states (line 76) "crystal structure of EBNA1-antibody complex…", I think this is misleading, as the structure solved is that of a Fab with an antigenic-EBNA1 peptide. Also in line 214, it should be stated that the complex is with monovalent Fab, and not divalent mAb. Although the methods section describes clearly that is a Fab-peptide complex, I think it is important to clarify this in the text, as both native EBNA1 and glialCAM form oligomers potentially affecting binding of divalent mAbs, compared to monovalent-Fab/peptide binding (see also comment #3).

Response 4.2 We revised the passages in the text to reflect the accurate components of the structure. The passage in the abstract was changed to "...the crystal structure of the EBNA1-peptide epitope in complex with the autoreactive Fab fragment…". We changed the

text in the second passage to: "…we solved the crystal structure of EBNA1$_{AA386-405}$ in complex with the MS39p2w174-Fab…".

Comment 4.3 mAb MS39p2w174 binds native glialCAM with an order of magnitude higher affinity than the phosphorylated glialCAM derived peptides. What do the authors think is the reason for this? Could it be oligomerization of the native protein? It is worth including some discussion about this after that related to the effect of phosphorylation (after line 433)



Revision Fig. 11. Size estimation of GlialCAM ECD and ICD. a,**b**, FPLC runs of **a**, ECD (dark blue) and **b**, ICD proteins (dark blue) overlayed with protein standard (cyan) with indicated molecular weights.

Comment 4.4 Regarding interferometry experiments. The authors only present dissociation constants (KD), but it would be of interest to show "on and off" kinetic constants, since they already have these data, to get insight on binding differences. Also, I couldn't find anywhere the number of biological binding experiments performed. Finally, please include in the text errors associated to KD determination.

Response 4.4 The reported K_D (in M) is not the dissociation constant but the equilibrium constant between the antibody and the antigen, which is described as the ratio of association constant K_{on} (in $M^{-1} * s^{-1}$) / dissociation constant K_{off} (in s^{-1}). It takes into account association and dissociation kinetics and is therefore the most extensive and valuable parameter to report[30]. We now include standard errors in the text when K_D values are mentioned. We have also changed the y-axes of our biolayer-interferometry experiments from µM to M to make them more easily accessible to the reader.

Comment 4.5 Regarding anisotropy correction of diffraction data, it would be informative to include in the crystallographic table the resolution limits along the three axes. Also data completeness before staraniso (I assume the reported completeness -96.1/78.0%- is after) to show that incompleteness does not come from insufficient sampling of reciprocal space.

Response 4.5 The resolution limits for the three dimensions in reciprocal space as applied by STARANISO are 2.23 Å (a*) x 3.69 Å (b*) x 2.19 Å (c*). STARANISO computed an ellipsoid post-fitted by least squares to the cutoff surface, removing points where the fit was poor. Note that the cutoff surface is unlikely to be perfectly ellipsoidal, so this is only an estimate.

We also amended completeness values at several steps of processing. Completeness of the uncorrected data is 96% (78%), as indicated in the initial crystallography table.
After ellipsoidal correction, the "spherical" completeness (i.e. the completeness within the sphere that contains the ellipsoid, after removing reflections outside of the ellipsoid) is 54.7% (13.1%). The ellipsoidal completeness (i.e., the amount of expected reflections within the ellipsoid that are actually measured) is 92.5% (72.4%). In addition, while data to 2.19 Å resolution had to be included to calculate the contours of the ellipsoid, the final refinement was performed using data to 2.5 Å resolution. At this resolution, completeness of uncorrected data in the highest shell is 99%.

We have changed the color palette of the structure in the updated Fig. 2f-j in order to make it easier to discern peptide residues from the heavy and light chain.

CRYSTALLOGRAPHIC DATA

Table 1. Data collection and refinement statistics

EBNA1 peptide 386-405/MS39p2w174 Fab	
Data collection	
Beamline	SSRL BL12-2
Wavelength (Å)	0.97946
Space group	I222
Cell dimensions	
a, *b*, *c* (Å)	119.66, 137.56, 179.00
α, β, γ (°)	90.00, 90.00, 90.00
Matthews coefficient (Å^3/Da)[a]	3.7
Solvent content (%)	66.8
Anisotropy	0.72
Resolution (Å)[b]	45.14(2.19)
R_{merge}[c]	0.16(5.92)
$I / \sigma I$ ratio[d]	5.5(0.7)
Uncorrected completeness (%)[e,f]	96.1(78.0)
Spherical completeness (%)[g]	54.7(13.1)
Ellipsoidal completeness (%)[h]	92.5(72.4)
Reflections (total/unique)	51,233(3,637)
Redundancy[j]	3.2(2.0)
Refinement	
Resolution (Å)	45.14-2.50
No. reflections/test set	36,574/1,912
R_{work} / R_{free}[k]	21.1/25.2
Mean B value (Å^2)	56.4
F_{obs}-F_{calc} correlation[l]	0.90
No. atoms	
Protein	6,778
Ligand/ion	4 glycerol/1 chlorine
Water	24
B-factors	
Protein	46 (overall)
Ligand/ion	73 (glycerol)/45 (chlorine)
Water	52
Ramachandran statistics[m]	
Most favored/allowed regions (%)	100
Disallowed regions (%)	0
R.m.s. deviations	
Bond lengths (Å)	0.33
Bond angles (°)	0.55

[a]Ratio of the volume of the asymmetric unit to the molecular weight of all protein in the asymmetric unit

[b]Value in parentheses is for the highest-resolution shell: 2.24 – 2.19 Å.

[c]Reliability factor for symmetry-related reflections calculated as: $R_{merge} = \Sigma_{hkl} \Sigma j=1 \text{ to } N \mid I_{hkl} - I_{hkl}(j) \mid / \Sigma_{hkl} \Sigma j=1 \text{ to } N\ I_{hkl}(j)$, where N is the redundancy of the data. In parentheses, the cumulative value at the highest-resolution shell

[d]Ratio of mean intensity to the mean standard deviation of the intensity over the entire resolution range

[e]Fraction of measured reflections to possible observations at the resolution range

[f]Completeness of the uncorrected data set

[g]Completeness within the sphere that contains the ellipsoid, after removing reflections outside of the ellipsoid

[h]The amount of expected reflections within the ellipsoid that are actually measured

[j]Number of measurements of individual, symmetry unique reflections

[k]Average deviation between the observed and calculated structure factors calculated as: R_{work} = Σ_{hkl} ||F_{obs}| - |F_{calc}|| / Σ_{hkl} |F_{obs}|, where the F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes of reflection hkl. R_{free} is equal to R_{factor} but for a randomly selected 5.0 % subset of the total reflections that were held aside throughout refinement for cross-validation
[l]Correlation coefficient between observed and calculated structure factor amplitudes
[m]According to Molprobity for non-proline and non-glycine residues

Comment 4.6 The authors mention in the abstract that results in the manuscript could guide developments of MS-related therapies. It is a bit disappointing that there is no discussion about this in the text. Is it possible that EBNA1 and/or glialCAM derived peptides could serve to neutralize auto-antibodies? Please, include some hypothesis about the therapeutic implications of the work in the discussion, or remove the sentence in the abstract.

Response 4.6 We thank the reviewer for pointing this out. Unfortunately, despite myriads of promising experimental therapeutic approaches, there are still no approved antigen-specific therapies for autoimmunity. The main B cell targeting therapeutics to date are anti-CD20 antibodies, which are highly effective for MS[31,32]. Interestingly, as latent EBV resides in memory B cells, B cell depleting antibodies might contribute to eradication of EBV, which could be a so-far little regarded mechanism contributing to the therapeutic outcome.

We believe that there are several therapeutic approaches that could be developed based on the connection between EBV and MS and based on the molecular mimicry mechanism described in our manuscript: (i) EBV vaccine. Having realized that EBV infection is a prerequisite for MS development, efforts should be increased to develop an effective vaccine against EBV, which might eradicate MS. Knowledge of protein regions with the potential to generate molecular mimicry against self proteins will help guide vaccine development and exclude these regions from vaccine approaches. (ii) Antigen-specific therapies, targeting B cell epitopes. Blocking antibodies from binding to their autoantigens could be achieved for example with attenuated antibodies that lack ADCC and CDC. (iii) Tolerizing approaches. Multiple antigen-specific tolerization approaches have been tested, which include DNA-immunization[33], oral tolerance[34], and novel approaches like CAR-T cells directed against antigen-specific T cells[35]. Several of these approaches have been unsuccessful in the past but the identification of novel pathogenic antigens and epitopes would re-vitalize their promise. We should mention that autoreactivity against larger sets of antigens has stifled several tolerizing approaches, as MS likely cannot be explained by reactivity to a single antigen. Nevertheless, our identification of EBNA1-GlialCAM as the driving antigens in a subset of MS now enables development of next-generation tolerizing therapies for this subset of MS.

Our specific antigen pair EBNA1 and GlialCAM open multiple paths to study autoimmunity and tolerance. Critical aspects of follow-up studies include the contribution of GlialCAM phosphorylation to the breakdown of tolerance, mechanisms that explain pathogenicity of antibody reactivity to intracellular antigens, and the clinical characterization of patients with anti-GlialCAM antibodies.

Comment 4.7 Line 294, reference 24 does not correspond to the PDB ID mentioned (J Mol Biol 1998)

Response 4.7 We thank the reviewer for pointing this out. We revised reference 24 (now reference 29): new reference 30: Bochkarev, A., Bochkareva, E., Frappier, L. & Edwards, A. M. The 2.2 Å structure of a permanganate-sensitive DNA site bound by the Epstein-Barr virus origin binding protein, EBNA1. J. Mol. Biol. 284, 1273–1278 (1998). We also reviewed all other references in the manuscript to make sure they are correct.

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Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have responded appropriately to previous comments and provided substantial further data supporting their conclusion that up to 25% of MS cases are likely to involve autoimmune cross reaction of antibodies to EBNA1 with the GlialCAM protein.

The results are important, very clearly presented and suitable for publication in Nature.

However, the current title is wrong, since there is no EBNA1 in EBV virions

The title of the paper should be changed to read.
Clonally Expanded B Cells in Multiple Sclerosis Bind EBV EBNA1 and GlialCAM

Referee #2 (Remarks to the Author):

I am largely satisfied by the authors' response, and recommend publication.
Specifically:
2.1: The investigators have excluded possible misdiagnosis of neuromyelitis optica spectrum disorders (NMOSD), with one possible exception, case #37, the one with highest CSF cell number. The proportion of B/T cells has not been given.
2.3: Immunofluorescence staining with antibody MS39p2w174 shows tight junction staining, resembling GlialCAM expression of cell cultures in high density.

Referee #3 (Remarks to the Author):

This is one of the most thorough and professionally done revisions and response letters I have ever seen! And I have been doing this job for a long time! I feel that the points raised in my review (#3) and also the points by the other referees have been fully addressed.

Congratulations to the authors for superb execution and rigor. I have a couple of minor gripes concerning the MS analysis and structural analysis but I do not believe in asking for endless revisions. But would like to make the suggestion -and this is a suggestion and not a request-that authors de-emphasize Table 2 by moving it to the SI. I would leave this to the authors discretion.

This is an excellent paper!

Referee #4 (Remarks to the Author):

For the most part, the authors have addressed properly the concerns I had on the manuscript. There remains only one concern related to their binding experiments:
Indeed, the reported KD is an equilibrium dissociation constant that in biolayer interferometry experiments is calculated as the ratio of two kinetic constants koff/kon (not the other way around). I agree with the authors that KD values are very informative binding parameters and should be reported. In my view, kon and koff are also informative parameters and provide insights

on the kinetic mechanism of the binding reaction. The authors already have kon and koff values from there interferometry experiments, and I don’t see the reason to not arrange them in a table and report them in the manuscript. I strongly suggest to include such table, so that readers can have access to all mechanistic information collected during the experiments. Finally, I still cannot find the number of biological replicates performed in the binding experiments. Please, state clearly in figure legends or methods what kind of replicates and how many of them are being averaged.

Author Rebuttals to First Revision:

Point-by-Point Response to Reviewers' Comments (2nd Revision) **January 6, 2021**

Manuscript: Clonally Expanded B Cells in Multiple Sclerosis Bind EBV EBNA1 and GlialCAM

Nature manuscript 2021-06-09836B

Authors: Tobias V. Lanz, R. Camille Brewer, Peggy P. Ho, Jae-Seung Moon, Kevin M. Jude, Daniel Fernandez, Ricardo A. Fernandes, Alejandro M. Gomez, Gabriel-Stefan Nadj, Christopher M. Bartley, Ryan D. Schubert, Isobel A. Hawes, Sara E. Vazquez, Manasi Iyer, J Bradley Zuchero, Bianca Teegen, Jeffrey E. Dunn, Christopher B. Lock, Lucas B. Kipp, Victoria C. Cotham, Beatrix M. Ueberheide, Blake T. Aftab, Mark S. Anderson, Joseph L. DeRisi, Michael R. Wilson, Rachael J.M. Bashford-Rogers, Michael Platten, K. Christopher Garcia, Lawrence Steinman, William H. Robinson

Referee #1 (Remarks to the Author):

The authors have responded appropriately to previous comments and provided substantial further data supporting their conclusion that up to 25% of MS cases are likely to involve autoimmune cross reaction of antibodies to EBNA1 with the GlialCAM protein.

The results are important, very clearly presented and suitable for publication in Nature.

However, the current title is wrong, since there is no EBNA1 in EBV virions

The title of the paper should be changed to read.
Clonally Expanded B Cells in Multiple Sclerosis Bind EBV EBNA1 and GlialCAM

Response to Referee #1. We have updated the title according to the Reviewer's recommendation.

Referee #2 (Remarks to the Author):

I am largely satisfied by the authors' response, and recommend publication.

Specifically:

Comment 2.1 The investigators have excluded possible misdiagnosis of neuromyelitis optica spectrum disorders (NMOSD), with one possible exception, case #37, the one with highest CSF cell number. The proportion of B/T cells has not been given.

Response 2.1 In order to include additional details of the clinical diagnoses and CSF counts, we amended the first paragraph of the methods section, now also mentioning diagnostic criteria of NMOSD. We also added a paragraph on the patient collective and CSF cell counts in the Supplementary Discussion.

Unfortunately, we are unable to accurately determine B and T cell ratios, as CSF cells were magnetically separated with anti-CD19 beads before fluorescent staining and sorting, hence the high numbers of B cells depicted in Extended Data Figure 1a,b. We noticed to our regret that the magnetic separation step was not previously mentioned in our manuscript, and we apologize for this. We have amended the methods section to now include these details.

Comment 2.3 Immunofluorescence staining with antibody MS39p2w174 shows tight junction staining, resembling GlialCAM expression of cell cultures in high density.

Response 2.3 We thank the Reviewer for the comments. The immunofluorescence stainings are included in the current manuscript in Extended Data Figure 8.

Referee #3 (Remarks to the Author):

This is one of the most thorough and professionally done revisions and response letters I have ever seen! And I have been doing this job for a long time! I feel that the points raised in my review (#3) and also the points by the other referees have been fully addressed.

Congratulations to the authors for superb execution and rigor. I have a couple of minor gripes concerning the MS analysis and structural analysis but I do not believe in asking for endless revisions. But would like to make the suggestion -and this is a suggestion and not a request-that authors de-emphasize Table 2 by moving it to the SI. I would leave this to the authors discretion.

This is an excellent paper!

Response to Referee #3. We thank the Reviewer for their positive evaluation and endorsement of our work. We agree with the Reviewer's opinion and moved Extended Data Table 2 (Overview of HLA loci) to the Supplementary Information section (new Supplementary Table 1). We included our HLA prediction analysis as new Supplementary Figure 2, and describe both briefly in the Supplementary Discussion. Concomitantly, we moved Extended Data Tables 3-5 (overview of proteins and peptides) to the Supplementary Information section (new Supplementary Tables 2-4), as well as the crystallographic table (new Supplementary Table 5) and the new table showing K_D, K_{ON}, and K_{OFF} values. Extended Data Table 1 (patient collective) and Extended Data Table 5 (phage display data, new Extended Data Table 2) remained the Extended Data section.

Referee #4 (Remarks to the Author):

For the most part, the authors have addressed properly the concerns I had on the manuscript. There remains only one concern related to their binding experiments:
Indeed, the reported KD is an equilibrium dissociation constant that in biolayer interferometry experiments is calculated as the ratio of two kinetic constants koff/kon (not the other way around). I agree with the authors that KD values are very informative binding parameters and should be reported. In my view, kon and koff are also informative parameters and provide insights on the kinetic mechanism of the binding reaction. The authors already have kon and koff values from there interferometry experiments, and I don't see the reason to not arrange them in a table and report them in the manuscript. I strongly suggest to include such table, so that readers can have access to all mechanistic information collected during the experiments. Finally, I still cannot find the number of biological replicates performed in the binding experiments. Please, state clearly in figure legends or methods what kind of replicates and how many of them are being averaged.

Response to Referee #4. We agree with the Reviewer that K_{on} and K_{off} values are important for the reader to understand the binding kinetics. We have included all values in Supplementary Table 6. We revised all figure legends to include proper statistics and replicates. The data shown in the Fig. 2 k,l, and Fig. 3 e,f,k,l are averages of 3-4 technical replicates, each representative of 3 independent experiments. We supply the values of the binding curves in the source data files for Fig. 2 and Fig. 3.

antibody	ligand	KD (M)	KD Error	kon ($M^{-1}s^{-1}$)	kon Error	koff (s^{-1})	koff Error	*n*
MS39p2w174	EBNA1 protein	1.99E-09	6.25E-10	4.07E+04	1.61E+04	7.32E-05	1.06E-05	4
MS39p2w174 germline	EBNA1 protein	4.19E-09	7.62E-10	2.94E+04	1.65E+04	1.14E-04	4.53E-05	4
MS39p2w174	GlialCAM protein	1.91E-10	1.73E-11	2.61E+05	5.01E+04	5.00E-05	1.21E-05	3
MS39p2w174 germline	GlialCAM protein	1.05E-08	4.12E-09	4.28E+04	1.39E+04	4.12E-04	4.79E-05	3
MS39p2w174	EBNA1 AA386-405	2.67E-09	7.83E-11	2.40E+05	5.28E+03	6.40E-04	1.24E-05	4
MS39p2w174	GlialCAM AA370-389	3.02E-07	3.10E-08	7.15E+04	6.39E+03	2.16E-02	1.09E-03	4
MS39p2w174	GlialCAM AA370-389 pSer376	6.10E-09	2.69E-10	2.80E+05	1.10E+04	1.71E-03	3.40E-05	4
MS39p2w174	GlialCAM AA370-389 pSer376/377	3.73E-09	1.50E-10	3.23E+05	1.14E+04	1.20E-03	2.33E-05	4

Supplementary Table 6. K_D, K_{on}, and K_{off} values for antibody-ligand pairs. Values correspond to **Fig. 2 k**,**l** (MS39p2w174 and Germline with EBNA1 protein), **Fig. 3 e**,**f** (MS39p2w174 and Germline with GlialCAM protein), and **Fig. 3 k**,**l** (MS39p2w174 with EBNA1 AA386-405, GlialCAM AA370-389, and phosphorylated GlialCAM AA370-389). Measurements are representative of at least 3 independent experiments. In each individual experiment, values are averages of n replicates in serial dilutions.

In our previous version of our crystal structure, we presented polar interactions <3.4Å. As this length would include hydrogen-bonds with relatively low energy, we have now set the distance to <3.2Å and revised figure 2h-i accordingly. Only one hydrogen-bond was longer than 3.2Å (between peptide R396 and HC D107) and has been deleted in the revised figure.