AUTOIMMUNITY

CD4$^+$ T cells reactive to Epstein-Barr virus late lytic antigens are enriched in individuals with multiple sclerosis

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Multiple sclerosis (MS) pathogenesis is linked to Epstein-Barr virus (EBV), but the underlying immune mechanisms remain unclear. Using an optimized T cell assay, we demonstrate that CD4$^+$ T cells from individuals with MS predominantly target EBV viral particle components, specifically the late lytic capsid and glycoprotein antigens, rather than latent antigens. In contrast, the Epstein-Barr nuclear antigen 1 (EBNA1) primarily activated CD8$^+$ T cells. EBV-specific CD4$^+$ T cell responses were twofold higher in individuals with untreated MS compared with healthy controls, whereas responses to other herpesviruses remained similar. Anti-CD20 therapy initiation in treatment-naïve participants reduced these responses, a finding validated in an independent cohort, and eliminated viral shedding in saliva. Our results establish preferential CD4$^+$ T cell reactivity to EBV late lytic antigens as a key feature of MS, providing a framework for developing EBV-targeted therapies, including vaccines and antivirals.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) and the leading cause of neurological disability in young adults (*1*). As the incidence of MS continues to rise, a better understanding of its etiology and novel therapeutic approaches are needed (*2*). Epidemiological studies have provided compelling evidence establishing Epstein-Barr virus (EBV) as a key environmental risk factor in MS development (*3*, *4*). Nearly all individuals with MS (99.9%) are seropositive for EBV (*4*), the risk in EBV-seronegative individuals approaches zero (*5*), and critically, EBV infection precedes both MS onset and CNS damage, supporting a causal relationship (*3*).

CD4$^+$ T cell–mediated immune responses are central contributors to MS pathogenesis (*6*, *7*). Such T cells are found in elevated numbers in the cerebrospinal fluid (CSF) of individuals with MS (*8*) and are present during early acute demyelinating lesion formation (*9*). CD4$^+$ T cells mediate their effects partly through the CD40-CD40L pathway, which enables them to provide help to other immune cells. The therapeutic relevance of this pathway in MS was demonstrated by frexalimab, a nondepleting anti-CD40L antibody that reduced gadolinium-enhancing lesions in a phase 2 trial (*10*). Although CD40L is expressed by multiple immune cell types and therapeutic benefit may partly arise from effects on B cells or other antigen-presenting cells, this pathway is central to CD4$^+$ T cell–dependent immune responses.

Multiple lines of evidence implicate T cell responses to EBV in MS pathogenesis. Although direct detection of EBV in the CNS remains challenging because of the low abundance of infected cells (*11*–*13*), T cells capable of recognizing EBV antigens have been found in the CNS of individuals with MS (*14*–*16*). Genetic evidence points specifically to CD4$^+$ T cells: The human leukocyte antigen (HLA) class II molecule DR15 (*DRA/DRB1*15:01*), which presents peptide antigens to CD4$^+$ T cells, is the major risk factor for the disease (*17*–*19*), and MS-associated genetic loci that are regulated by EBV transcription factors specifically disrupt the CD40-CD40L pathway (*20*). The critical role of peripheral CD4$^+$ T cells in driving organ-specific inflammation is well established in other autoimmune conditions, most notably celiac disease, which serves as a compelling model for understanding MS (*7*). Together, these findings suggest that identifying the major EBV antigens recognized by CD4$^+$ T cells may shed light on MS pathogenesis. The efficacy of frexalimab, despite the limited penetrance of monoclonal antibodies across the blood-brain barrier, suggests that modulating peripheral T cell responses can affect CNS inflammation, potentially by preventing the activation and migration of pathogenic T cells before they enter the CNS.

The precise targets of peripheral EBV-specific CD4$^+$ T cells remain debated, with some studies reporting broad reactivity across lytic and latent antigens (*21*–*23*) and others showing preferential recognition of lytic antigens (*24*–*26*). Studies using lymphoblastoid cell lines to stimulate CD4$^+$ T cells have yielded mixed results in individuals with MS, likely complicated by the heterogeneous permissiveness for lytic reactivation across different lymphoblastoid cell line clones (*14*, *27*–*29*). Much of the field has focused on EBNA1 (Epstein-Barr nuclear antigen 1), a latent viral protein that demonstrates cross-reactivity with several CNS antigens (*30*–*35*). Less attention has been given to lytic antigens, despite evidence that individuals carrying the major MS genetic risk allele *DRB1*15:01* respond strongly to two

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EBV virion glycoproteins (*24*). In addition, B cells from individuals with MS show heightened spontaneous lytic reactivation (*28*). More broadly, studies of other viruses have shown that antigens derived from viral particles serve as potent and selective stimulators of $CD4^+$ T cells (*36*).

Current disease-modifying therapies (DMTs) for MS modulate immune responses through diverse mechanisms, with several affecting memory B cells, the primary EBV reservoir (*37*). Anti-CD20 therapies, dimethyl fumarate (DMF), and teriflunomide have all been shown to reduce EBV-specific cellular immune responses, potentially by depleting infected cells or modulating antigen presentation (*38*, *39*). Emerging therapeutic strategies under investigation include Bruton's tyrosine kinase inhibitors, which target B cell signaling pathways essential for EBV persistence and reactivation, and direct antiviral agents (*40*–*43*). Nevertheless, how these interventions, both approved DMTs and investigational approaches, affect EBV-specific cellular immunity remains incompletely characterized. A deeper understanding of the $CD4^+$ T cell response to specific EBV antigens could clarify whether future therapeutic agents require CNS penetrance for efficacy and identify which viral epitopes should be prioritized as targets for antiviral or immunomodulatory interventions.

Using enzyme-linked immunospot (ELISPOT) assays across healthy controls (HCs), participants with treatment-naïve MS, and participants with MS receiving DMTs, we aimed to identify which viral proteins elicit these responses, how DMTs affect EBV-specific immunity, and whether responses correlate with viral shedding.

RESULTS

$CD4^+$ T cell responses to EBV lytic antigens are elevated in individuals with MS

We generated latent and lytic lysates from the B95.8 cell line using phorbol 12-myristate 13-acetate (PMA) (Fig. 1A). Intact protein complexes were purified by size exclusion chromatography, and antigen expression was confirmed by immunoblot (Fig. 1B). Latent lysates expressed EBNA1, whereas lytic lysates also expressed early lytic antigens (Z and EA-D) and late lytic antigens (gp350 and viral capsid antigen VCA p18) (Fig. 1B).

Using an interferon-γ (IFN-γ) ELISPOT assay, we observed that lytic lysates induced significantly higher numbers of spot-forming cells (SFCs) compared with latent lysates in both HCs ($P < 0.0001$) and participants with MS ($P < 0.0001$) (Fig. 1, C and D). This

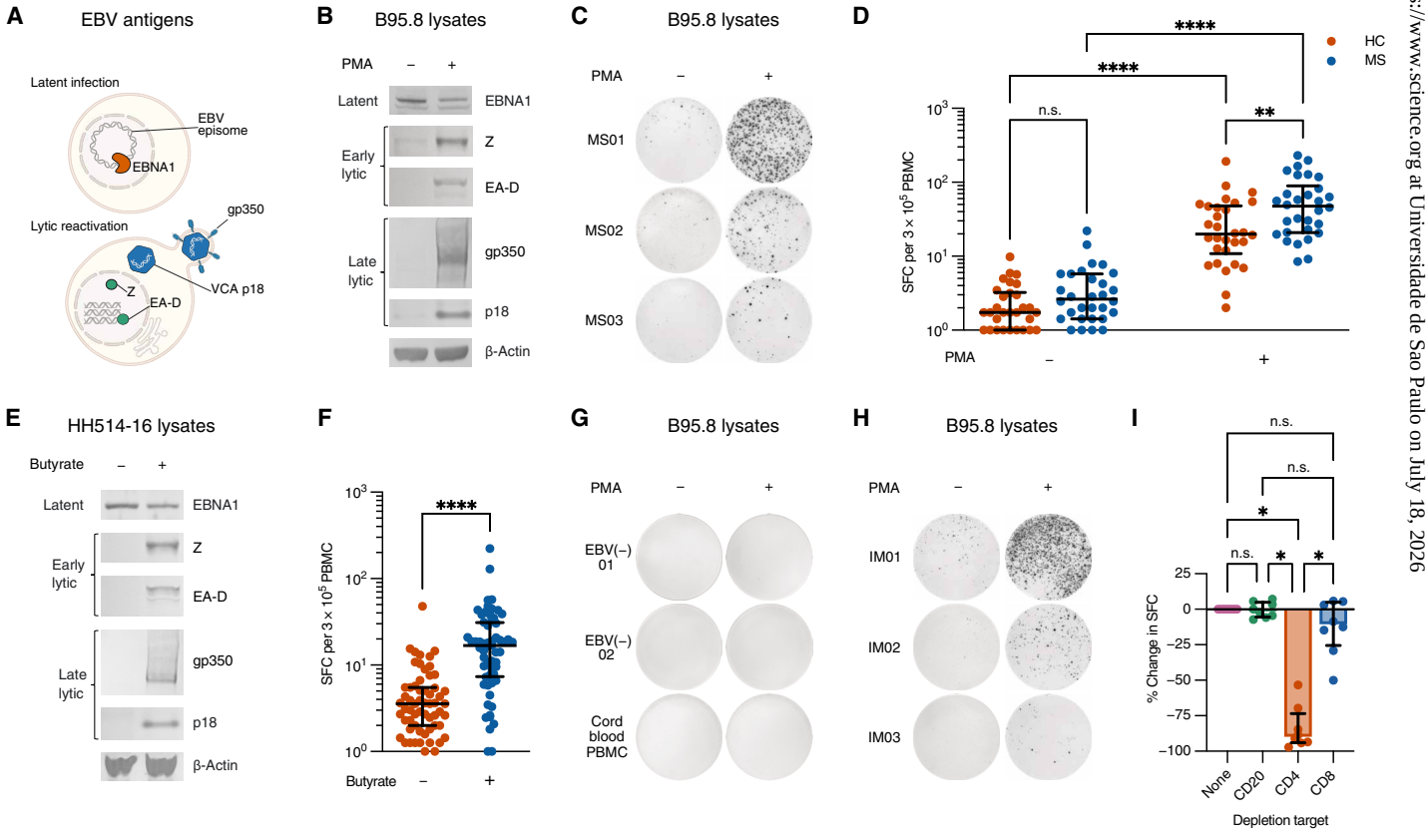

Fig. 1. $CD4^+$ T cell responses to EBV lytic antigens are elevated in individuals with MS. (**A**) Schematic of B95.8 antigens classified as latent or lytic. (**B**) Immunoblots of B95.8 lysates without (PMA−) or with (PMA+) lytic induction. (**C**) Representative IFN-γ ELISPOT wells from three participants with MS (MS01 to MS03) stimulated with B95.8 lysates. (**D**) SFCs per 3×10^5 PBMCs comparing responses to PMA− and PMA+ lysates in HCs ($n = 30$) and untreated participants with MS ($n = 30$, discovery cohort). Each data point represents the mean of two technical replicates. (**E**) Immunoblots of HH514-16 lysates without (butyrate−) or with (butyrate+) lytic induction. (**F**) SFCs comparing responses to butyrate− and butyrate+ HH514-16 lysates ($n = 60$ participants with MS, testing cohort). (**G**) ELISPOT assays from two EBV-seronegative individuals and one cord blood sample. (**H**) ELISPOT assays from three participants with infectious mononucleosis (IM01 to IM03). (**I**) Percentage changes in SFCs after CD20, CD4, or CD8 depletion ($n = 8$, discovery cohort). Data are presented as medians ± IQR. Data were analyzed by the linear mixed effects model with the Bonferroni adjustment (D), paired Student's *t* test (F), or Friedman test with pairwise Wilcoxon signed-rank tests and Bonferroni adjustment (I); $^*P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$; n.s., not significant.

response to lytic antigens was significantly elevated in participants with untreated MS, who demonstrated ~2-fold higher T cell reactivity compared with HCs ($P = 0.0035$) (Fig. 1D). Given the role of T helper 17 (T_H17) cells in MS, we also performed interleukin-17A (IL-17A) ELISPOT assays. We did not observe IL-17A responses to EBV lysates in either participants with MS or HCs (fig. S1), in line with previous studies examining $CD4^+$ T cell responses to cytomegalovirus (CMV) and human herpesvirus 6 (*44*, *45*). This suggests that the dominant, measurable response to these antigens is T_H1-mediated.

We validated this finding in our independent testing cohort using HH514-16 cells, an EBV-positive cell line that does not require PMA for lytic induction, confirming that the preferential response to lytic antigens is not an artifact of PMA stimulation (Fig. 1, E and F). The assay's specificity was confirmed using peripheral blood mononuclear cells (PBMCs) from two EBV-seronegative individuals and one donor's cord blood mononuclear cells, all of which showed no response to either lysate (Fig. 1G). We also tested three participants with infectious mononucleosis (IM) to assess responses during primary infection. These individuals exhibited a similar pattern of high-magnitude, preferential T cell reactivity to lytic antigens (Fig. 1H), with responses of nearly identical magnitude to those observed in participants with MS (Fig. 1, C and H). This aligns with previous studies reporting that the set point of response to lytic antigens is established during primary infection and maintained thereafter (*21*). Clinical characteristics for these control individuals are detailed in fig. S2.

To define the T cell subset mediating this dominant lytic response, we performed selective cell depletion experiments. Our depletion strategy was validated using known HLA class II– and class I–restricted EBV peptides (fig. S3 and tables S1 and S2). Depletion of $CD4^+$ T cells reduced the response to lytic B95.8 lysates by a median [interquartile range (IQR)] of 90% (73%, 94%) ($P = 0.047$), whereas depletion of $CD8^+$ T cells did not significantly reduce the response ($P = 0.66$) (Fig. 1I). Depletion of $CD20^+$ B cells did not affect the response (Fig. 1I), confirming that antigen presentation by B cells was not required. These findings demonstrate that $CD4^+$ T cells are the predominant population recognizing the antigens in lytic lysates in participants with MS. To further validate our findings, we quantified IFN-γ^+ $CD4^+$ T cells by flow cytometry (fig. S4A), and we observed a strong correlation with our ELISPOT results (fig. S4B). Control experiments confirmed that residual detergent from lysate preparation did not affect T cell responses (fig. S5).

EBNA1 induces lower $CD4^+$ T cell responses than total EBV lytic antigens and primarily activates $CD8^+$ T cells in individuals with MS

B95.8 lysates contain both latent and lytic antigens. Even without PMA induction, B95.8 cells are leaky and express some lytic antigens, making it difficult to determine whether the low-level responses to PMA− lysates (Fig. 1D) were driven by latent antigens or residual lytic antigen expression. To isolate the response to latent antigens, we tested lysates from Raji cells, which express latent antigens [EBNA1, EBNA2, EBNA3A, EBNA3B, and latent membrane protein 1 (LMP1)] but cannot complete the lytic cycle (Fig. 2A). Raji cell lysates, when tested at the same protein concentration matched to B95.8 lytic lysates (1:320), elicited minimal T cell responses in participants with MS (Fig. 2, B and C), consistent with latent antigens being poor drivers of $CD4^+$ T cell responses. We did not test higher concentrations because control experiments demonstrated that residual PMA causes nonspecific T cell activation at concentrations above 1:160 (fig. S6). To assess EBNA1-specific responses directly, we compared responses to Raji lysates versus EBNA1 peptides but observed no correlation (Fig. 2D). EBNA1 is of particular interest because this antigen has been frequently implicated in MS pathogenesis because of demonstrated molecular mimicry with CNS antigens (*30*–*33*), prompting us to further investigate EBNA1-specific T cell responses.

To measure $CD4^+$ T cell responses to full-length EBNA1 protein, which requires processing and presentation via HLA class II, we implemented an approach using human embryonic kidney 293 (HEK293) cells overexpressing EBNA1 (HEK-EBNA1) (Fig. 2E). Unlike Raji cells, which express multiple latent antigens, HEK-EBNA1 cells express only EBNA1 and avoid PMA artifacts, allowing us to test at a 32-fold higher protein concentration. HEK-EBNA1 lysates elicited significantly higher responses than HEK-null lysates ($P = 0.021$), although the magnitude of the specific response was low (1 SFC per 3×10^5 PBMCs) (Fig. 2, F and G). The relationship between this response and the EBNA1 peptide response did not reach statistical significance ($P = 0.14$) (Fig. 2H). In participants who responded to EBNA1 peptides (>5 SFCs), the median (IQR) response to EBNA1 protein lysate was 94% (78%, 99%) lower than the peptide response, consistent with $CD8^+$ T cells driving most of the response to EBNA1 peptides.

To confirm which T cell subset mediated the EBNA1 peptide response, we performed cell depletion experiments. $CD8^+$ T cell depletion decreased this response by a median (IQR) of 86% (75%, 92%) ($P = 0.0015$), whereas $CD4^+$ T cell depletion had a small but significant effect ($P = 0.038$) (Fig. 2, I and J). This demonstrates that EBNA1-specific T cells in MS are predominantly $CD8^+$ T cells rather than $CD4^+$ T cells.

$CD4^+$ T cells responding to EBV lytic antigens primarily recognize proteins from viral particles, not early lytic antigens

Lytic antigens can be divided into early antigens (e.g., Z and EA-D), whose expression is independent of viral DNA replication, and late antigens (e.g., gp350 and VCA p18), which form viral particles and require DNA replication for expression (Fig. 1A). We next examined whether early or late antigens drive the $CD4^+$ T cell response. To address this, we used tenofovir alafenamide (TAF), a nucleoside analog that inhibits viral DNA replication, thereby preventing the expression of late lytic antigens but sparing early antigens (*46*). We induced lytic reactivation in B95.8 cells using PMA/butyrate, with or without TAF at a 20 μM concentration selected to inhibit viral replication by 95% on the basis of dose-response curves (fig. S7). Control experiments confirmed that PMA (fig. S6, A and B), butyrate, and TAF (fig. S6C) had no off-target effects on T cell responses to defined EBV peptides at the concentrations used.

TAF treatment inhibited viral replication and blocked late lytic antigen expression but preserved early antigen expression (Fig. 3, A and B). We observed a significant reduction in T cell responses to lysates prepared from TAF-treated B95.8 cells compared with untreated lytic lysates ($P < 0.0001$) (Fig. 3C). The results were paralleled by total antibody responses to the lysates (Fig. 3D). These findings indicate that late lytic antigens, components of the viral particle, were the primary T cell and antibody targets in the total lytic B95.8 lysate.

To confirm that viral particles drive the $CD4^+$ T cell response, we used an orthogonal approach: HEK293 cells containing the EBV p2089 *BZLF1* knockout bacterial artificial chromosome, which release viral particles into the medium upon *BZLF1* transfection (Fig. 3E and fig. S8). Control experiments confirmed that TAF does not directly

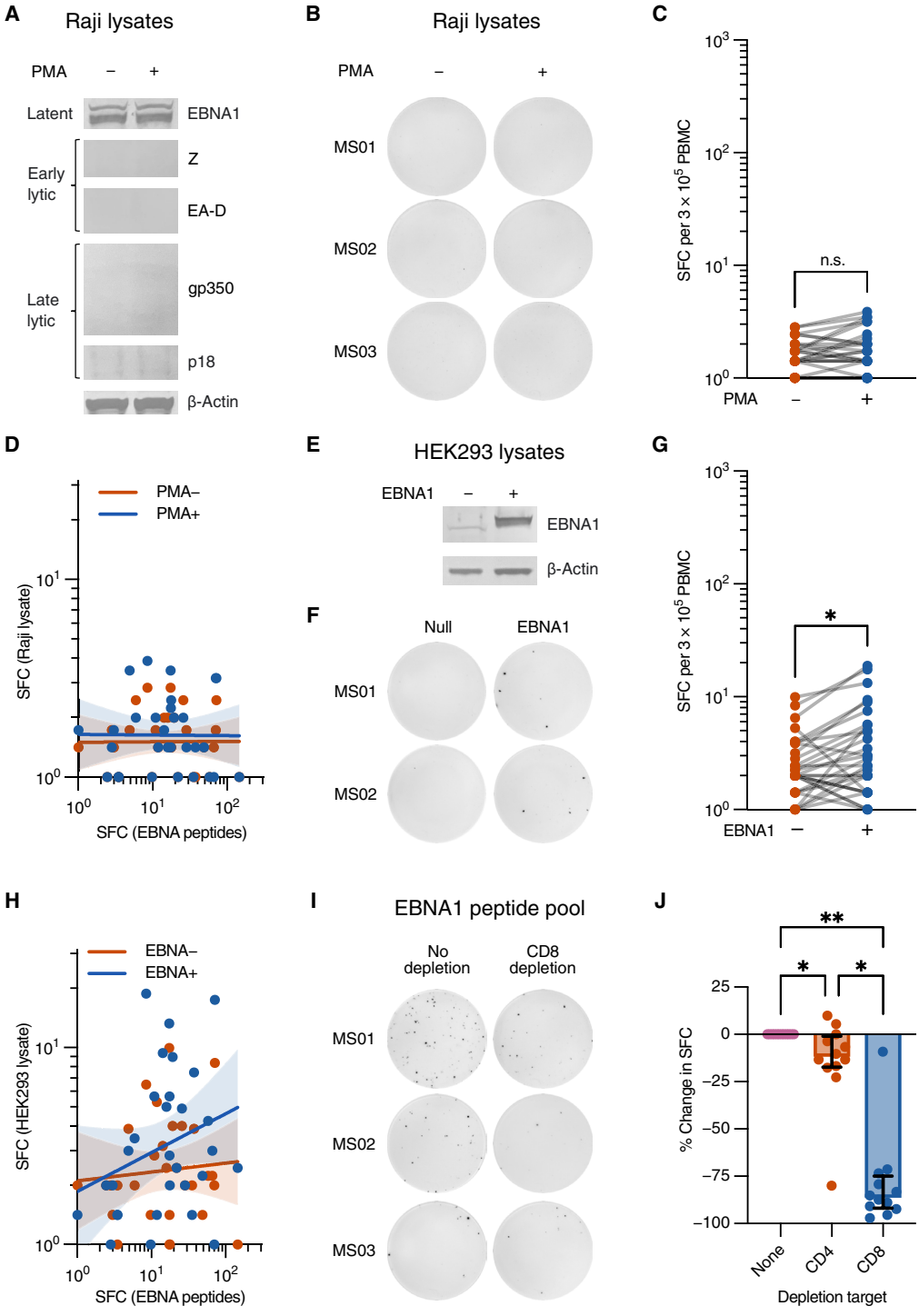

Fig. 2. EBNA1 induces lower CD4$^+$ T cell responses than total EBV lytic antigens and primarily activates CD8$^+$ T cells in individuals with MS. (**A**) Immunoblots of Raji cell lysates with and without PMA treatment. (**B**) Representative ELISPOT wells from three participants with MS stimulated with Raji lysates (PMA− versus PMA+). (**C**) Quantification of SFCs for Raji lysates between PMA− and PMA+ conditions ($n = 30$, discovery cohort). (**D**) Correlation of SFCs using EBNA1 peptides versus Raji lysates (PMA− and PMA+) ($n = 30$, discovery cohort). Shaded regions represent 95% confidence intervals. (**E**) Immunoblots confirming EBNA1 expression in HEK cell lysates. (**F**) Representative ELISPOT wells from two participants with MS comparing null versus EBNA1-expressing HEK cell lysates. (**G**) Quantification of SFCs with HEK cell lysates ($n = 30$, discovery cohort). (**H**) Correlation of SFCs using EBNA1 peptides versus HEK293 cell lysates expressing EBNA1 (blue) or control (orange) ($n = 30$, discovery cohort). Shaded regions represent 95% confidence intervals. (**I**) Representative ELISPOT wells showing responses to EBNA1 overlapping peptide pool with and without CD8 depletion. (**J**) Percentage changes in SFCs after CD4 or CD8 depletion ($n = 12$ participants with MS). Data are presented as medians ± IQR. Data were analyzed by the paired Wilcoxon signed-rank test (C), linear regression [(D) and (H)], paired Student's t test (G), or Friedman test with pairwise Wilcoxon signed-rank tests and Bonferroni adjustment (J); $^*P < 0.05$; $^{**}P < 0.01$; n.s., not significant.

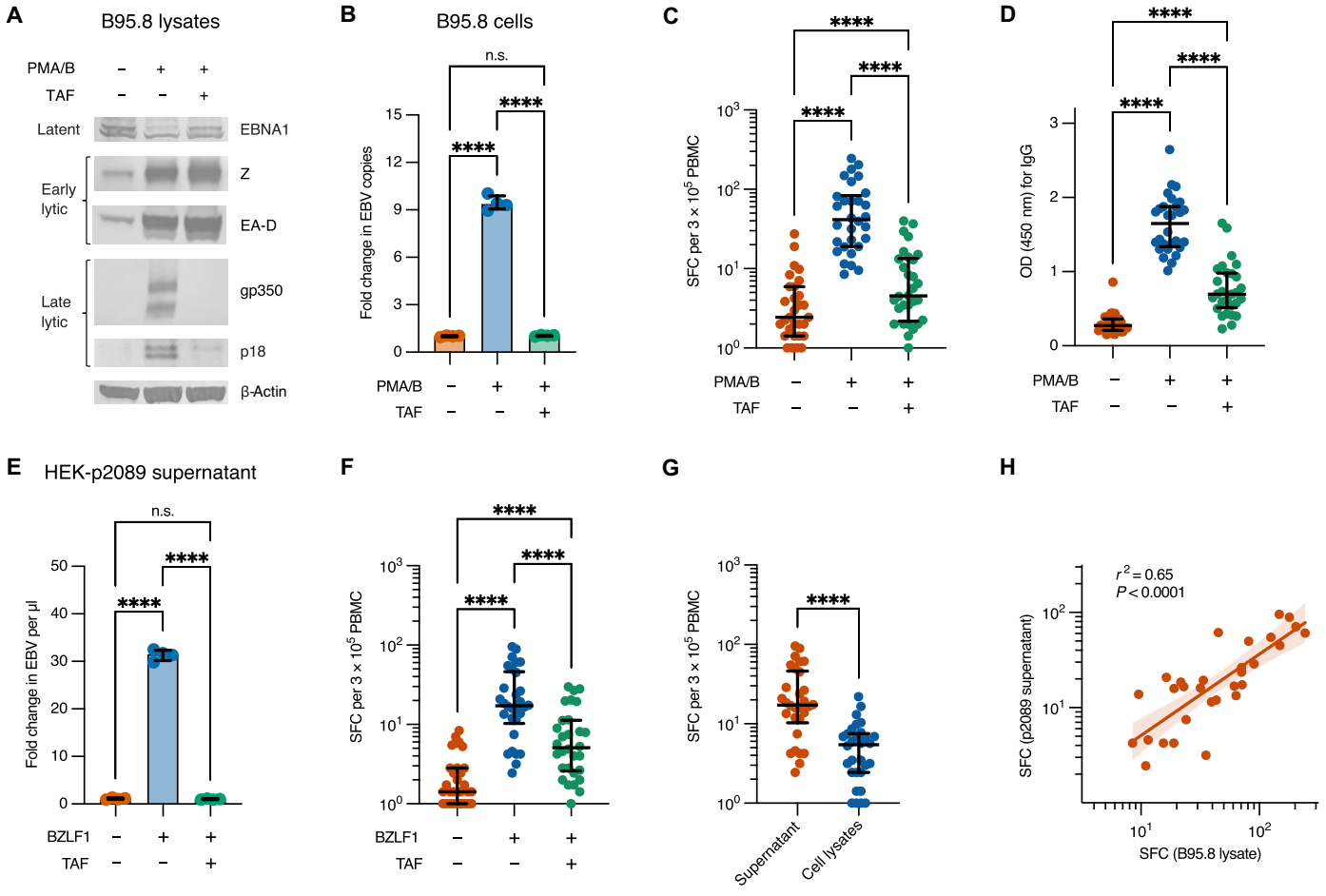

Fig. 3. CD4$^+$ T cells responding to EBV lytic antigens primarily recognize proteins from viral particles, not early lytic antigens. (**A**) Immunoblots of B95.8 lysates treated with PMA/butyrate (PMA/B) with or without TAF (20 μM). (**B**) Fold change in EBV DNA copies in B95.8 cells across treatment conditions. (**C**) SFCs per 3 × 10^5 PBMCs for B95.8 lysates under different conditions ($n = 30$, discovery cohort). Each data point represents the mean of two technical replicates. (**D**) IgG antibody responses to B95.8 lysates ($n = 28$ participants with MS). OD, optical density. (**E**) Viral production in HEK-p2089 cells expressing *BZLF1* with or without TAF (0.75 μM) ($n = 4$ replicates per condition). (**F**) T cell responses to HEK-p2089 supernatants ($n = 30$, discovery cohort). (**G**) Comparison of T cell responses to supernatants versus cell lysates from *BZLF1*-expressing HEK-p2089 cells ($n = 30$, discovery cohort). (**H**) Correlation between responses to B95.8 lysates and p2089 supernatants ($n = 30$, discovery cohort). Shaded regions represent 95% confidence intervals. Data are presented as medians ± IQR. Data were analyzed by the linear mixed effects model with the Bonferroni adjustment [(B) to (E)], Friedman test with pairwise Wilcoxon signed-rank tests and Bonferroni adjustment (F), paired Student's *t* test (G), or simple linear regression (H); ****$P < 0.0001$; n.s., not significant.

impair T cell function (fig. S6C). Supernatants containing viral particles strongly stimulated T cell responses, and these responses were inhibited by TAF treatment (Fig. 3F) at a 0.75 μM concentration selected to inhibit viral replication by 95% on the basis of dose-response curves (fig. S7A). The T cell response to the supernatant was significantly higher than the response to the cell lysate from the same producer cells ($P < 0.0001$) (Fig. 3G), indicating that viral particles are the primary targets. The correlation between T cell responses to B95.8 lysates and p2089 supernatants was strong ($r^2 = 0.65$, $P < 0.0001$) (Fig. 3H), suggesting that both antigen sources stimulate the same T cell populations.

EBV-specific T cells recognizing late lytic antigens are altered by DMTs

Having established that CD4$^+$ T cells primarily recognize EBV viral particles and that responses are elevated in untreated MS, we next asked whether this elevation is specific to EBV and whether responses are affected by DMTs. We compared T cell responses to EBV, CMV, and varicella-zoster virus (VZV) lysates across HCs, untreated participants with MS, and participants with MS receiving different DMTs (Fig. 4, A to C). Statistically significant differences between HCs and untreated participants with MS were observed only for EBV ($P = 0.011$) and not for CMV ($P = 1.00$) or VZV ($P = 1.00$) (Fig. 4, A to C; lysate validation in fig. S9).

Certain DMTs altered EBV-specific responses. Anti-CD20 therapies and DMF/diroximel fumarate showed significantly reduced responses to EBV lysates by 3.3- and 2.9-fold compared with those in untreated participants with MS, respectively ($P < 0.0001$ and $P = 0.013$), bringing them to levels similar to HCs (Fig. 4A). In contrast, natalizumab increased responses by 3.2-fold ($P = 0.005$) (Fig. 4A). Anti-CD20 and DMF did not appreciably alter responses to CMV or VZV, whereas natalizumab only significantly increased responses to VZV ($P = 0.0011$) (Fig. 4, B and C), consistent with a more selective impact on EBV-specific immunity.

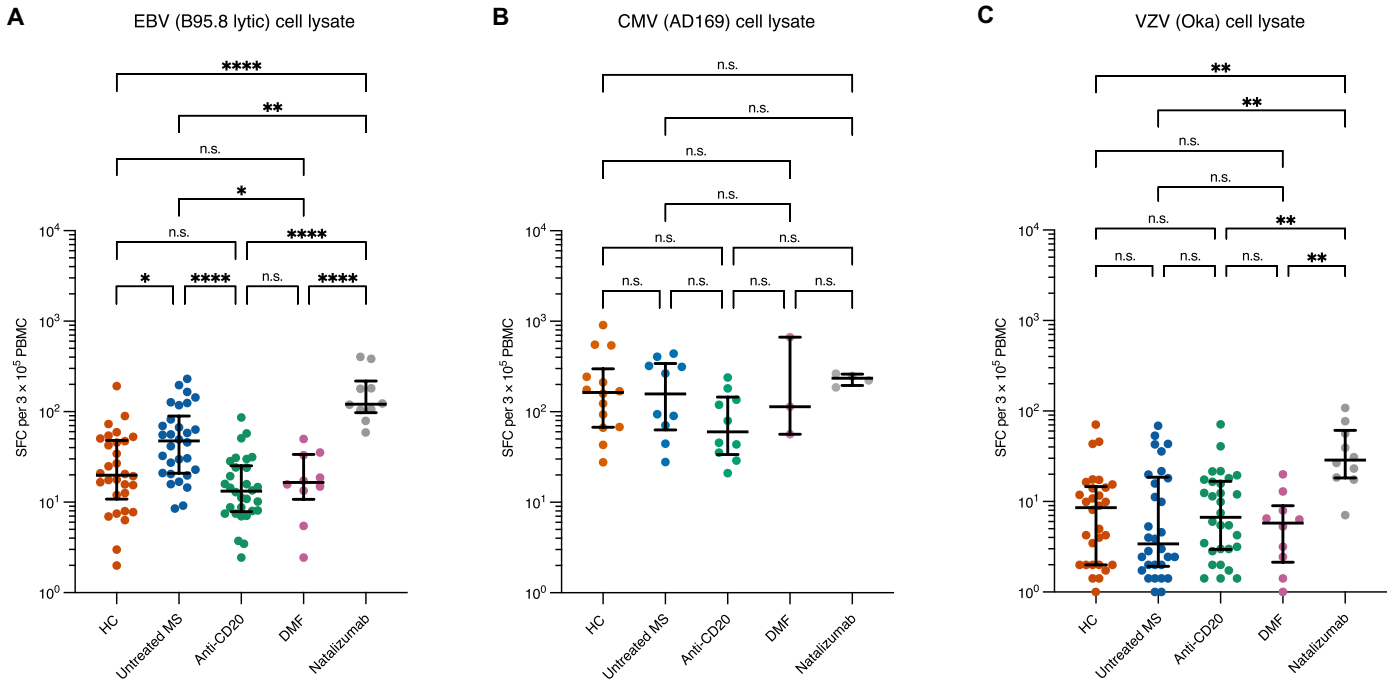

Fig. 4. EBV-specific T cells recognizing late lytic antigens are altered by disease-modifying therapies. (**A**) Comparison of T cell responses to B95.8 EBV lytic lysates among HCs ($n = 30$), untreated participants with MS ($n = 30$), and participants with MS on different disease-modifying therapies [anti-CD20 ($n = 30$), DMF ($n = 10$), or natalizumab ($n = 10$); all discovery cohort]. Each data point represents the mean of two technical replicates. (**B**) T cell responses as in (A) using CMV (AD169) lysates in seropositive individuals. (**C**) T cell responses as in (A) for VZV (Oka) lysates. Data are presented as medians ± IQR. Data were analyzed by the one-way ANOVA with Tukey's post hoc test [(A) and (B)] or Kruskal-Wallis test with Dunn's test and Holm adjustment (C); $^*P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$; n.s., not significant.

We also tested $CD8^+$ T cell responses using EBV and CMV HLA class I peptide pools (tables S2 and S3) and the EBNA1 overlapping peptide pool, with results shown in fig. S10. We next examined antibody responses to EBNA1, VCA, and B95.8 lytic lysates. Immunoglobulin G (IgG) responses to EBNA1 and B95.8 lytic lysates, but not VCA, were significantly higher in participants with MS compared with HCs ($P = 0.032$ and $P = 0.0029$, respectively) (fig. S11, A to C), further supporting our observation that $CD4^+$ T cell responses to late lytic antigens are increased in participants with MS, consistent with a previous study (*47*). Unlike $CD4^+$ T cell responses, antibody levels to B95.8 lytic lysates were similar in anti-CD20–treated versus untreated participants with MS (fig. S11B). This finding aligns with a published humoral analysis of the same testing cohort in this paper, which reported only minimal changes in EBNA1 IgG and VCA IgG during anti-CD20 therapy (*48*), underscoring the contrast between the relatively static humoral response and the dynamic cellular immune response during B cell depletion. In addition, B95.8 lytic antibodies were more strongly associated with MS than either EBNA1 or VCA ($P = 0.0026$) (fig. S11D).

In two independent longitudinal cohorts, B cell depletion uniquely affects EBV-specific T cells targeting late lytic antigens

To investigate the dynamics of EBV-specific T cell responses after B cell depletion, we performed longitudinal analyses of treatment-naïve participants with MS before and 6 months after initiating anti-CD20 therapy in a testing cohort ($n = 60$). Responses to EBV B95.8 lytic lysates after anti-CD20 treatment were reduced by 2.5-fold ($P < 0.0001$) (Fig. 5A). This effect was more modest for CMV lysates (in the subset of seropositive individuals) and for *Escherichia coli* lysates, which each decreased by 1.5- and 1.6-fold, respectively ($P = 0.026$ and $P = 0.00014$) (Fig. 5, B to D). These results were validated in a second independent longitudinal cohort sampled ~1 year after the last anti-CD20 dose ($n = 9$), using VZV as an alternative herpesvirus control because its near-universal seroprevalence (>95%) allowed analysis without serological testing (Fig. 5, E to H). Whereas VZV and *E. coli* responses showed no significant reduction in this cohort ($P = 0.79$ and $P = 0.82$, respectively), the reduction in EBV-specific responses remained significant ($P = 0.011$) and was similar in magnitude to the testing cohort despite the longer interval since treatment.

In both cohorts, the reduction in EBV-specific responses was significantly greater than for each of the other antigens tested. In the testing cohort, EBV-specific reductions exceeded those for both CMV ($P = 0.00041$) and *E. coli* ($P = 0.00022$), and in the validation cohort, they exceeded those for both VZV ($P = 0.012$) and *E. coli* ($P = 0.015$). The reductions in the non-EBV antigens did not differ significantly from each other in either cohort ($P = 0.34$ and $P = 1.00$, respectively) (Fig. 5, D and H). This difference was particularly evident in the validation cohort, where the reduction remained statistically significant only for EBV ($P = 0.011$) (Fig. 5E) despite substantial time for B cell repopulation, whereas VZV and *E. coli* responses had recovered (Fig. 5, F and G). These findings suggest that B cell depletion therapy has a more pronounced impact on T cells that recognize EBV viral particles than would be expected from a general reduction in B cell–dependent antigen presentation.

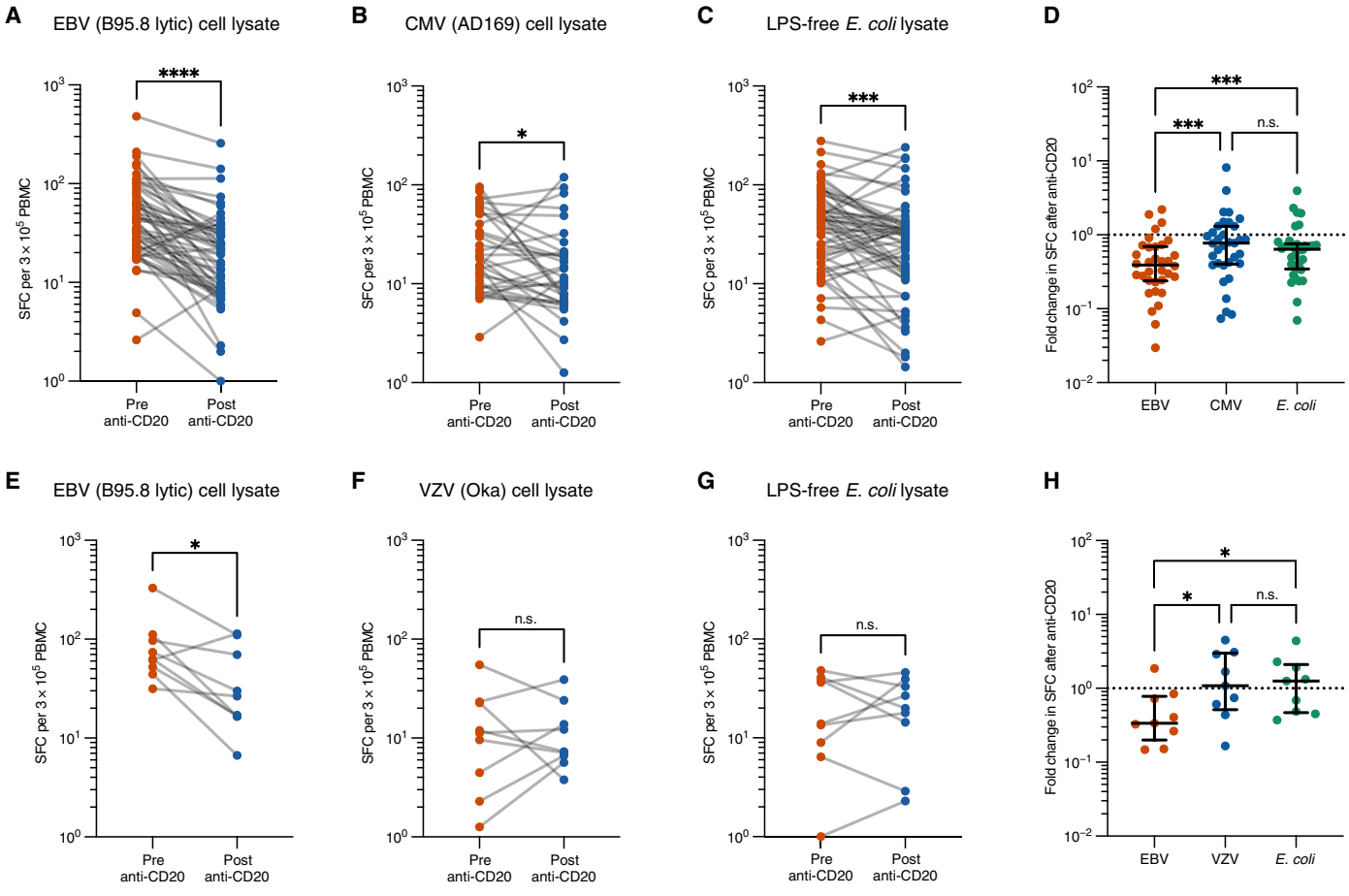

Fig. 5. In two independent longitudinal cohorts, B cell depletion uniquely affects EBV-specific T cells targeting late lytic antigens. (**A**) Comparison of EBV B95.8 lysate–specific T cell responses before and after initiating anti-CD20 therapy ($n = 60$, testing cohort). Each data point represents the mean of three technical replicates. (**B**) Analysis for CMV (AD169) lysate responses in seropositive individuals ($n = 34$). (**C**) Analysis of responses to lipopolysaccharide (LPS)–free *E. coli* lysate ($n = 60$). (**D**) Fold change in SFCs after anti-CD20 treatment for EBV, CMV, and *E. coli* in CMV-seropositive individuals. (**E**) EBV B95.8 lysate–specific T cell responses before and after initiating anti-CD20 therapy ($n = 9$, validation cohort). Each data point represents the mean of three technical replicates. (**F**) Analysis for VZV (Oka) lysate responses ($n = 9$). (**G**) Analysis of responses to LPS-free *E. coli* lysate ($n = 9$). (**H**) Fold change in SFCs after anti-CD20 treatment for EBV, VZV, and *E. coli*. Data are presented as medians ± IQR. Data were analyzed by the paired Student's *t* test [(A) to (C) and (E) to (G)], Friedman test with pairwise Wilcoxon signed-rank tests and Bonferroni adjustment (D), or linear mixed effects model with Bonferroni adjustment (H); $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s., not significant.

EBV shedding in saliva is similar in individuals with MS and HCs but is markedly reduced by B cell depletion

Given the increased EBV-specific T cell responses in participants with MS, we asked whether this reflected higher levels of viral shedding in saliva. We collected saliva samples over 1 month and found no significant difference in the frequency of EBV-positive samples ($P = 0.88$) or the average viral load during shedding events ($P = 0.86$) between HCs and participants with MS not receiving anti-CD20 therapy (Fig. 6, A and B); as expected, shedding frequency and viral load were correlated (Fig. 6C). This finding aligns with a previous study in a large cohort of adults reporting no difference in salivary shedding between participants with MS and controls (*49*).

In contrast, participants receiving anti-CD20 therapy showed a reduction in EBV shedding. Whereas 100% of HCs and participants with MS not on anti-CD20 had detectable EBV shedding over 1 month, 0% of participants on anti-CD20 ($n = 5$, treated for 0 to 14 months) had any detectable EBV shedding (Fig. 6A). Consistent with this, EBV viral load in B cells was significantly reduced in anti-CD20–treated participants ($P < 0.0001$) (Fig. 6D). The only participant with detectable EBV in B cells was on ofatumumab for 6 months and did not have confirmed B cell depletion at the time of sampling. These findings demonstrate that B cell depletion reduces systemic EBV burden and eliminates detectable salivary shedding.

Given that we used TAF (a tenofovir prodrug) to block late lytic antigen expression in vitro, and several clinical trials are evaluating tenofovir-based antivirals as add-on therapies in MS, we conducted an exploratory analysis of individuals on long-term tenofovir for non-HIV indications. Although limited by sample size, EBV shedding and T cell responses persisted despite >3 years of treatment ($n = 2$ and $n = 5$, respectively; fig. S12), in contrast with the effects of anti-CD20.

Recombinant antigen screening reveals coordinated expansion of EBV lytic responses in MS

To define the specific EBV antigens driving the $CD4^+$ T cell response and eliminate potential PMA artifacts, we identified PEP005

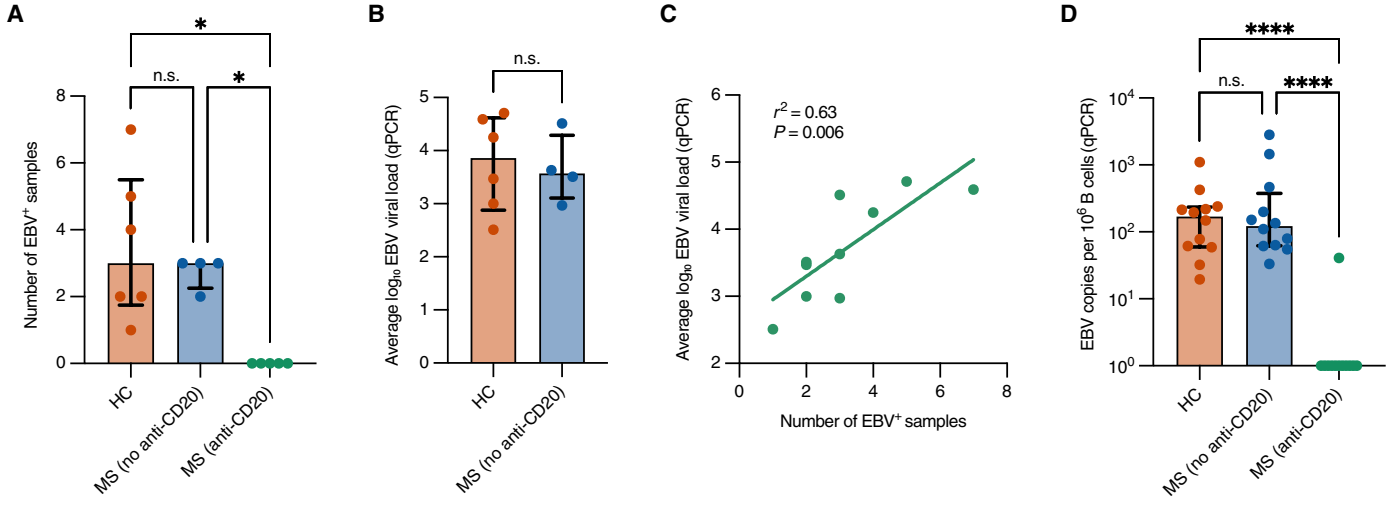

Fig. 6. EBV shedding in saliva is similar in individuals with MS and healthy controls but is markedly reduced by B cell depletion. (**A**) Numbers of EBV-positive samples in healthy controls (HC; $n = 6$), participants with MS without anti-CD20 ($n = 4$; three untreated and one natalizumab), and anti-CD20–treated participants with MS ($n = 5$) in eight samples collected over 1 month. (**B**) Average $\log_{10}$ EBV viral loads in saliva, measured by quantitative polymerase chain reaction (qPCR), during shedding events in HCs and participants with MS without anti-CD20. (**C**) Correlation between numbers of EBV-positive samples and average $\log_{10}$ EBV viral loads at shedding. (**D**) EBV copies per 10^6 B cells in HCs, participants with MS without anti-CD20 (all untreated), and participants with MS on anti-CD20 ($n = 12$ in each group). Undetectable values are displayed as 10^0. Data are presented as medians ± IQR. Data were analyzed by the Kruskal-Wallis test with Dunn's test and Holm adjustment [(A) and (D)], Student's *t* test (B), or linear regression (C); $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$; n.s., not significant.

(ingenol-3-angelate) as an alternative lytic inducer. Unlike those treated with PMA, PEP005-treated lysates showed no nonspecific T cell activation (fig. S13, A and B) but comparable late lytic antigen (gp350) expression (fig. S13C). Dose-response experiments confirmed that PEP005-induced lysates could be used at a concentration that saturated antigen-specific T cell activation (fig. S13, D and E).

To comprehensively map the antigenic targets of this response, we screened PBMCs from participants with MS and HCs ($n = 12$ each) against a complete library of all 28 known EBV viral particle proteins (table S4). To control for expected HLA-dependent effects on responses to individual antigens, we included similar proportions of HLA-DR15–positive and HLA-DR15–negative individuals in each group. All proteins were produced in HEK 293 or 293T cells to ensure proper folding and posttranslational modifications. Although His-tag purification was considered, some antigens such as gB cannot be purified without introducing mutations that alter protein conformation. We therefore used cell lysates directly, with null-transfected lysates as controls. Expression was confirmed by immunoblot for capsid (Fig. 7A), tegument (Fig. 7B), and glycoprotein (Fig. 7C) antigens. The sum of T cell responses to individual recombinant proteins correlated strongly with the response to whole viral lysate ($r = 0.97$, $P < 0.0001$), confirming that our panel captures most of the antigen-specific response (Fig. 7D).

The heatmap, displaying IFN-γ ELISPOT responses (SFC per 3×10^5 PBMCs) to each recombinant antigen on a log scale, revealed that the pattern of immunodominance was similar between participants with MS and HCs (Fig. 7E). Examination of individual antigens showed that the $CD4^+$ T cell response was dominated by three proteins: the major capsid protein encoded by *BcLF1* and the glycoproteins gB (*BALF4*) and gH (*BXLF2*) (Fig. 7F). Individuals with DR15 showed responses to the capsid antigen encoded by *BORF1*, consistent with a previously described DR15-restricted epitope (Fig. 7E) (*21*). Responses to the immediate-early (IE) antigens Z and R (encoded by *BZLF1* and *BRLF1*) using overlapping peptide pools correlated with total lytic lysate responses ($r = 0.61$, $P = 0.0015$; Fig. 7G); however, these responses could not be elicited using whole recombinant proteins (fig. S14, A to C), consistent with $CD8^+$ T cell responses to intracellular antigens rather than $CD4^+$ responses to exogenous viral particles. These IE responses were also elevated in MS ($P = 0.0021$) (fig. S14D). The correlation between IE and viral particle responses indicates a coordinated expansion of T cells targeting antigens across the lytic cycle in MS.

Participants with MS also recognized a broader diversity of antigens than HCs ($P = 0.0011$) (Fig. 7H). Viral particle antigens classified as high abundance on the basis of a prior mass spectrometry analysis of purified virions (≥1000 area units) (*50*) were more likely to elicit responses ($P < 0.0001$) (Fig. 7I). At the category level, participants with MS showed significantly higher responses to capsid ($P = 0.029$) and glycoprotein antigens ($P = 0.0033$), whereas tegument responses were similar to those of HCs ($P = 0.10$) (Fig. 7J).

DISCUSSION

Our results show that $CD4^+$ T cells in participants with MS preferentially recognize late lytic antigens that comprise viral particles rather than latent or early lytic antigens, a pattern that also aligns with our EBV-specific antibody findings. The EBV-specific $CD4^+$ T cell response was twofold higher in untreated participants with MS compared with HCs, whereas responses to other herpesviruses remained similar, suggesting a specific role for EBV in MS immunopathology. Comprehensive screening against individual recombinant EBV proteins confirmed that this response is directed primarily to capsid and glycoprotein antigens. Anti-CD20 therapy reduced EBV-specific $CD4^+$ T cell responses and eliminated detectable viral shedding, consistent with B cells serving as the source of antigens driving these responses.

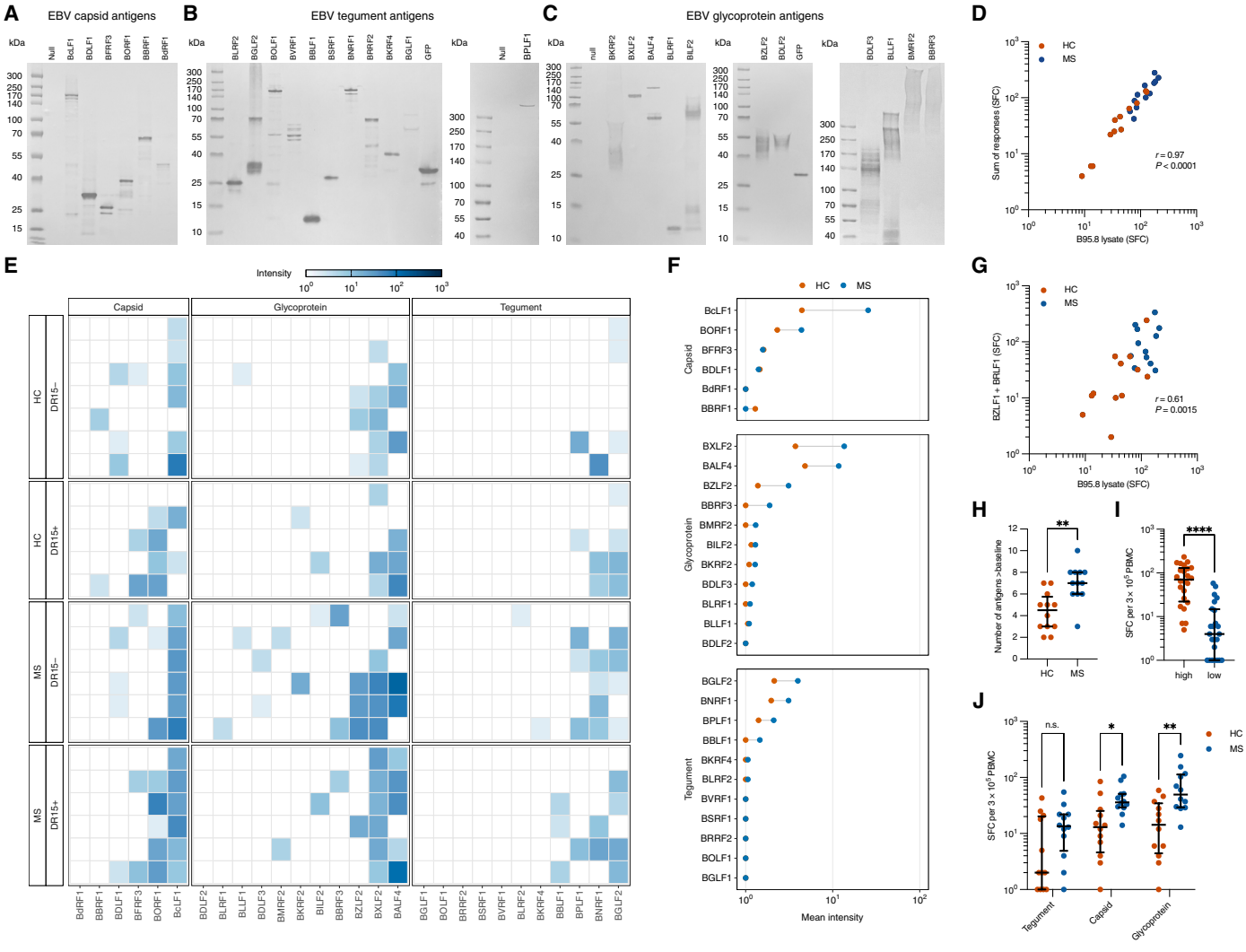

Fig. 7. Recombinant antigen screening reveals coordinated expansion of EBV lytic responses in MS. (**A** to **C**) Immunoblot analysis of recombinant EBV capsid (A), tegument (B), and glycoprotein (C) antigens. (**D**) Correlation between the sums of responses to individual particle antigens and B95.8 lysate responses ($n = 12$ participants with MS and 12 HCs). (**E**) Heatmap showing IFN-γ ELISPOT responses (SFC per 3×10^5 PBMCs) to individual viral particle antigens in HC and participants with MS ordered by DR15 status. (**F**) Comparison of mean T cell responses to individual capsid, glycoprotein, and tegument antigens between HCs (orange, $n = 12$) and participants with MS (blue, $n = 12$). (**G**) Correlation between responses to overlapping peptides from immediate-early antigens (*BZLF1* + *BRLF1*) and B95.8 lysate responses ($n = 12$ participants with MS and 12 HCs). (**H**) Number of antigens eliciting detectable responses above baseline in HCs versus participants with MS ($n = 12$ participants with MS and 12 HCs). (**I**) Comparison of T cell responses to high-abundance (≥1000 area units by mass spectrometry) versus low-abundance (<1000 area units) viral particle antigens, classified on the basis of Johannsen *et al.* (*50*) ($n = 24$). (**J**) Comparison of total responses to tegument, capsid, and glycoprotein antigen classes between HCs and participants with MS ($n = 12$ participants with MS and 12 HCs). Data are presented as medians ± IQR. Data were analyzed by the Spearman correlation [(D) and (G)], Student's *t* test (H), Mann-Whitney test (I), or linear mixed effects model with Bonferroni adjustment (J); $^*P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$; n.s., not significant.

The predominance of $CD4^+$ T cell responses to EBV viral particles in MS marks a shift from previous EBNA1-focused research (*30*–*35*). In contrast with viral particles, EBNA1 primarily activated $CD8^+$ T cells in our assays, consistent with its intracellular localization. Although molecular mimicry between EBNA1 and CNS antigens has been proposed, our findings suggest that the EBNA1-specific $CD4^+$ T cell population is relatively limited. These results align with the classical immunological distinction, in which intracellular antigens (EBNA1) are presented via HLA class I to $CD8^+$ T cells, whereas extracellular antigens (viral particle components) are presented via HLA class II to $CD4^+$ T cells. Prior studies have demonstrated similar $CD4^+$ T cell–selective activation by viral particles from CMV, influenza, parainfluenza, and human herpesvirus 6 (*36*, *45*), suggesting that immunodominant recognition of extracellular virion components may represent a general feature of $CD4^+$ T cell immunity to viral infection.

Current DMTs had divergent effects on EBV-specific immunity. Anti-CD20 therapies and DMF reduced EBV-specific responses by ~3-fold, bringing them to levels comparable to those of HCs. These reductions were relatively selective for EBV, with less pronounced effects on responses to CMV and VZV, suggesting a particular impact on EBV-specific immunity rather than generalized immunosuppression. In contrast, natalizumab increased EBV-specific responses by threefold, likely reflecting the accumulation of virus-specific T cells

in peripheral blood during α4-integrin blockade. This expansion may contribute to the increased disease activity sometimes observed after natalizumab withdrawal, given that pooled T cells could migrate into the CNS once the blockade is removed.

Anti-CD20 therapy also eliminated detectable EBV shedding in saliva, suggesting that B cells serve as the primary viral reservoir. This contrasts with a previous study reporting that rituximab does not affect oral shedding in patients with lymphoma, although concurrent cytotoxic chemotherapy in that setting may have confounded results through loss of T cell–mediated control, and CD20-expressing tumor cells may have acted as an antibody sink, reducing rituximab availability for normal B cells (*51*). The pronounced effect of B cell–targeting therapies on both EBV-specific T cell responses and viral shedding suggests that depleting infected B cells simultaneously removes both the antigen source and key antigen-presenting cells. The effects seen with DMF align with emerging evidence that memory B cell depletion may represent a common mechanism through which diverse MS therapies exert their beneficial effects (*37*, *52*, *53*).

Our findings on $CD4^+$ T cell responses to extracellular EBV particles in MS demonstrate notable parallels to celiac disease immunopathogenesis. In celiac disease, $CD4^+$ T cells recognize the foreign antigen gluten, with ~1 in 10,000 gluten-specific T cells detected using ELISPOT assays for IFN-γ (*54*); our assays revealed a comparable frequency of EBV-specific $CD4^+$ T cells in MS. Both diseases show similar reductions in antigen-specific T cells upon removing the driving antigen. Dietary gluten elimination reduces gluten-specific $CD4^+$ T cells by 75% (*55*), correlating with clinical and histological remission; in our study, anti-CD20 therapies similarly decreased EBV-specific $CD4^+$ T cells by 60 to 70%. These parallel responses support the concept that foreign antigen–driven $CD4^+$ T cell responses represent a shared mechanism in autoimmune pathogenesis, with important therapeutic implications. Although downstream effector mechanisms in celiac disease remain incompletely understood, this has not hindered therapeutic development targeting the foreign antigen or antigen presentation. Similarly, our identification of a dominant $CD4^+$ T cell response to EBV particles in MS suggests that targeting the viral antigen source may be an effective therapeutic strategy, even if downstream CNS inflammatory mechanisms remain to be fully characterized.

The observation that the size of the EBV-specific $CD4^+$ T cell compartment appears to be established during primary infection, as noted in a prior longitudinal study of individuals with IM (*21*), has important mechanistic implications. Participants with MS and HCs exhibited comparable levels of EBV shedding in saliva and similar viral loads in blood despite marked differences in T cell responses, suggesting that ongoing viral reactivation maintains rather than expands the established T cell compartment. We propose that a more profound primary infection expands the $CD4^+$ T cell compartment specific for viral particles and establishes a broader antigenic repertoire, both of which persist as an immune set point throughout life. Subsequent reactivation events may provide survival signals sufficient to maintain this compartment without requiring sustained viremia. Although participants with MS could experience higher frequencies of lytic reactivation in lymphoid tissues, this would likely be reflected in saliva or blood as more frequent or higher-level shedding, which we did not observe.

Our findings have important implications for EBV-targeted therapeutic strategies in MS. For vaccines, our data suggest caution with approaches targeting viral particle antigens, which could expand the pathogenic $CD4^+$ T cell compartment. An alternative strategy would be to boost $CD8^+$ T cell responses against IE or early lytic antigens, which are expressed within hours of reactivation before viral particle assembly (*56*). Enhanced surveillance against cells expressing these antigens could halt the lytic cycle before pathogenic $CD4^+$ T cell antigens are produced. For antivirals, our study establishes important benchmarks for clinical trial design. Dosing should aim to achieve suppression of EBV-specific responses comparable to that observed with DMTs, which may require higher doses than those used for other indications. Dose optimization could be initially addressed in healthy individuals, avoiding the confounding effects of concurrent DMTs. Last, our observation that the $CD4^+$ T cell set point appears modifiable in the absence of viral shedding raises an intriguing preventive possibility: Treatment during or after primary infection could prevent subsequent MS development because this T cell compartment may never repopulate to its original baseline in the absence of the frank viremia seen during primary infection.

An important limitation of our study is that we analyzed only peripheral immune responses rather than those in the CNS. However, peripheral responses are relevant given that frexalimab reduces MS disease activity despite limited CNS penetrance by disrupting peripheral CD40-CD40L interactions (*10*). On the basis of our findings, we propose that anti-EBV strategies may be effective even without CNS penetrance if they can substantially reduce or eliminate the source of viral particles driving peripheral T cell activation. A second limitation of our study is that we focused on saliva, which may not be the major reservoir of EBV viremia, given that the virus has been detected throughout the gastrointestinal tract (*57*–*59*). This broader distribution highlights the need for systemic tissue penetrance of therapeutics. In addition, although we observed more pronounced and sustained reductions in EBV-specific responses compared with other antigens, the modest sample sizes in some comparisons, particularly in the validation cohort, limit definitive conclusions about selectivity, and larger studies are needed to confirm these findings. Last, our study does not address whether EBV-specific $CD4^+$ T cell responses predict clinical outcomes, an important question for future longitudinal studies.

Together, our findings establish EBV viral particle antigens as the dominant targets of $CD4^+$ T cell responses in MS and demonstrate that these responses are modifiable by current therapies. By identifying a readily measurable, peripherally accessible immune response linked to disease, this work provides a foundation for the rational design and monitoring of EBV-targeted vaccines and antivirals for MS.

MATERIALS AND METHODS

Study design

We collected samples from participants in three locations: Boston, MA (USA), Bergen (Norway), and Baltimore, MD (USA). Each site operated under separate ethical approvals. All participants at each site provided written informed consent before sample collection. Details for each cohort are provided in tables S5 and S6.

For the Boston cohort (used for biomarker discovery and development), participants were enrolled at Mass General Brigham sites under three institutional review board (IRB)–approved protocols (2023P000922, 2023P002060, and 2023P000828). This cohort included five groups: (i) participants with relapsing-remitting MS meeting 2017 McDonald criteria for diagnosis recruited from outpatient clinics and inpatient neurology services (n = 80, of whom 30 were

treatment-naïve and 50 were on DMTs), (ii) age-matched HCs selected from the general population ($n = 30$), (iii) participants with IM from the inpatient medicine service ($n = 3$), (iv) individuals with documented negative EBV serologies from inpatient and outpatient settings ($n = 2$), and (v) individuals taking tenofovir disoproxil fumarate/emtricitabine (TDF/FTC) recruited both from the general population and from gastroenterology services who were HIV-negative and not on immunosuppressive regimens, treated for at least 3 years ($n = 5$). In the Boston cohort, participants with MS receiving anti-CD20 therapies included those on ocrelizumab, ofatumumab, and ublituximab. In addition, participants on DMF included both those on DMF and diroximel fumarate.

For the Bergen cohort (used for biomarker testing), treatment-naïve participants ($n = 60$) were previously enrolled in the Overlord-MS trial comparing rituximab and ocrelizumab (ClinicalTrials.gov ID: NCT04578639). The study protocol was approved by the Regional Committee for Medical and Health Research Ethics in Western Norway (66,391) and the Norwegian Medicines Agency.

For the Baltimore cohort (used for biomarker validation), participants ($n = 10$) were part of a pilot, open-label trial of ocrelizumab discontinuation (ClinicalTrials.gov ID: NCT04261790), which enrolled 10 adults with active relapsing-remitting MS at the Johns Hopkins University School of Medicine between August 2020 and April 2021. One of 10 individuals had insufficient cells in the post-treatment sample for our analyses. Participants received two standard courses of ocrelizumab, two 300-mg infusions two weeks apart, followed 6 months later by a single 600-mg infusion, and then discontinued therapy. Samples for the current analyses were collected at two time points: before the first infusion and 1 year after the final 600-mg dose.

Statistical analysis

Statistical tests were performed with Prism 10 (GraphPad software) and R statistical software (v4.5.0; R Core Team 2025). Continuous variables were log transformed to improve normality. Data distributions were tested for normality by visual inspection and Shapiro-Wilk tests to guide the selection between parametric and nonparametric statistical tests. For paired data, normality of differences was tested. When comparing independent groups, normally distributed data were analyzed by unpaired *t* tests (for two groups) or one-way analysis of variance (ANOVA) (for more than two groups). Significant ANOVA results were followed by Tukey's post hoc test. For non-normally distributed data from independent groups, the Mann-Whitney test (for two groups) or Kruskal-Wallis test (for more than two groups) was used. Significant Kruskal-Wallis results were followed by Dunn's test with Holm adjustment. When comparing dependent groups (repeated over time or across different assays or conditions per individual), paired *t* tests (for two groups) or linear mixed effects models (for more than two groups) were used when assumptions of normality were met. For linear mixed effects models, this was assessed by checking residual normality. Linear mixed effects models included random effects for the participants to account for within-person correlations. Significant linear mixed effects model results were followed by Bonferroni adjustment for pairwise comparisons. For non-normally distributed data from dependent groups or when linear mixed effects model assumptions were not met, the Wilcoxon signed-rank test (for two groups) or Friedman test (for more than two groups) was used. For Wilcoxon signed-rank tests comparing multiple conditions, Bonferroni adjustment was applied. Significant Friedman results were followed by pairwise Wilcoxon signed-rank tests with Bonferroni adjustment. Simple linear regression was used to assess linear relationships between continuous variables when residuals were normally distributed, reporting r^2 (coefficient of determination) and associated *P* values. Spearman correlation was used for non-normally distributed data or when assessing monotonic relationships. Univariable logistic regression was used to model the association between predictor variables and dichotomous outcomes. All comparisons were conducted using two-tailed tests; statistical significance was defined as $P < 0.05$. All significant statistical results are indicated in the figures with the following conventions: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$. Summary statistics are reported as medians with IQR. An overall summary of statistical analyses is presented in table S7. All individual level data for $n < 20$ are presented in data file S1.

Supplementary Materials

The PDF file includes:

Supplementary Methods
Figs. S1 to S14
Tables S1 to S8
Legends for data files S1 and S2

Other Supplementary Material for this manuscript includes the following:

Data files S1 and S2
MDAR Reproducibility Checklist

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CD4+ T cells reactive to Epstein-Barr virus late lytic antigens are enriched in individuals with multiple sclerosis

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Editor's summary

Epstein-Barr virus (EBV) has been nearly definitively linked to development of multiple sclerosis (MS); however, the exact roles of EBV itself and the immune response to it are only now being elucidated. Here, Bjornevik *et al.* investigated the CD4+ T cell response to EBV in individuals with MS, finding that these cells primarily reacted against EBV viral particle components. Moreover, these responses were reduced upon B cell depletion therapy, which has shown therapeutic benefit in MS. Although future work will determine whether these CD4+ T cells are pathogenic drivers of disease, these findings highlight the therapeutic potential of approaches aimed at EBV and EBV-specific immune responses. —Courtney Malo