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CD4 T cells restricted to DRB1*15:01 recognize two Epstein–Barr virus glycoproteins capable of intracellular antigen presentation

Natalia Drosu[a,b,1], Monique Anderson[a], Philippe A. Bilodeau[a], Shuhei Nishiyama[a], Takahisa Mikami[a], Natasha Bobrowski-Khoury[a], Jackson Cabot[a], David Housman[b,1], and Michael Levy[a]

Affiliations are included on p. 8.

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Both genetic and environmental factors contribute to multiple sclerosis (MS) risk. Infection with the Epstein–Barr virus (EBV) is the strongest environmental risk factor, and HLA-DR15 is the strongest genetic risk factor for MS. We employed computational methods and in vitro assays for CD4 T cell activation to investigate the DR15-restricted response to EBV. Using a machine learning-based HLA ligand predictor, the EBV glycoprotein B (gB) was predicted to be enriched in epitopes restricted to presentation by DRB1*15:01. In DR15-positive individuals, two epitopes comprised the major CD4 T cell response to gB. Surprisingly, the expression of recombinant gB in a DR15-homozygous B cell line or primary autologous B cells elicited a CD4 T cell response, indicating that intracellular gB was loaded onto HLA class II molecules. By deleting the signal sequence of gB, we determined that this pathway for direct activation of CD4 T cells was dependent on trafficking to the endoplasmic reticulum (ER) within the B cell. We screened seven recombinant EBV antigens from the ER compartment for immune responses in DR15-negative vs. DR15-homozygous individuals. In addition to gB, gH was a key CD4 T cell target in individuals homozygous for DR15. Compared to non-DR15 controls, DR15-homozygotes had significantly higher T cell responses to both gB and gH but not to EBV latent or lytic antigens overall. Responses to gB and gH were slightly elevated in DR15 homozygotes with MS. Our results link MS environmental and genetic risk factors by demonstrating that HLA-DR15 dictates CD4 T cell immunity to EBV antigens.

multiple sclerosis | Epstein–Barr virus | antivirals | HLA-DR15

Significance

Emerging evidence points to Epstein–Barr virus (EBV) infection as a prerequisite for multiple sclerosis (MS), but how EBV leads to MS several years later is unknown. The HLA-DR15 haplotype is the strongest genetic risk factor for MS, carrying an eightfold increased risk in homozygotes. We found DR15-restricted epitopes recognized by EBV-specific CD4 T cells that act as direct reporters of viral antigen presentation in B cells. Our results explain how immune responses to EBV reactivation may contribute to MS risk and enable molecular dissection and deep phenotyping of EBV-specific CD4 T cells in peripheral blood and the CNS. Our observations provide a clear rationale for effective treatment of MS by antiviral therapies or antigen-specific deletion of CD4 T cells that recognize EBV-infected B cells.

Epidemiological evidence has established that Epstein–Barr virus (EBV) is necessary to cause multiple sclerosis (MS) (1). However, it remains unclear why the vast majority of EBV-infected individuals never develop MS. Compelling hypotheses centered around molecular mimicry between the EBV nuclear antigen 1 (EBNA1) and several neuroglial proteins—including glialCAM, anoctamin 2, and alpha-crystallin B have been proposed (2–5). However, the extent to which mimicry to central nervous system (CNS) antigens drives disease activity has yet to be determined. Recent observations from T cell receptor (TCR) sequencing studies support the idea that patients with MS may experience more EBV lytic reactivation as CD8 T cells in MS have broader TCR diversity, and CNS T cells were found to be strongly biased toward EBV lytic antigens with a phenotype suggestive of recent priming (6). In support of this hypothesis, spontaneous lymphoblastoid cell lines (sLCLs) from patients with active MS were found to have higher EBV lytic gene expression, and CD4 T cell responses to sLCLs could be lowered by treatment of sLCLs with antivirals targeting the EBV DNA polymerase (7). While molecular mimicry to EBNA1 and poor control of EBV reactivation likely contribute to aspects of the disease, critical clues about MS—particularly around genetic susceptibility and its contribution to the immune repertoire in MS—remain incompletely addressed.

Genetic studies have consistently demonstrated an association between MS risk and the HLA-DR15 haplotype (8), which contains two HLA class II alleles in linkage disequilibrium, HLA-DRB1*15:01 and HLA-DRB5*01:01 (9). Individuals who are heterozygous for this haplotype have four times the risk of MS, and those who are homozygous have eight times the risk of MS (10). Prior studies have suggested that HLA-DR15 may lead to poor immune control of EBV or increased viral entry into B cells expressing HLA-DR15 as a co-receptor (11–13). While many other HLA-DP and -DQ class II alleles permit variable levels of entry of EBV into B cells (14, 15), these have not been associated with the same risk of MS as DR15. There is also no association between DR15 and infectious mononucleosis (16). An alternative explanation for the selection of HLA-DR15 alleles that has received less attention is the possibility of allele-restricted EBV antigen presentation critical to MS risk.

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[1]To whom correspondence may be addressed. Email: ndrosu@mgh.harvard.edu or dhousman@mit.edu.

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To date, the only autoimmune disease with an HLA class II association for which there exists an identified antigen is Celiac disease (CeD), where the strong association to HLA-DQ2 or DQ8 is due to the unique ability of these alleles to present peptides derived from gliadin (17, 18). HLA class II-restricted presentation of gliadin to CD4 T cells triggers a subsequent cascade of pathological immune activation (19). Although gluten-specific CD4 T cells are rare, even in active CeD lesions (20), they are distinctly dysfunctional. They initiate the inappropriate activation and amplification of downstream effector T cells, which destroy enterocytes by nonclassical autoimmunity (19, 21–24). A hypothesis by analogy would suggest that the link to HLA-DR15 and MS may be related to the presentation of EBV-derived epitopes to instigator CD4 T cells restricted to either DRB1*15:01 or DRB5*01:01 (25). These CD4 T cells could then inappropriately activate downstream effector T cells that destroy myelin. One example of a DR15-restricted EBV peptide previously defined in healthy donors is derived from the EBV triplex capsid protein encoded by *BORF1* (26). This and additional EBV peptides restricted to DR15 alleles have yet to be systematically examined in patients with MS.

Typically, viral proteins within infected cells are presented on HLA Class I molecules to CD8 cells; on the other hand, viral proteins outside of cells can be captured by antigen-presenting cells (APCs), where they are digested and loaded onto HLA Class II molecules, such as HLA-DR15, for presentation to CD4 cells. However, previous studies have also demonstrated that intracellular pathways exist to present the EBV antigens EBNA-1 or gp350 on HLA class II molecules (27, 28). Since EBV persists in memory B cells (29), we wanted to determine whether EBV and DR15 may be functionally linked by the DR15-restricted direct presentation of specific viral antigens. We focused on the ability of EBV proteins to access intracellular routes for HLA class II presentation in the context of the DR15 haplotype given the link to MS. We hypothesized that specific EBV-derived epitopes are DR15-enriched, accessible for endogenous loading onto HLA class II and that CD4 T cell response to this subset could differentiate patients with MS from HLA-matched healthy controls.

Results

Predicted EBV Epitopes Contain More MS Risk Allele-associated Outliers for DRB1*15 Than DRB5*01 Alleles and gB (*BALF4*) Is Preferentially Predicted to be Presented by DRB1*15:01. We first examined structural differences that may affect peptide binding to HLA molecules that confer an increased risk of MS. We compared the risk alleles DRB1*15:01 and DRB5*01:01 versus the most closely related HLA alleles DRB1*15:02 and DRB5*01:02, respectively (30). For the DRB1 alleles, a single amino acid substitution (V115G) in the peptide binding groove distinguishes *15:01 from *15:02 (*SI Appendix*, Fig. S1 *A* and *B*), which hinders the binding of peptides with bulky amino acids at position P1 (*SI Appendix*, Fig. S1 *E* and *G*). For the DRB5 alleles, three amino acids (D59G, D66N, and L67V) near the peptide binding groove differentiate *01:01 from *01:02, which create a decrease in negative surface charge (*SI Appendix*, Fig. S1 *C* and *D*) and a preference for more basic amino acids at P9 (*SI Appendix*, Fig. S1 *F* and *H*). The peptide binding motif differences between DRB1 alleles (*SI Appendix*, Fig. S1*I*) were more divergent than between DRB5 alleles (*SI Appendix*, Fig. S1*J*), suggesting that DRB1*15:01 may be more unique concerning the epitopes it can present.

To investigate these findings in the context of EBV epitopes, we performed computational predictions using MixMHC2pred, a recently published machine-learning algorithm based on deep motif deconvolution derived from extensive peptidomics combined with X-ray crystallographic data (31). We selected this algorithm specifically because it included the ability to predict binding to DRB1*15:01 and DRB1*15:02 as well as DRB5*01:01 and DRB5*01:02. We first generated a list of all 311,878 possible 12-18mer EBV peptides using the B95-8 reference proteome and then computed the odds scores for each peptide for each of the four alleles. We grouped our results by epitope to account for overlapping peptides with a shared 9-mer core. In line with our structural analyses, we found several MS-risk allele-associated outlier epitopes for DRB1*15 (Fig. 1*A*) but not DRB5*01 (Fig. 1*B*) alleles.

Surprisingly, the top two DRB1*15:01 epitopes (designated IHV and ILI by the first three amino acids of the 9-mer core) were both derived from the same antigen – the glycoprotein B (gB) encoded by the *BALF4* gene. When grouping epitopes by protein, we found a strong preference for epitopes derived from gB for DRB1*15:01 compared to *15:02 (Fig. 1*C*). The tegument protein encoded by *BBLF1* and the IL-10 homolog encoded by *BCRF1* also scored slightly higher for DRB1*15:01 (Fig. 1*C*). However, we observed little to no difference in predictions by protein when comparing DRB5 alleles (Fig. 1*D*). When we examined all 131 available HLA-DRB alleles, DRB1*15:01 was the highest-scoring allele for predicted gB epitopes (Fig. 1*E*). In contrast, we did not observe any allelic preference for 14 other EBV-encoded membrane proteins (*SI Appendix*, Fig. S2 *A–N*).

Predicted gB Peptides Demonstrate CD4 T Cell Responses in Individuals with DR15 and Are Naturally Processed from Recombinant gB Protein. Next, we wanted to determine whether the two predicted gB epitopes elicited responses in DR15-positive individuals. The presence of DR15 in these individuals was inferred by a qPCR assay for the SNP rs3135388-A, which is highly correlated ($r^2 = 0.98$) (32). Since CD4 T cell responses to EBV were previously shown to be rare in chronically infected individuals (26), we decided to enrich for CD4 T cells by depleting cells expressing CD8, CD56, CD19, CD123, CD235a, and TCRγδ from whole PBMCs. Using this strategy, the percentage of CD4 T cells of total CD3 T cells increased from 47.1 to 87.5% (Fig. 2*A* and *SI Appendix*, Fig. S3). We used ELISPOT assays to test the two peptides corresponding to the predicted gB epitopes (amino acids 103–116 and 599–613) (Fig. 2 *B* and *C*). Since it was previously shown that an EBV triplex capsid protein (*BORF1)* epitope was restricted to DRB1*15:01 (26), we included the *BORF1*-encoded 289–302 peptide in our assays (Fig. 2 *B* and *C*). By screening individuals for DR15, we identified four heterozygous individuals (DR15-1 to -4) who responded to both gB peptides and one individual (DR15-5) who did not respond to either peptide (Fig. 2 *B* and *C*). Individuals DR15-2 through -5 had an established diagnosis of relapsing-remitting MS (RRMS), while individual DR15-1 had not undergone clinical testing for MS.

To verify whether these responses could be elicited using naturally processed recombinant gB protein, we expressed the *BALF4* region encoding the soluble ectodomain of gB in 293T cells (Fig. 2*E*). Supernatants containing the antigen could elicit responses in all four individuals who reacted to the gB peptides but not in the nonresponder (Fig. 2*F*). The sum of responses to the two peptides was equal to that of recombinant gB, suggesting that these contained the dominant epitopes (Fig. 2*G*). While four other high-scoring predicted gB peptides restricted to DR15 were also tested (corresponding to amino acids 79–93, 174–189, 257–270, and 722–737) (*SI Appendix*, Table S1), responses to these were not observed. We then isolated fresh CD4 T cells from two donors and combined them with the EBV-negative DR15-homozygous B cell line, SUDHL-4 (Fig. 2 *H–J*). We only observed responses when gB or *BORF1*-encoded peptides were added in combination with

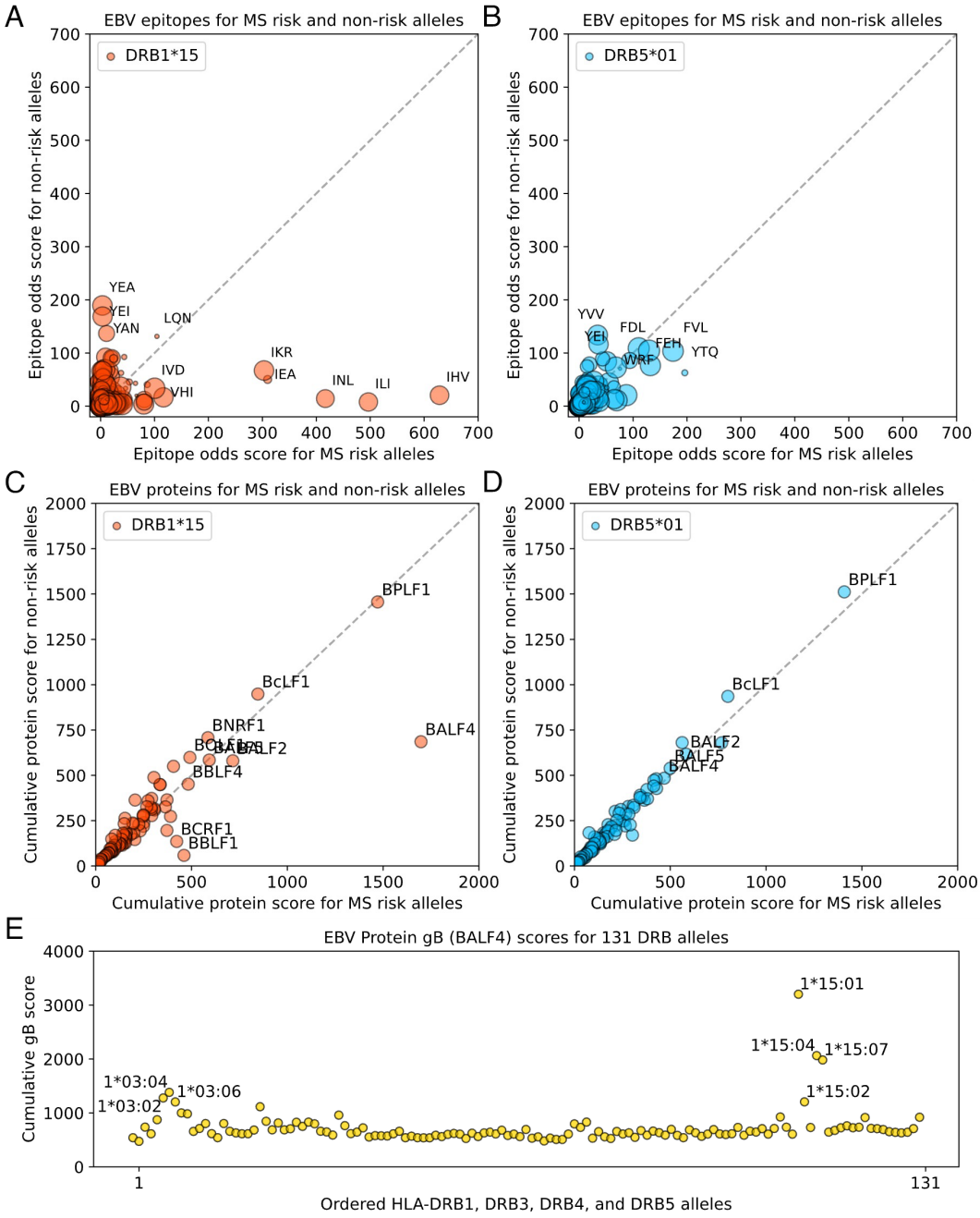

Fig. 1. Predicted EBV epitopes contain more MS risk allele-associated outliers for DRB1*15 than DRB5*01 alleles, and gB (*BALF4*) is preferentially predicted to be presented by DRB1*15:01. (*A*) Comparison of predicted EBV epitope odds scores for HLA-DRB1*15:01, which is associated with MS risk, versus *15:02. Each epitope is designated by the first three amino acids of the predicted 9-mer core. The diameter of each point demarking an epitope is directly proportional to the number of peptides with the same core. (*B*) Comparison of predicted EBV epitope odds scores as in *A*, except considering HLA alleles DRB5*01:01 versus *01:02 instead. (*C*) Cumulative odds scores grouped by EBV protein for DRB1*15:01 versus *15:02 alleles. Each point is annotated with the gene name. (*D*) Cumulative odds scores grouped by EBV protein for DRB5*01:01 versus *01:02 alleles. (*E*) The cumulative EBV glycoprotein B (gB) score for 131 HLA-DRB alleles with available peptide predictions. Alleles are displayed numerically from left to right, and the top seven alleles are annotated.

CD4 T cells and SUDHL-4 cells, demonstrating that SUDHL-4 cells could substitute for APCs for either antigen (Fig. 2 *I* and *J*).

Protein Expression in APCs Is Sufficient for Activating gB-Reactive CD4 T Cells but Not Triplex Capsid Protein-Reactive T Cells and Depends on Entry into the Endoplasmic Reticulum. Given that the number of EBV-infected cells in chronically infected individuals is exceedingly low, a sufficient level of circulating gB antigen is not likely to be present for uptake by rare gB-specific B cells. We tested the more likely explanation that EBV-infected B cells could present DR15-restricted antigens via an alternative mechanism that loads intracellular viral proteins like gB onto HLA class II molecules. As DR15 individuals could respond to both *BORF1* and *BALF4*-encoded peptides, and SUDHL-4 cells could present both antigens, we generated several constructs for expressing recombinant *BALF4* and *BORF1* variants (Fig. 3 *A* and *B*). We validated the expression of each construct in electroporated SUDHL-4 cells by Western blot and the efficiency by flow cytometry (Fig. 3 *C*–*F* and *SI Appendix*, Figs. S4, S6).

When we expressed *BALF4* in SUDHL-4 cells, we observed direct activation of CD4 T cells (Fig. 3 *G* and *H*). The supernatant from these cells did not contain a source of antigen to explain this

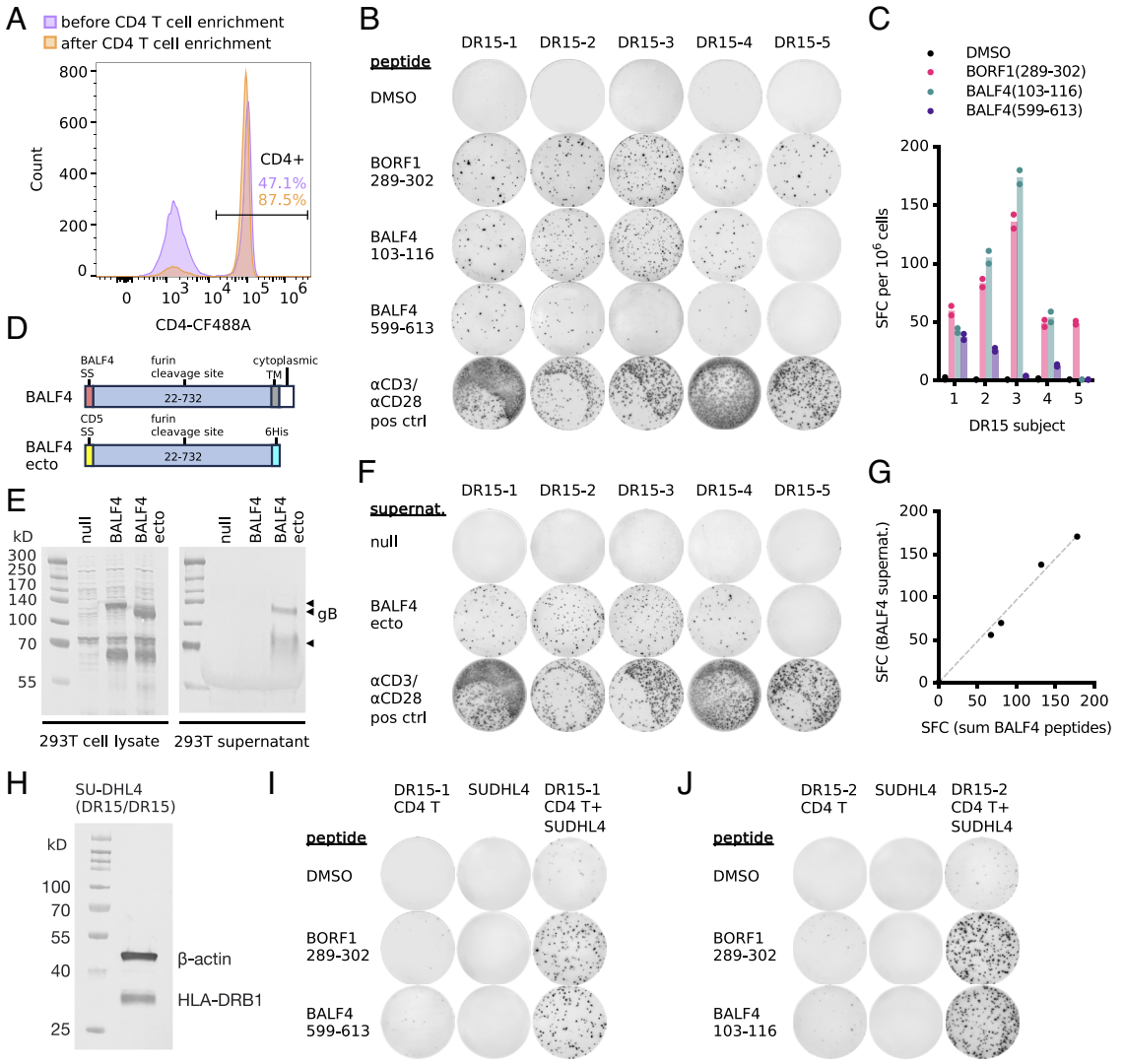

Fig. 2. Predicted gB peptides demonstrate CD4 T cell responses in individuals with DR15 and are naturally processed from recombinant gB protein. (*A*) Flow cytometric evaluation for CD4 enrichment of total CD3 T cells after magnetic bead-based depletion of cells expressing CD8, CD56, CD19, CD123, CD235a, and TCRγδ from whole PBMCs. (*B*) ELISPOT assays for IFNγ using 1 × 10^6 CD4-enriched PBMCs from five DR15-heterozygous donors. Responses were evaluated to triplex capsid antigen (*BORF1*) 289–302 and to the predicted gB (*BALF4*) peptides (a.a. 103–116 and 599–613). CD3/CD28 beads were used as a positive control on an aliquot of remaining cells. Assays were run in duplicate, and representative images are shown. (*C*) Quantification of part (*B*). (*D*) Schematic of the coding region of the gB full-length protein (*BALF4*) showing the signal sequence (SS) in red, extracellular domain in blue, transmembrane domain (TM) in gray and cytoplasmic domain in white. A second construct encoding the ectodomain is shown below with a C-terminal His-tag and a CD5 signal sequence (*BALF4*-ecto). (*E*) Western blot analysis of HEK293T cell lysates and supernatant collected 72 h. after transfection with pcDNA3.1-null, *BALF4*, or *BALF4*-ecto vectors. Blots were probed with a rabbit polyclonal antibody raised against gB. Arrows mark full-length and furin-cleaved gB. (*F*) ELISPOT assays for IFNγ as in part *B* using supernatants collected from the null and *BALF4*-ecto transfected HEK293T cells from part (*E*). (*G*) Quantification of mean responses from *F* versus *B*. Each point represents one individual. (*H*) Western blot of cell lysate from the DR15-homozygous B cell line SUDHL-4 probed with an anti-DRB1 antibody. Β-actin was used as a loading control. (*I*) ELISPOT assays for IFNγ using freshly isolated CD4 T cells from donor DR15-1 alone, SUDHL-4 cells alone, or both mixed with DMSO or peptides, as indicated. All assays were run in duplicate, and representative images are shown. (*J*) ELISPOT assays for IFNγ, as in part *I*, using CD4 T cells prepared from donor DR15-2.

activity (*SI Appendix*, Fig. S7), suggesting that the mechanism was endogenously mediated. This process was not affected by deleting the transmembrane domain or by the addition of an influenza hemagglutinin (HA) tag, indicating that trafficking to the membrane was not necessary (Fig. 3 *G* and *H*). However, the ability to activate CD4 T cells was abrogated when the signal sequence was deleted from *BALF4*, implying that translocation to the ER was required (Fig. 3 *G* and *H*). Since *BORF1* encodes a cytoplasmic protein that does not traffic to the ER, we were curious whether the expression of *BORF1* could activate CD4 T cells as well. Consistent with a pathway dependent on the ER, the expression of *BORF1* was insufficient to activate CD4 T cells (Fig. 3 *G* and *H*). Adding the region encoding the signal sequence from *BALF4* to drive the *BORF1* capsid protein to the ER led to the activation of CD4 T cells (Fig. 3 *G* and *H*). While this process was more efficient when we added a LAMP1 sorting signal to direct the *BORF1* capsid protein to the endosome (33), it was still possible to directly activate CD4 T cells without this step (Fig. 3 *G* and *H*). We also used primary autologous B cells to rule out the possibility that SUDHL-4 cells were abnormal in their ability to mediate endogenous antigen presentation on HLA class II (Fig. 3*I* and *SI Appendix*, Fig. S5). Expression of *BALF4* in primary B cells was sufficient to activate autologous CD4 T cells, indicating that this process occurs in physiologic lymphocytes (Fig. 3*I*).

The ER-Resident Domains of gB and gH Elicit Higher Immune Responses in DR15-Homozygous Individuals Than in Non-DR15 Individuals. We then generated a list of EBV antigens that are known to traffic to the ER, including transmembrane and secreted proteins (Fig. 4*A*). This list was compiled by including the 13

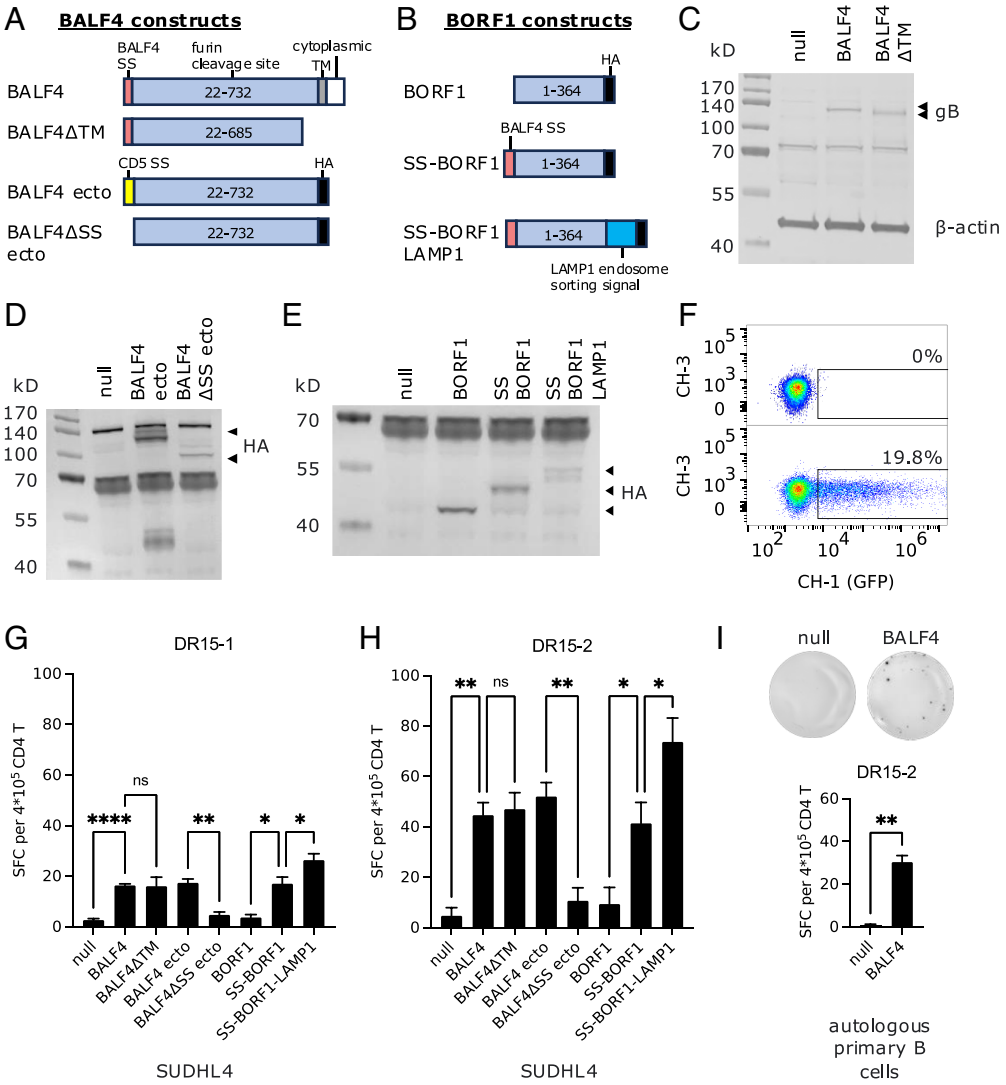

Fig. 3. Protein expression in APCs is sufficient for activating gB-reactive CD4 T cells but not triplex capsid protein-reactive T cells and depends on entry into the endoplasmic reticulum. (*A*) Schematic of constructs encoding the coding region of full-length gB (*BALF4*), truncated gB missing the transmembrane domain (*BALF4*ΔTM), a C-terminal HA-tagged gB ectodomain with a CD5 signal sequence (*BALF4*-ecto), and without a signal sequence (*BALF4*-ectoΔSS). These constructs were designed for insertion into pcDNA3.1 vector for expression in mammalian cells. (*B*) As in *A*, schematic of the coding region of the triplex capsid subunit (*BORF1*) full-length protein with a C-terminal HA tag, with the addition of the *BALF4* signal sequence (SS-*BORF1*), and with a C-terminal human LAMP1 endosome sorting signal (SS-*BORF1*-LAMP1). (*C*) Western blot of SUDHL-4 cell lysates from cells electroporated with null, *BALF4*, or *BALF*ΔTM plasmids. Cells were harvested 16 h after electroporation, and blots were probed with an anti-gB polyclonal antibody (indicated by arrows). B-actin was used as a loading control. (*D*) Western blot as in *C* using plasmids encoding null, *BALF4*-ecto, or *BALF4*-ectoΔSS constructs. Blots were probed with an anti-HA antibody. (*E*) Western blot as in *C* for plasmids encoding null, *BORF1*, SS-*BORF1*, or SS-*BORF1*-LAMP1 constructs. Blots were probed with an anti-HA antibody. (*F*) Flow cytometric evaluation of electroporation efficiency for SUDHL-4 cells expressing GFP (channel 1) 16 h after electroporation with a pcDNA3.1-GFP vector versus nonspecific channel 3. (*G*) Quantification of ELISPOT assays for IFNγ using freshly isolated CD4 T cells from donor DR15-1 in combination with SUDHL-4 cells 16 h after electroporation with each construct as indicated. Each bar represents the mean and SD obtained from three independent experiments. One-way ANOVA ($P < 0.05$) was followed by multiple hypothesis testing between the five groups indicated. Statistical significance is highlighted by *P* values: $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, ns: nonsignificant. (*H*) Quantification of ELISPOT and analysis as in *G* using freshly isolated CD4 T cells from donor DR15-2. (*I*) Quantification of ELISPOT assays for IFNγ using freshly isolated CD4 T cells from donor DR15-2 in combination with autologous primary B cells 16 h after electroporation with pcDNA3.1-null or *BALF4* plasmid. Each bar represents the mean and SD obtained from three independent experiments. *P* values highlight statistical significance: $^{**}P < 0.01$.

known EBV-encoded glycoproteins (34), two latent membrane proteins, and the secreted viral IL-10. We selected seven proteins that contained significant intra-ER regions for further study as we hypothesized that these regions would be accessible for HLA class II loading. We used recombinant C-terminally His-tagged ectodomains to screen for T cell responses to each antigen, validated by Western blot (Fig. 4*B*). We attempted to make all proteins recombinantly but could not detect the expression of gp78, gp150, or gN. Furthermore, we did not select the viral IL-10 as this protein shares extensive amino acid homology with human IL-10 (35). To compare the DR15-specific immune responses to each antigen, we collected blood from eight healthy participants who were DR15-null, ten healthy participants who were DR15-homozygous, and eight participants who were DR15-homozygous and had a diagnosis of RRMS, all of whom were EBV positive (Table 1). All participants were typed for HLA-DRB1 by sequence-based typing and the alleles are listed in *SI Appendix*, Table S2. We then assayed responses by ELISPOT for IFNγ using CD8-depleted PBMCs. As expected, 0/8 of the DR15-null healthy participants responded to gB above baseline (Fig. 4*C*). In comparison, DR15-homozygous healthy individuals had significantly higher responses to gB, with 9/10 individuals showing responses above baseline (Fig. 4*C*). We also observed DR15-dependent responses to gH, where 8/10 individuals reacted versus 0/8 in the DR15-null group (Fig. 4*C*). The gH epitope(s) were mapped to two dominant epitopes also restricted

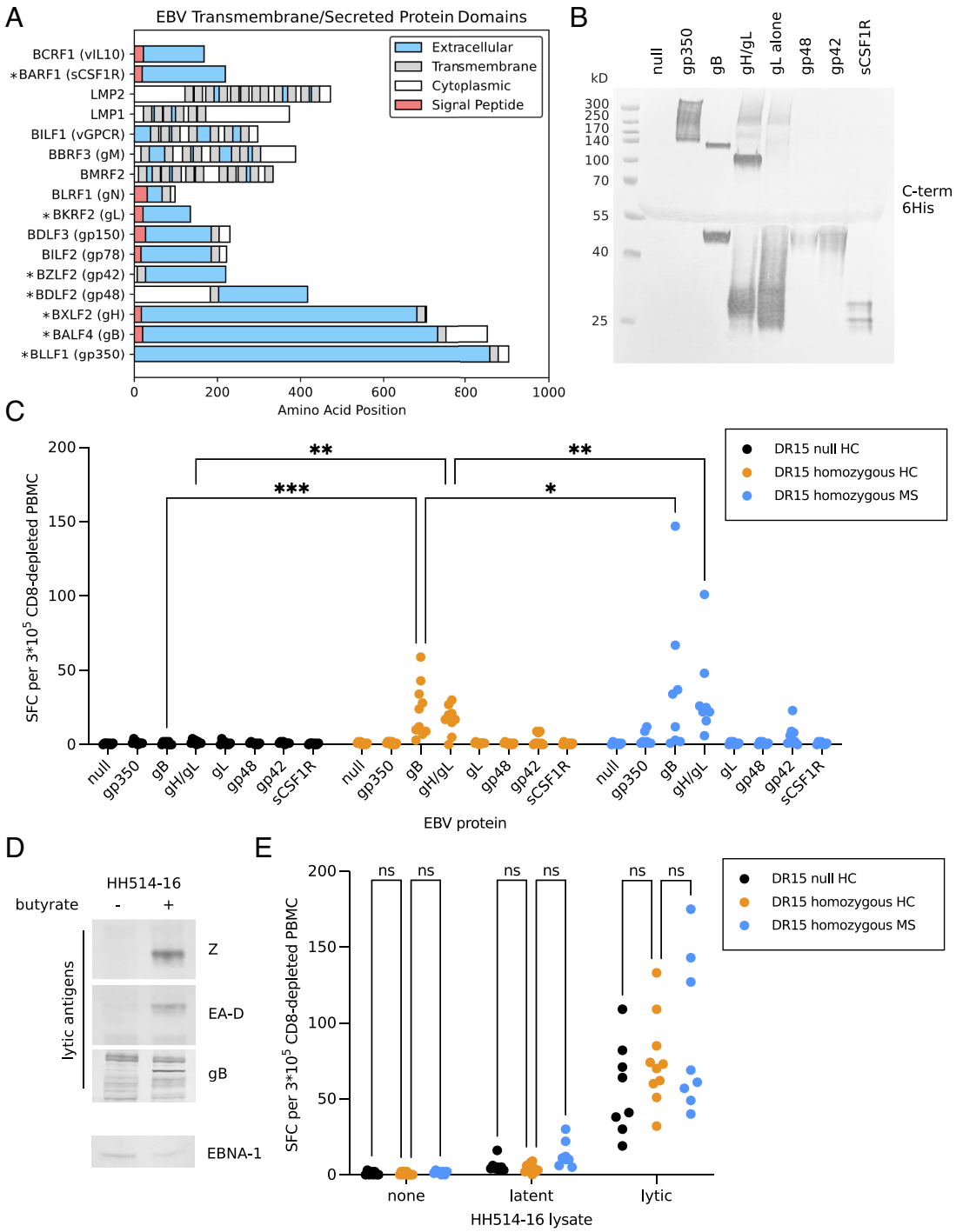

Fig. 4. The ER-resident domains of gB and gH elicit higher immune responses in DR15-homozygous individuals than in non-DR15 individuals. (*A*) Schematic of the EBV-encoded transmembrane and secreted proteins that can access the ER compartment. Signal peptides are shown in red, extracellular domains (intra-ER) in blue, cytoplasmic domains in white, and transmembrane regions in gray. Proteins marked with an asterisk were selected for recombinant ectodomain production in HEK-293T cells. (*B*) Western blot of the seven C-His tagged recombinant protein ectodomains selected from part A produced in HEK-293T cells. Blots were probed with an antibody recognizing the C-terminal His tag. (*C*) Quantification of ELISPOT assays for IFNγ using CD8-depleted PBMCs from 8 DR15-null healthy donors, 10 DR15-homozygous healthy donors, and 8 DR15-homozygous donors with MS. Responses to each recombinant EBV antigen were assayed after 18 h. Two-way ANOVA ($P < 0.05$) was followed by comparing responses between either healthy controls (DR15 null versus DR15 homozygous) or DR15 homozygotes (healthy controls versus MS) without correction for multiple hypothesis testing. Those reaching statistical significance are displayed and highlighted by *P* values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (*D*) Western blot analysis of HH514-16 cell lysates from latent or lytic cells induced with 3 mM sodium butyrate for 72 h. Blots were probed with EBV ZEBRA (Z), EA-D, and gB antibodies. (*E*) Quantification ELISPOT assays as in *C* using lysates from part (*D*). Responses to either media alone, latent antigens, or lytic antigens from HH514-16 cell lysates were assayed after 18 h. Two-way ANOVA ($P < 0.05$) was followed by comparing specific groups of interest indicated on graph, without correction for multiple hypothesis testing; ns: nonsignificant.

to DRB1*15:01 (*SI Appendix*, Fig. S8). While responses were slightly higher in patients with MS, these could not be uniquely distinguished from CD4 T cell responses of healthy HLA-matched controls (Fig. 4*C*). This finding suggests that the identity of the EBV antigens that generate a CD4 T cell immune response is dictated by the presence of DR15 but is not specific to MS.

Table 1. Characteristics of DR15-null and homozygous participants

	DR15 null healthy controls	DR15 homozygous healthy controls	DR15 homozygous RRMS
Number of participants	8	10	8
Age	35.1 ± 9.4	38.6 ± 7.3	49.5 ± 15.5
Sex (M:F)	6:2	1:9	1:7
History of mononucleosis	2	1	4
Family history of MS	0	0	2
EBNA1 IgG+	8	10	8
VCA IgG+	8	10	8
Treatment: anti-CD20	N/A	N/A	4
Treatment: natalizumab	N/A	N/A	1
Treatment: none	N/A	N/A	3

Finally, we wanted to determine whether DR15 homozygotes had higher responses to EBV in general. We prepared lysates from the human EBV-positive cell line HH514-16, which can be induced by butyrate to express both early and late lytic antigens (Fig. 4*D*). Using a mixture of antigens derived from either latent or lytic HH514-16 cells, we did not observe a difference in the magnitude of T cell responses between DR15-null and DR15-homozygous healthy controls (Fig. 4*E*). Similarly, we did not observe a difference between DR15-homozygous healthy controls and HLA-matched patients with MS (Fig. 4*E*). In all groups, the T cell response was heavily skewed toward lytic antigens instead of latent antigens (Fig. 4*E*), suggesting that lytic antigens are the dominant targets of CD4 T cells in chronically infected carriers. These results indicate that the DR15-dependent response to EBV is limited to specific lytic epitopes, including from gB and gH.

A Proposed Model for Nonclassical Direct Activation of CD4 T Cells by EBV-Infected B Cells Acting as APCs. In the classical immunological model for CD4 T cell activation by B cells (Fig. 5*A*), an exogenous antigen is internalized from the extracellular space. Given our findings, we propose a direct activation model of DR15-restricted CD4 T cells where an EBV antigen (gB) is endogenously loaded onto HLA class II (Fig. 5*B*). While the classical model requires B cell receptor (BCR) specificity for a given antigen (Fig. 5*A*), the specificity of the BCR is not important if the antigen is instead concentrated by intracellular production (Fig. 5*B*). In this alternative model, providing CD4 T cell help to EBV-expressing B cells could produce antibodies to independent antigens of any specificity (Fig. 5*B*). This may explain why oligoclonal bands in the CSF of people with MS are often directed against seemingly random targets (Fig. 5*B*).

Discussion

Antigen presentation of a subset of EBV proteins may explain the association between MS and HLA-DR15. Our study indicates that in EBV-infected individuals with DR15, CD4 T cell responses are frequently directed to two late lytic EBV glycoproteins—gB and gH. Functionally, these CD4 T cells are special because they are predicted to recognize EBV-infected B cells directly after lytic reactivation.

Mechanistically, EBV-reactive CD4 T cells in MS may be driving the inappropriate licensing of myelinolytic CD8 T cells that

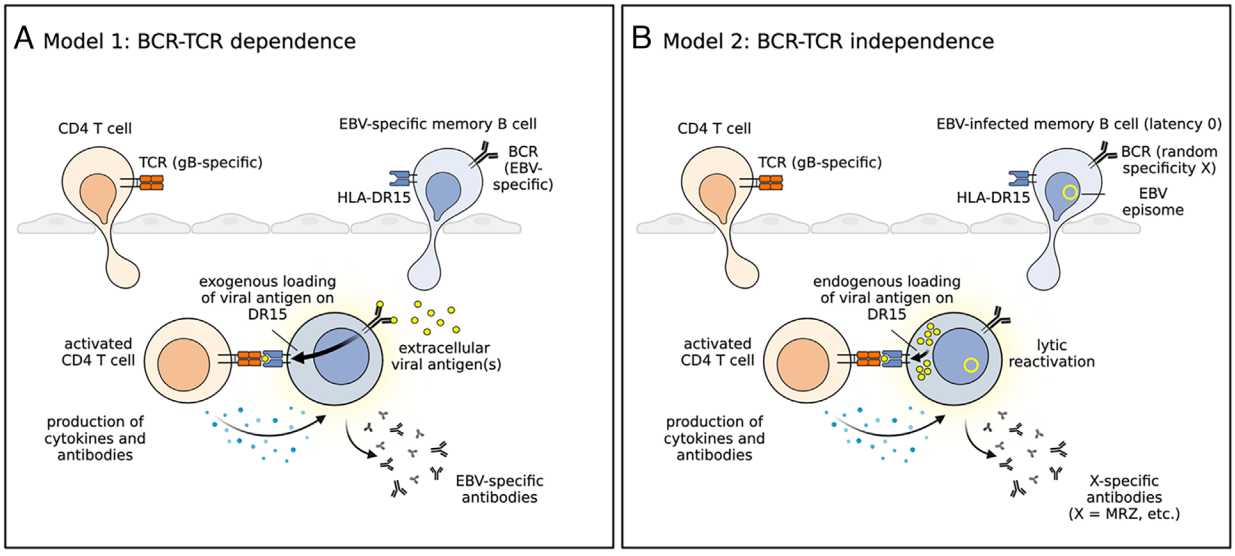

Fig. 5. A proposed model for nonclassical direct activation of CD4 T cells by EBV-infected B cells acting as APCs. (*A*) In the classical model, memory B cells can internalize gB from the extracellular space via their BCR, with subsequent processing and presentation of a gB peptide on HLA-DR15. CD4 T cells would then recognize the antigen via a gB peptide-specific TCR and activate the B cell for plasma cell differentiation to produce an EBV-specific antibody. (*B*) In a nonclassical model, CD4 T cells can be activated directly by EBV-infected B cells endogenously expressing gB—for example, during lytic reactivation from latency. In this model, the antigen would be endogenously loaded onto HLA-DR15, breaking the TCR's dependence on the BCR. Memory B cells receiving CD4 T cell help may instead produce a random antibody with specificity X, which matches the BCR of the EBV-infected B cell. One such possibility for X would be MRZ (measles—M, rubella—R, or varicella zoster—Z), one of the most specific tests for MS.

infiltrate the CNS, just like gluten-reactive CD4 T cells license enterocyte killing by intraepithelial lymphocytes in CeD (21, 23). While these interactions are not necessarily based on mimicry, they still lead to cell type-specific targeting. Unbiased single-cell profiling of the CSF compartment previously demonstrated that patients with MS have a unique CNS-restricted subcluster of CD4 T cells (36). The antigenic specificity of this cluster should be characterized in detail to determine whether these cells recognize latent or lytic EBV antigens endogenously presented on HLA class II molecules. While our studies did not find a signature for DR15-restricted EBV-reactive T cells in peripheral blood specific to MS, we cannot rule out the possibility that these T cells are uniquely present in the CSF of patients with MS. Furthermore, our results comparing patients with MS to healthy controls are limited by the fact that most patients were on immunosuppressive therapies.

Given our findings, we suggest that having the DR15 allele increases the probability of generating CD4 T cell responses to EBV antigens that can be directly recognized when endogenously synthesized in B cells. The specificity of the EBV response in MS may derive from an EBV antigen in the CNS. One possibility for how this could occur is if EBV selectively reactivates when B cells traffic to the CNS in patients with MS but not in controls. CSF TCR profiling studies support this hypothesis, as nearly all EBV-reactive T cells in MS CSF were found to recognize lytic antigens and demonstrated recent activation (6). The possibility of endogenous antigen presentation may explain why the antigenic targets of oligoclonal bands—a hallmark of MS—have been elusive for decades (37). Treatment studies with natalizumab indicate that T cells in the CNS are essential for maintaining oligoclonal bands, suggesting that B cells likely receive co-stimulatory signals from CD4 T cells (38). If this occurs upon recognition of an endogenous EBV antigen presented on HLA class II, the resulting antibody response would be predicted to be both clonal since primary EBV infection is predominantly a lymphoproliferative process—and seemingly random. Since EBV may be eliminated from B cells after plasma cell differentiation and proliferation (39, 40), testing this hypothesis would involve examining whether the BCR sequences of latently infected peripheral blood B cells match the sequences of oligoclonal bands in the CNS of patients with MS.

Our studies suggest two approaches to therapies for MS. One possibility is antigen-specific deletion of EBV-reactive CD4 T cells that recognize reactivated B cells. Methods for achieving this goal have previously been discussed in the context of CeD for removing gluten-reactive T cells (41). These could include toxin-conjugated tetramers or immunization with subsequent depletion of cells expressing an activation marker like CD40L. A broader approach would be to use antivirals targeting the EBV DNA polymerase, as these block the expression of late lytic viral antigens that depend on the viral preinitiation complex for transcription (42). As part of this subset, gB, gH, and nearly all viral proteins transported to the ER are strongly suppressed by antivirals (34). Interestingly, a recent case series of patients with MS suggested that prodrugs of tenofovir, an antiviral targeting the EBV DNA polymerase, may help control disease activity (42, 43). Mechanistically, antiviral therapies could act on EBV-infected B cells by blocking the presentation of specific pathogenic antigens to CD4 T cells. The number or activation profile of these CD4 T cells could serve as a biomarker to establish the therapeutic efficacy of antivirals in clinical trials.

One important limitation of our study is that cellular processes for loading HLA class II may be more complex during lytic reactivation than the expression of single antigens in EBV-negative cells. While we observed that antigen presentation of gB was dependent on trafficking to the ER, other cellular pathways intersect with internal routes for HLA class II loading. In particular, EBNA-1 has been reported to be presented on HLA class II by autophagy (28), and several viral capsid scaffold proteins interact with autophagic membranes to facilitate replication (44). The subset of EBV antigens that can be endogenously loaded onto HLA class II, specifically DR15, should be defined in infected B cells during latency and lytic reactivation by biochemical means.

In summary, our findings enable the rational design of antigen-specific immunological studies and therapies to understand better the role of EBV antigen presentation in patients with MS. Detailed phenotyping of DR15-restricted CD4 T cells in the CSF of patients with MS during active disease should follow. Furthermore, clinical studies are warranted to decrease either EBV antigen production or antigen-specific CD4 T cells.

Methods

Detailed methods are described in *SI Appendix*.

Blood Collection and Processing from Study Participants. Blood was collected from study participants at Mass General Brigham centers under the approved IRB protocols 2023P000922 and 2023P002060. Healthy controls were selected using the Mass General Brigham Biobank, a large-scale research initiative designed to collect genetic information alongside health information from participants. For healthy DR15 homozygotes, we initially searched for the presence of the SNP rs3135388 A/A. This prescreening step was required as only ~1% of the general population is homozygous for DR15. We then searched for subjects in the highest Charlson Comorbidity Index 10-y Survival Probability category as classified by the Mass General Brigham Biobank. Subjects who met these two criteria were asked to participate in the study, and eight subjects were recruited in this way. One subject also had a healthy identical twin who chose to participate in the study. The tenth subject had rs3135388 G/G but was of Japanese descent and was included after sequence-based HLA typing. DR15 negative healthy controls were selected from the general population, and HLA was ascertained by sequence-based typing. Similarly, patients with relapsing-remitting MS were recruited from the Mass General Brigham MS clinic and screened for the SNP rs3135388 with validation by sequence-based typing for those with genotype A/A. All study participants were informed about the research and consented before blood collection. The blood samples were processed within 8 h of collection. Peripheral blood mononuclear cells (PBMCs) were isolated from 60 to 80 mL whole blood collected in Acid Citrate Dextrose tubes by density gradient centrifugation using SepMate-50 PBMC isolation tubes (Stemcell Technologies) according to the manufacturer's instructions. PBMCs were cryopreserved in AIM V medium with AlbuMAX supplement (Thermo Fisher) containing 10% DMSO and stored at −140 °C. Serum was isolated from 4 mL blood collected in SST tubes by centrifugation, then stored at −80 °C. Demographic information for study participants is listed in Table 1.

Cell Lines and Maintenance in Culture. The SUDHL-4 cell line was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, where it was previously HLA typed for DRB1. The HH514-16 Burkitt lymphoma and B95-8 cell lines used in this study were a kind gift from Dr. George Miller. All suspension cells were maintained in RPMI-10% FBS with penicillin (100 U/mL), streptomycin (100 μg/mL), and Amphotericin-B (1 μg/mL). HEK-293T cells were obtained from ATCC and maintained in DMEM-10% FBS supplemented with penicillin and streptomycin.

Data, Materials, and Software Availability. All study data are included in the manuscript and/or *SI Appendix*.

Author affiliations: [a]Department of Neurology, Division of Neuroimmunology & Neuroinfectious Disease, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114; and [b]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

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