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blood advances

Differential antibody response to EBV proteome following EBVST immunotherapy in EBV-associated lymphomas

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Key Points

- This study investigated antibody responses to the EBV proteome in patients with EBV-positive tumor who received EBVST infusions.
- Nonresponders (progressors) exhibited elevated IgG antibody responses compared with responders (nonprogressors) at 3 months after infusions.

Epstein-Barr virus (EBV) is associated with a diverse range of lymphomas. EBV-specific T-cell (EBVST) infusions have shown promise in safety and clinical effectiveness in treating EBV-associated lymphomas; however, not all patients respond to T-cell immunotherapies. To identify EBV antigen–specific antibody responses associated with clinical outcomes, we comprehensively characterized antibody responses to the complete EBV proteome using a custom protein microarray in 56 patients with EBV-associated lymphoma who received EBVST infusions in phase 1 clinical trials. Responders (nonprogressors) and nonresponders (progressors) had distinct antibody profiles against EBV. Twenty-five immunoglobulin G (IgG) antibodies were significantly elevated in higher levels in nonresponders than in responders at 3 months after EBVST infusion. Ten of these remained significant after adjustment for sex, age, and cancer type, including LMP2A (4 variants), BGRF1/BDRF1 (2 variants), LMP1, BKRF2, BKRF4, and BALF5. Random forest analysis identified these 10 IgG antibodies as key predictors of clinical response. Paired analyses using blood samples collected at both before infusion and 3 months after EBVST infusion indicated an increase in the mean antibody level for 6 other anti-EBV antibodies (IgG [BGLF2, LF1, and BGLF3]; IgA [BGLF3, BALF2, and BBLF2/3) in nonresponders. Overall, our findings suggest that these EBV-directed antibodies as potential serological markers for predicting clinical responses to EBVST infusions and as therapeutic targets for immunotherapy in EBV-positive lymphomas. These trials were registered at www.clinicaltrials.gov as #NCT01555892 (Cytotoxic T-Lymphocytes for EBV-positive Lymphoma [GRALE]), #NCT02973113 (Nivolumab With Epstein Barr Virus Specific T Cells [EBVSTS], Relapsed/Refractory EBV Positive Lymphoma [PREVALE]), and #NCT02287311 (Most Closely Matched 3rd Party Rapidly Generated LMP, BARF1, and EBNA1 Specific CTL, EBV-Positive Lymphoma [MABEL]).

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Supplemental data are available with the online version of this article. Original data or related materials are available on request from the corresponding author, Denise L. Doolan (d.doolan@imb.uq.edu.au).

The full-text version of this article contains a data supplement.

Introduction

Epstein-Barr virus (EBV) is associated with a broad spectrum of hematologic malignancies, including Burkitt lymphoma, Hodgkin lymphoma, a subset of non-Hodgkin lymphomas including extranodal natural killer/T-cell lymphoma, and posttransplant lymphoproliferative disease (PTLD).[1] EBV-associated lymphomas are characterized by the presence of viral antigens that serve as potential targets for T-cell immunotherapy.[2] Specifically, T cells designed to target and respond to EBV-specific antigens (known as EBV-specific T cells [EBVSTs]) can exert immunostimulatory effects by secreting cytokines and chemokines upon target antigen recognition that recruit and activate local immune responses to viral and nonviral antigens associated with the tumor.[3,4]

EBV-associated malignancies exhibit specific expression patterns of a small set of EBV proteins that determine their EBV latency types.[5,6] Among these, type 3 latency emerges only under conditions of severe immunosuppression and is more susceptible to adoptive T-cell therapy, likely because of the potent immunogenicity of type 3 viral antigens.[7-9] The first EBVSTs in the 1990s were donor derived (lymphoblastoid cell line-activated) and administered in the hematopoietic stem cell transplant setting, an immunosuppressed patient setting. EBVSTs directed against viral antigens generated from off-the-shelf donors have effectively treated EBV-associated PTLD in the hematopoietic stem cell transplant setting.[10-12]

Type 1/2 latency tumors express EBV proteins including EBV nuclear antigen (EBNA1) and latent membrane proteins (LMP1 and LMP2), but these are less immunogenic compared with type 3 tumors, making them potentially challenging to target with EBVSTs.[2] However, T-cell immunotherapy against the LMP1 and/or LMP2 antigens has been shown to be durable, safe, and effective approach with minimal toxicity in cases of Hodgkin lymphoma and natural killer/T-cell lymphoma (ie, type 2 tumors).[4,13,14] Despite these promising clinical results, achieving long-term remission of type II latency lymphoma remains a significant challenge. Certain patient subgroups exhibit partial or no responses, often followed by relapses during T-cell immunotherapy.[15] Previous research on EBVST immunotherapy under immunosuppressed conditions revealed instances of epitope spreading.[2] This phenomenon, in which the immune profile (ie, viral antigens eliciting immune responses) extended beyond the initially targeted EBV antigens, was observed in patients who achieved positive clinical responses.[4] We hypothesized that antibody (Ab) levels would decrease in responders, reflecting reduced antigen load, and increase or remain stable in nonresponders due to persistent or expanding antigen presence.

To test this hypothesis and identify which EBV-directed humoral immune responses are the most indicative of clinical responses to EBVST infusions in patients with EBV-associated lymphoma, this study analyzed both immunoglobulin G (IgG) and IgA Ab responses against the complete EBV proteome. We used a custom EBV protein microarray to measure both IgG and IgA Abs against 202 EBV protein sequences in plasma samples from a cohort of 56 patients with EBV-associated lymphomas treated with autologous or allogeneic (third-party) EBVST infusions enrolled in phase I clinical trials conducted by the Baylor College of Medicine, Houston, TX.

Unlike gene expression analyses in tumors, which often require invasive procedures such as biopsies, measuring Abs in the blood offers noninvasive informative markers for assessing patient-level EBV response, providing valuable insights into the host immune response. Hence, Abs could serve as potential indicators for patient monitoring and clinical management of EBV-associated lymphomas. In addition, these Ab-directed EBV antigens could guide the development of immunotherapy targets for treating EBV-associated lymphomas.

Materials and methods

Participants and samples

Archived plasma samples were selected from patients diagnosed with EBV-positive lymphomas receiving infusions of autologous or allogeneic (third-party) peptide-stimulated EBVSTs collected from 1 of 3 multicenter phase 1 clinical trials: GRALE (NCT01555892 in which autologous EBVSTs were infused), PREVALE (NCT02973113 in which autologous EBVSTs were infused with Nivolumab) and MABEL (NCT02287311 in which banked allogeneic EBVSTs were infused), all conducted at the Baylor College of Medicine. These studies were approved by the institutional review boards of the Baylor College of Medicine, the National Cancer Institute, representative institutes, and affiliated hospitals and were conducted under an Investigational New Drug application to the US Food and Drug Administration. Written informed consent was obtained from all participants. All laboratory testing of archived samples was conducted under a protocol approved by the James Cook University Human Research Ethics Committee (H7696).

The 56 patients selected for our study were all diagnosed with EBV-positive lymphoma, treated with EBVSTs per clinical trial protocols, and had residual banked blood samples available for Ab testing. They were categorized into 2 groups: responders (nonprogressors; complete or partial response recorded at last follow-up; n = 36), and nonresponders (progressors or stable disease recorded at last follow-up; n = 20). The samples were collected at the pre–EBVST infusion time point and 3 post–EBVST infusion time points (2 weeks, 4 weeks, and 3 months).

EBV custom protein microarray

Plasma samples were probed using a custom EBV protein microarray targeting IgA and IgG Abs against 202 EBV protein sequences, representing the complete EBV proteome, as previously described.[16-20] Briefly, our comprehensive EBV protein microarray contains 199 EBV protein sequences, representing nonredundant open reading frames and predicted splice variants from 86 EBV proteins generated from 5 different type 1 and type 2 EBV strains (AG876, Akata, B95-8, Mutu, and Raji). After polymerase chain reaction (PCR) amplification of the 199 nonredundant open reading frames from purified EBV cell line DNA, each sequence was cloned into a linearized, proprietary T7 expression vector (Antigen Discovery Inc). The sequences were then expressed using an *Escherichia coli* cell-free protein system and printed onto nitrocellulose slides (ie, microarrays). Also included on the array were peptide sequences representing 3 synthetic EBV peptides (VCAp18, EBNA-1, and EA p47), which are considered putative cancer biomarkers.[16] These 202 EBV protein/peptide sequences featured in our custom EBV protein microarrays are listed in supplemental Table 2.

Table 1. Baseline characteristics of all individuals and by treatment response status (responders vs nonresponders) to EBVST treatment

Characteristic	Overall, N = 56 (%)	Treatment response, n (%)		*P* value*
		Responders, n = 36 (64%)	Nonresponders, n = 20 (36%)	
Sex				
Female	17 (30%)	10 (28%)	7 (35%)	.6
Male	39 (70%)	26 (72%)	13 (65%)	
Age groups				
0-30 years	24 (43%)	17 (47%)	7 (35%)	.6
31-60 years	21 (38%)	12 (33%)	9 (45%)	
61-100 years	11 (20%)	7 (19%)	4 (20%)	
Ethnic groups				
Hispanic	12 (21%)	9 (25%)	3 (15%)	.5
Non-Hispanic	44 (79%)	27 (75%)	17 (85%)	
Diagnosis				
T-cell lymphomas	13 (23%)	8 (22%)	5 (25%)	.7
Hodgkin disease	19 (34%)	14 (39%)	5 (25%)	
Burkitt/large cell lymphoma	13 (23%)	7 (19%)	6 (30%)	
Other	11 (20%)	7 (19%)	4 (20%)	

*Pearson χ^2 test; Fisher's exact test.

Furthermore, each microarray includes the following controls: (1) synthetic peptides routinely used for EBV serology (VCAp18, EBNA1, and EaD); (2) purified IgG (6 serial 1:10 dilutions, starting at 0.1 μg/mL) as positive controls to account for nonviable hybridization; (3) anti-IgG Ab (0.1 μg/mL) to confirm secondary Ab reactivity; (4) 4 "no DNA" (nontranslated protein) spots included to correct for person-specific background as a measure of non-EBV reactivity; and (5) 2 buffer-only negative controls. Each protein sequence cloned into the pXT7 expression vector incorporates N-terminal 10× histidine (HIS) and C-terminal hemagglutinin (HA) tags to confirm the expression of full-length protein sequences on the array using anti-HIS and anti-HA Abs for quality control (by the supplier on 1-3 slides per print batch). The correlation of antipolyhistidine and anti-hemagglutinin tag responses between microarrays was determined to confirm minimal within-batch variability.

Plasma samples from each study participant (Table 1) were tested in a blinded manner with respect to response status, as described previously.[16,18-20] Briefly, Ab responses were detected with biotin-conjugated goat anti-human IgG (1:1000 dilution) or IgA (1:500 dilution) secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA) and visualized with streptavidin-conjugated SureLight P3 (Columbia Biosciences, Columbia, MD; 1:200 dilution) Ab. After probing, air-dried probed slides were scanned on an Axon GenePix 4300A (Molecular Devices). Raw fluorescence intensities were corrected for spot-specific background using Axon GenePix Pro 7 software, and data were variant log transformed using variance stabilizing normalization transformation in Gmine.[21] The array output was then standardized, referred to as the standardized signal intensity (SSI), to the person-specific background using the individual's cutoff (mean ± 1.5 standard deviation of the 4 "no DNA" spots). Positivity was defined as a SSI >1.0, and output was further categorized into positive (1) and negative (0) responses.

Nineteen duplicate samples were included for assessment of reproducibility, and a cutoff coefficient of variation of 30% was selected.[18-20] We excluded array spots with coefficients of variation ≥30% from the analysis, leaving 74 IgG and 202 IgA markers for comparison between responders and nonresponders.

Quantification of EBV DNA in peripheral blood mononuclear cells by real-time quantitative PCR

EBV DNA was quantified in peripheral blood mononuclear cells (PBMC) samples from responders and nonresponders collected at preinfusion and postinfusion time points (2 weeks, 4 weeks, and 3 months) using real-time quantitative PCR targeting specific EBER regions of the EBV genome, as detailed in the supplemental Materials.

Statistical analysis

All statistical analyses were performed using R Statistical Software (v4.2.1; R Core Team 2022).[22] In addition to reporting nominal *P* values from primary statistical tests, the Benjamini and Hochberg false discovery rate (FDR; FDR = 5%) method was applied to account for multiple tests.

Differences in the distribution of demographic and clinical variables (sex, age group, ethnic group, and diagnosis) between responders and nonresponders were assessed using a χ^2 test or Fisher exact test, with the latter applied when the number of participants in any group was <5. Differences in EBV DNA level (log10 transformed) between responders and nonresponders were assessed at each time point (preinfusion and 2 weeks, 4 weeks, and 3 months after infusion) using Mann-Whitney *U* tests.

Differences in the mean SSI for IgG and IgA Abs between the responders/nonprogressors (n = 36) and nonresponders/progressors (n = 20) were assessed using unpaired *t* tests at each

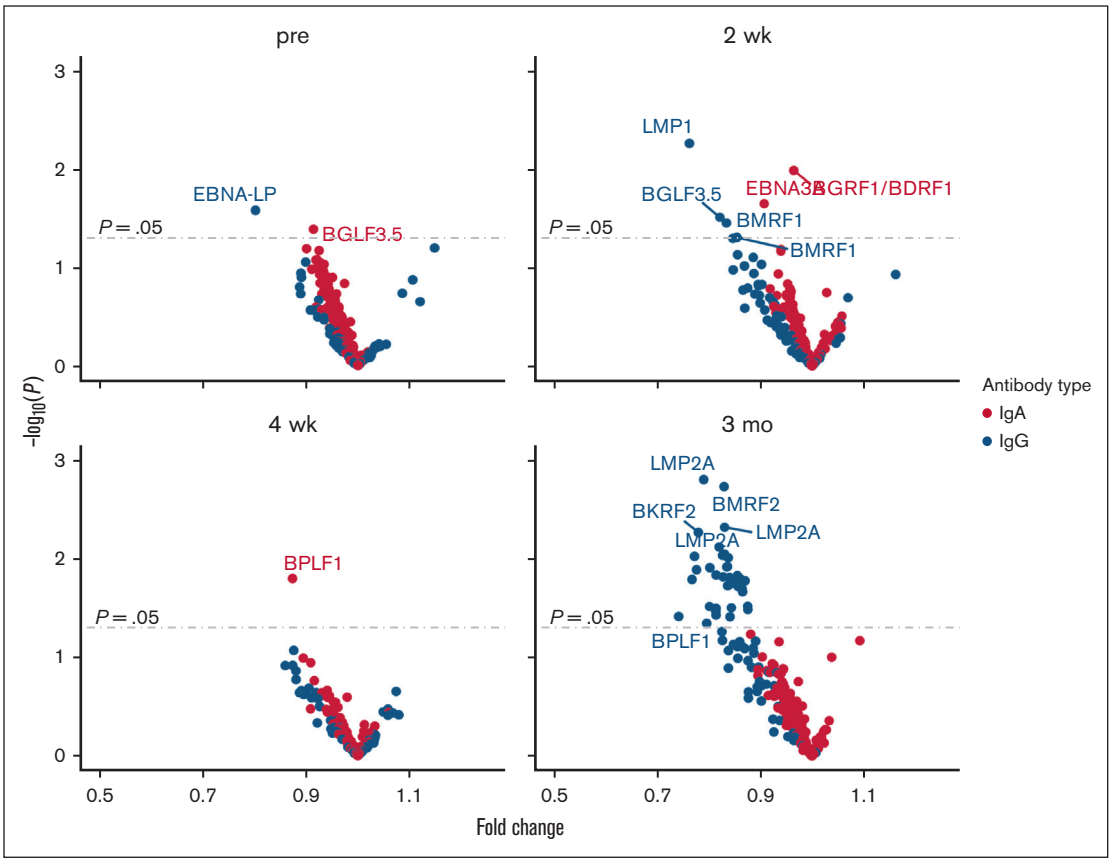

Figure 1. Differential Ab responses between responders and nonresponders to EBVST immunotherapy. The volcano plots show the difference in IgA and IgG levels (SSI) between responders (n = 36) and non-responders (n = 20) to EBVST immunotherapy at preinfusion and 2-week (2 wk), 4-week (4 wk), and 3-month (3 mo) postinfusion time points. The x-axis represents the fold change (ratio of SSI for responders vs nonresponders), whereas the y-axis shows the corresponding *t* test log 10 (*P* values.) IgA Abs are shown in red, and IgG Abs are shown in blue. Abs with a coefficient of variation (CV) <30% are included in the analysis (IgA = 202; IgG = 74). The dashed lines represent the nominally significant *P* value threshold.

blood collection time point. The odds ratios (ORs) and 95% confidence intervals (CIs) for the association between detection of each anti-EBV Ab (ie, categorized into binary responses; positive = 1; negative = 0) and treatment response status (responders/nonprogressors = 1; nonresponders/progressors = 0) were calculated using logistic regression models adjusted for sex, age at enrolment, and diagnosis type (T-cell lymphomas, Hodgkin lymphoma, B-cell lymphomas, and other lymphomas). In addition to the adjusted logistic regression models, the continuous SSI output at the 3-month postinfusion time point was analyzed with random forest models[23] using the R package random forest (v4.7.1)[24] to rank IgG or IgA Abs based on their importance in differentiating samples into the defined groups (responders/nonresponders). Specifically, Abs at each time point were ranked using the mean decrease in Gini index and mean decrease in accuracy metrics, which order variables according to their importance in improving prediction model purity and accuracy, respectively. Spearman rank correlation was used to evaluate the relationship between EBV DNA levels and specific EBV Ab responses at 3 months after infusion.

The total change in Ab response between the preinfusion and 3-month postinfusion time points (Ab_{3mo}–Ab_{pre}) was evaluated among responders (nonprogressors) and nonresponders (progressors) in patients with matched blood samples at both time points (n = 41 samples for IgG [30 responders and 11 nonresponders]; n = 42 samples for IgA [30 responders and 12 nonresponders]). ORs and 95% CIs for the association between each anti-EBV Ab variable (ie, Ab_{3mo}–Ab_{pre} change in SSI) and treatment response status were calculated using logistic regression models adjusted for sex, age at enrolment, and diagnosis type (T-cell lymphomas, Hodgkin lymphoma, B-cell lymphomas, and other lymphomas).

Results

Population characteristics

All patients included in the EBVST immunotherapy study were diagnosed with EBV-associated lymphoma (n = 56) and were classified as either responders (nonprogressors; n = 36) or nonresponders (progressors or stable disease; n = 20) at the end of clinical trial follow-up (Table 1). No differences in sex (*P* = .6), age (*P* = .6), race/ethnicity (*P* = .8), or lymphoma diagnosis category (*P* = .7) were observed between responders and nonresponders.

Significantly higher peripheral blood EBV DNA level was observed in nonresponders at 3 months after infusion than in responders (*P* = .012; supplemental Figure 1). At earlier time points (before

infusion, 2 weeks, and 4 weeks), EBV DNA level was also higher in nonresponders, but these differences were not statistically significant ($P > .05$).

EBV proteome-wide analysis of virus-directed humoral response before and after EBVST infusions

We assessed differences in the mean SSI for 74 IgG and 202 IgA Abs between responders (n = 36) and nonresponders (n = 20) to EBVST immunotherapy using unpaired *t* tests at preclinical treatment (baseline) and at 3 time points after treatment (2 weeks, 4 weeks, and 3 months; Figure 1; Table 2). Before treatment, nonresponders had higher levels of IgG Abs against EBV nuclear antigen leader protein (EBNA; nominal $P = .03$; *t* test) and IgA Abs for lytic gene BGLF3.5 (nominal $P = .04$; *t* test) than responders (Table 2).

Two weeks after immunotherapy treatment, nonresponders exhibited elevated levels of IgG Abs against LMP1 (nominal $P = .01$; *t* test), BGLF3.5 (nominal $P = .03$; *t* test), and 2 variants of BMRF1 (nominal $P = .04$; and $P = .05$; *t* test), as well as IgA Abs against BGRF1/BDRF1 (nominal $P = .01$; *t* test) and EBNA3A (nominal $P = .02$; *t* test). At 4 weeks after treatment, nonresponders had higher level of IgA Abs against BPLF1 (nominal $P = .02$; *t* test).

At 3 months after EBVST infusion, nonresponders exhibited more pronounced IgG responses, whereas IgA responses did not show similar differences (Figure 1). Specifically, 36 IgG Abs were higher among nonresponders (nominal $P < .05$; *t* test; Table 2). After adjusting for multiple testing using the Benjamini and Hochberg FDR, 25 IgG Abs at the 3-month postinfusion time point reached borderline significance (FDR = 0.058). No other marker/time point combinations reached significance after FDR correction.

Logistic regression models adjusted for sex, age, and diagnosis type were then applied to the Abs with a nominal *P* value <.05 (supplemental Table 1). At 3 months after treatment, a total of 10 IgG Abs (8 of the 36 IgG Abs identified by *t* test and 2 additional IgG Abs identified by logistic regression) were present at higher levels in nonresponders (nominal $P < .05$; logistic regression; supplemental Table 1). Random Forest analysis was then applied to identify the most important Abs for predicting clinical outcomes using the mean decrease in Gini and mean decrease in accuracy metrics[24] at the 3-month post–EBVST infusion time point. Abs scoring high for both metrics, indicating their status as important predictors of clinical response outcome, were visualized on a multiway importance plot (Figure 2). The 10 Abs significantly elevated at the 3-month time point (by nominal *P*-value) in the logistic regression models were also identified as important predictors in the random forest model, demonstrating robust agreement between the 2 methods. These IgG Ab responses were directed against LMP2A (4 fragments), BGRF1/BDRF1 (2 fragments), LMP1, BKRF2, BKRF4, and BALF5; and were visualized on a multiway importance plot (Figure 2).

Correlation analysis between these 10 EBV-specific Abs and EBV DNA level revealed moderate to strong positive correlations in nonresponders, particularly for 1 fragment of LMP2A (Spearman $\rho = 0.608$; $P = .04$), 1 fragment of BGRF1/BDRF1 ($\rho = 0.587$; $P = .049$), and BKRF4 ($\rho = 0.629$; $P = .032$; supplemental Table 3). In contrast, these correlations were not observed in responders, for whom the associations were weak and not statistically significant ($P > .1$).

Changes in EBV-directed Ab response during EBVST infusion treatment

Total change in Ab response between before infusion and 3 months after infusion (Ab_{3mo}–Ab_{pre}) was evaluated among responders (nonprogressors) and nonresponders (progressors). Only individuals with paired IgG (n = 41) and paired IgA data (n = 42) at both time points were included in the analysis.

Total change in 6 anti-EBV Abs (3 IgA [BGLF3, BALF2, and BBLF2/3] and 3 IgG [BGLF2, LF1, and BGLF3]) was associated with treatment response (nominal $P < .05$; logistic regression) when adjusted for sex, age, and diagnosis (Table 3); however, none of them retained significance after FDR correction. Ribbon plots in Figure 3 show the changes in average responses per each Ab marker. Notably, the nonresponder group exhibited increased mean Ab responses at 3 months compared with before treatment for all 6 Ab markers, indicating the elicitation of robust Ab responses for EBVST infusions in individuals who did not clinically respond to treatment (nonresponders) with progressive or stable disease.

One distinctive observation was that the consistent association between total change in both IgG ($P = .046$; OR, 0.075; 95% CI, 0.004-0.713) and IgA ($P = .036$; OR, 0.011; 95% CI, 0, 0.564) Ab responses to BGLF3 and clinical nonresponse. BGLF3 IgG was also elevated at the 3-month time point in the nonresponder group in univariate analyses (nominal $P < .05$; *t* test) and was identified as a key predictor by random forest metrics (supplemental Table 1).

Discussion

EBV-positive tumors express viral latency–associated antigens that can be targeted for T-cell immunotherapy. The adoptive transfer of EBVSTs has proven to be a promising treatment option for immunogenic type 3 latency-derived PTLD, commonly occurring in transplant recipients with compromised immune systems.[12] Although previous studies have explored peripheral autoantibodies against tumor-associated proteins as markers for cancer and predictors of clinical outcomes,[25-27] recent research on EBVSTs has primarily focused on a limited set of viral antigens expressed during type 2 latency, such as LMP1, LMP2, and EBNA1.[28]

Broadening the spectrum of viral antigens by proteome-wide profiling offers a promising approach for identifying EBV-directed antigens that can best predict clinical responses for EBV-associated lymphoma. This strategy can also identify an expanded set of target antigens for EBVSTs, potentially improving complete response rates and achieving nonprogressive disease. To address this, we used a custom protein microarray consisting of predicted sequences from the complete EBV proteome to measure IgG and IgA Ab responses. To the best of our knowledge, this is the first report comprehensively evaluating patterns of anti-EBV Ab responses in patients with EBV-positive lymphoma treated with EBVST infusions.

Overall, our findings reveal that patients with positive clinical outcomes (responders/non-progressors) to EBVST immunotherapy were characterized by a low level of anti-EBV Ab responses, whereas patients who did not respond to treatment (nonresponders) with progressive or stable disease had notably higher

Table 2. EBV proteins on microarrays (microarray sequence, protein name, and EBV life cycle) for IgG and IgA Abs with evidence of differential levels between responders and nonresponders to EBVST immunotherapy, by time point

Array sequence	Protein name	Life cycle	*t* test *P* value	FDR adj *P* value	Responder group mean	Nonresponder group mean	IgG/ IgA
Pre-EBVST immunotherapy							
YP_001129440.1-20824-20955	EBNA-LP	Latent	.03	.998	1.04	1.29	IgG
YP_001129483.1-112496-112035	BGLF3.5	Late lytic	.04	.903	1.15	1.26	IgA
2 weeks after EBVST immunotherapy							
YP_001129485.1-117754-118890	BGRF1/BDRF1	Late lytic	.01	.998	0.97	1.01	IgA
AFY97906.1-168167-168081	LMP1	Latent	.01	.411	0.99	1.30	IgG
YP_001129463.1-80447-82888	EBNA3A	Latent	.02	.998	1.23	1.36	IgA
YP_001129483.1-112496-112035	BGLF3.5	Late lytic	.03	.719	0.76	0.92	IgG
AFY97929.1-67486-68700	BMRF1	Early lytic	.04	.719	1.83	2.19	IgG
YP_001129454.1-67745-68959	BMRF1	Early lytic	.05	.719	1.97	2.30	IgG
4 weeks after EBVST immunotherapy							
YP_001129449.1-59370-49906-3	BPLF1	Late lytic	.02	.999	0.86	0.99	IgA
3 months after EBVST immunotherapy							
YP_001129436.1-871-951	LMP2A	Latent	<.01	.058	1.10	1.39	IgG
YP_001129455.1-68964-70037	BMRF2	Glycoprotein	<.01	.058	1.14	1.38	IgG
YP_001129436.1-360-458	LMP2A	Latent	<.01	.058	1.09	1.31	IgG
YP_001129472.1-98500-98913	BKRF2	Glycoprotein	.01	.058	0.92	1.18	IgG
YP_001129436.1-540-788	LMP2A	Latent	.01	.058	0.94	1.15	IgG
YP_001129484.1-113481-112483	BGLF3	Late lytic	.01	.058	1.31	1.58	IgG
YP_001129506.1-154125-153187	BILF1	Glycoprotein	.01	.058	1.14	1.37	IgG
YP_001129479.1-107679-108896	BBRF3	Glycoprotein	.01	.058	1.53	1.97	IgG
YP_401715.1-160908-158851	BALF3	Late lytic	.01	.058	1.23	1.46	IgG
CAA24827.1-122341-120929	BGLF5	Early lytic	.01	.058	0.91	1.09	IgG
YP_001129461.1-76771-77259	BLRF2	Late lytic	.01	.058	2.18	2.61	IgG
CAA24860.1-102116-101445	BZLF2	Glycoprotein	.01	.058	1.95	2.43	IgG
YP_001129485.1-117754-118890	BGRF1/BDRF1	Late lytic	.01	.058	0.87	1.11	IgG
YP_001129466.1-90630-89959	BZLF2	Glycoprotein	.01	.058	2.02	2.47	IgG
YP_001129500.1-136454-135636	BVLF1	Late lytic	.01	.058	1.24	1.45	IgG
YP_001129504.1-151808-150519	LF2	Early lytic	.02	.058	1.14	1.38	IgG
YP_001129436.1-1026-1196	LMP2A	Latent	.02	.058	1.05	1.24	IgG
AFY97988.1-166888-166706	BNLF2A	Late lytic	.02	.058	1.17	1.36	IgG
YP_001129440.1-35441-35473	EBNA-LP	Latent	.02	.058	0.83	1.09	IgG
YP_001129470.1-94844-96457	BRRF2	Late lytic	.02	.058	2.15	2.54	IgG
YP_001129496.1-131574-129454	BXLF2	Glycoprotein	.02	.058	1.35	1.54	IgG
YP_001129498.1-133398-134144	BXRF1	Late lytic	.02	.058	1.12	1.33	IgG

FDR correction method used was Benjamini and Hochberg. The table is ordered by *t* test *P* values (lowest to highest). *t* test *P* values < .05 are considered nominally significant for this analysis, with FDR-corrected values considered statistically significant.

adj, adjusted.

Table 2 (continued)

Array sequence	Protein name	Life cycle	*t* test *P* value	FDR adj *P* value	Responder group mean	Nonresponder group mean	IgG/ IgA
YP_001129486.1-115415-114405	BGLF2	Early lytic	.02	.058	1.16	1.39	IgG
YP_001129439.1-9659-10171	BcRF1	Late lytic	.02	.058	1.12	1.31	IgG
YP_001129512.1-166530-167195	BARF1	Early lytic	.02	.059	1.13	1.31	IgG
CAA24838.1-61507-62037	BFRF3	Late lytic	.02	.061	2.25	2.59	IgG
YP_001129448.1-49335-49865	BFRF3	Late lytic	.03	.078	2.37	2.70	IgG
CAA24829.1-124938-125915	BGRF1/BDRF1	Late lytic	.03	.078	0.92	1.15	IgG
YP_001129488.1-117560-116883	BDLF4	Early lytic	.03	.078	1.48	1.75	IgG
YP_001129438.1-1736-5692-2	FGAM	Other/unknown	.03	.078	1.35	1.65	IgG
AFY97924.1-49199-49729	BFRF3	Late lytic	.03	.078	2.27	2.59	IgG
YP_001129489.1-117772-117539	BDLF3.5	Glycoprotein	.03	.078	0.91	1.11	IgG
AFY97906.1-168167-168081	LMP1	Latent	.04	.082	1.09	1.34	IgG
YP_001129440.1-20824-20955	EBNA-LP	Latent	.04	.082	0.97	1.31	IgG
YP_001129454.1-67745-68959	BMRF1	Early lytic	.04	.082	1.98	2.36	IgG
CAA24839.1-71527-62078-3	BPLF1	Late lytic	.05	.093	0.76	0.96	IgG

FDR correction method used was Benjamini and Hochberg. The table is ordered by *t* test *P* values (lowest to highest). *t* test *P* values < .05 are considered nominally significant for this analysis, with FDR-corrected values considered statistically significant.
adj, adjusted.

anti-EBV Abs, particularly at 3 months after treatment. Abs serve as indicators of exposure, and elevated Ab responses in individuals who did not respond to EBVST infusions suggest that they are experiencing uncontrolled EBV infection. Although we did not directly measure EBV DNA levels in circulation, we measured EBV DNA levels in PBMCs to estimate the frequency of EBV-infected cells. Significantly higher EBV DNA level was observed in nonresponders than responders at 3 months after infusion, reflecting a higher number of infected cells or increased viral replication within these cells in nonresponders.

Our data are consistent with previous reports showing persistently high EBV viremia levels in patients with PTLD who poorly responded to immunotherapy[11,29] but offer a more detailed assessment of a specific set of EBV antigens, whose lack of control is associated with poor clinical outcome.

IgG Ab targeting multiple LMP2A sequences differed by clinical response. LMP2A is a facilitator of B-cell survival, promoting viral persistence and supporting B-cell activation and transformation.[30-33] LMP1 expression is identified as a surrogate marker of EBV in Hodgkin lymphoma and, as an oncogene, contributes to cell proliferation and survival in EBV-associated malignancies while suppressing apoptosis.[34-36] As such, elevated expression in nonresponders is biologically plausible and warrants further refined targeting efforts in EBVST therapeutic approaches.

Multiple associations between elevated Ab response and nonresponders/progressors were also observed for BGRF1/BDRF1, a gene set encoding DNA packaging terminase subunit, which is believed to play a significant role in capsid assembly and/or stability and in nuclear egress.[37,38]

In addition, noteworthy antibodies are the 3 IgG (BGLF2, LF1, and BGLF3) and 3 IgA (BGLF3, BALF2, and BBLF2/3) identified as nominally elevated between the preinfusion and 3-month postinfusion time points, as well as being associated with nonresponders. Interestingly, BGLF3 demonstrated consistent association with clinical response, not just in relation to Ab change but also with elevated average Ab level measured 3 months after EBVST infusion. BGLF3 is part of a viral preinitiation complex essential for regulating the expression of late EBV gene expression.[39]

Collectively, our findings revealed a trend of consistently elevated Ab responses against a subset of oncogenic latent, early, and late lytic cycle antigens of EBV in patients with progressive or stable disease (nonresponders of EBVST infusions). These results align with previous studies demonstrating that several EBV oncogenic and lytic transcripts are abundantly expressed in EBV-positive lymphomas, including peripheral T-cell lymphoma,[40] Burkitt lymphoma,[41] Hodgkin lymphoma,[42] and other EBV-associated malignancies, such as gastric cancers[43] and nasopharyngeal carcinoma.[44,45] Interestingly, some of those EBV-specific Ab responses demonstrated a moderate to strong positive correlation with EBV DNA levels in nonresponders, particularly for LMP2A, BGRF1/BDRF1, and BKRF4. This relationship suggests that elevated virus-infected cells may drive the production of specific Abs targeting viral latency and lytic antigens, either directly or indirectly, by contributing to virus replication in the oropharynx (or in other compartments), possibly as a compensatory mechanism for the immune system's inability to control the infection. In contrast,

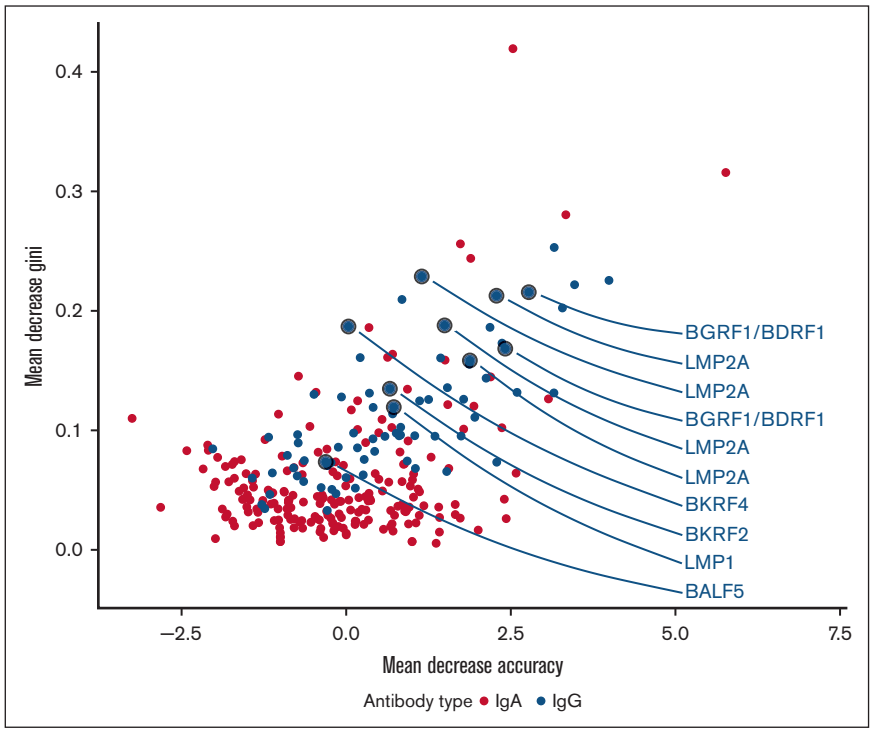

Figure 2. Multiway variable importance plot of IgA and IgG responses at 3 months after infusion (mean decrease accuracy and mean decrease Gini). This figure illustrates the importance of individual Abs in differentiating between responders and nonresponders, as assessed using the random forest model. The x-axis represents the mean decrease in accuracy, and the y-axis represents the mean decrease in Gini. Higher values on both axes indicate greater importance in the model. Abs in the upper right of the plot are identified as important by both metrics, indicating more substantial contributions to model performance. Notably, Abs that were nominally significant at the 3-month time point ($P < .05$) in the logistic regression models are also among the most important variables identified by the random forest as the most important variables, demonstrating robust agreement across methods.

these correlations were weak and not statistically significant in responders, further suggesting that a controlled immune response, rather than excessive Ab production, is critical for favorable clinical outcomes.

Overall, these findings underscore the importance of broadening the target antigen–specific repertoire of EBVSTs for immunotherapy, because targeting a wider range of latent and lytic antigens may enhance the ability to reactivate the immunosuppressive tumor microenvironment, promote epitope spreading, and improve clinical response in patients with EBV-associated lymphomas.

Although the identified EBV antigens were associated with differential Ab responses and correlated with the elevated presence of circulating virus-infected cells in nonresponders, their potential as therapeutic targets for immunotherapy requires further study. These antigens may guide the design of EBV-specific T-cell therapies or vaccines, but additional research is needed to confirm their expression in tumors and their immunogenicity across diverse populations. Such work is critical for establishing their clinical utility.

The contribution of B cells and B-cell–mediated Ab responses, representing the humoral arm of the adaptive immune system, to antitumor activity and immunotherapies has not been extensively explored in immune-oncology research.[46] Although B cells are known to play roles in tumor immunology, including antigen presentation and production of tumor-specific Abs,[47,48] their function in facilitating antitumor responses, including Ab-dependent cell cytotoxicity and complement cascade activation, are not fully elucidated.[49-51] Th2-mediated immunity, characterized by the production of interleukin-4 (IL-4), IL-5, and IL-13, has been traditionally considered to facilitate tumor growth by promoting angiogenesis and inhibiting cell-mediated immunity, with several studies supporting that humoral immunity can have a negative impact on antitumor immunity.[52,53] In this study, elevated EBV-specific Ab levels in nonresponders suggest a Th2-skewed immune response, which may have compromised the Th1-mediated cellular immunity necessary for effective viral and tumor control. Th1 responses, marked by interferon gamma production and cytotoxic T-cell activation, are critical for the elimination of EBV-infected and tumor cells.[3,7] The observed Ab elevation in nonresponders, together with the moderate to strong positive correlations between these Abs and EBV DNA levels, supports the hypothesis that persistent viral activity and immune dysregulation are driving a Th2-dominant response in these individuals. This Th2 polarization may undermine the efficacy of EBVSTs, which rely on robust Th1-driven cellular immunity to target and destroy EBV-infected cells.

Further investigation into the cytokine profiles and immune cell subsets in these patients would be valuable to explore strategies for improving therapeutic outcomes. One limitation of our study is the small sample size, which reflects the constraints of phase 1 clinical study designs. As such, it is crucial to validate and extend our initial observations in larger, independent patient cohorts from other clinical trials involving EBVST immunotherapy. Additionally, incorporating separate cohorts of patients treated with R-CHOP (rituximab, cyclophosphamide, doxorubicin, Oncovin [vincristine], and prednisone) or other therapeutic modalities, such as targeted therapies or alternative chemotherapy regimens, would provide valuable insights into the specificity of the identified Ab responses. Such analyses could determine whether these responses are unique to EBVST immunotherapy or also observed in patients undergoing conventional treatments. This would ensure the robustness and generalizability of our findings across different treatment contexts.

Table 3. ORs for EBV-directed Abs with evidence of an association between clinical response and the Ab change between preinfusion and 3-month postinfusion (Ab_{3mo}–Ab_{pre}) time points

Array sequence	Protein name	Life cycle	IgG/IgA	Logistic regression *P* value	*P* value (FDR)	OR (95% CI)
YP_001129484.1-113481-112483	BGLF3	Late lytic	IgA	.036	.889	0.011 (0-0.564)
YP_001129510.1-165796-162410-2	BALF2	Early lytic	IgA	.036	.889	0 (0-0.154)
AFY97950.1-104968-104363	BBLF2/3	Early lytic	IgA	.043	.889	0 (0-0.324)
YP_001129486.1-115415-114405	BGLF2	Early lytic	IgG	.042	.359	0.06 (0.002-0.681)
YP_001129505.1-153178-151769	LF1	Other/unknown	IgG	.045	.359	0.036 (0.001-0.7)
YP_001129484.1-113481-112483	BGLF3	Late lytic	IgG	.046	.359	0.075 (0.004-0.713)

FDR correction method used was Benjamini and Hochberg. The table is ordered by *P* value (lowest to highest). Logistic regression *P* values < .05 were considered nominally significant for this analysis.

We also acknowledge the existence of several compartments of EBV-infected cells in the body, including EBV-infected B cells in lymphoid tissues, EBV-infected epithelial cells in the oropharynx, and EBV-infected tumor cells. The Ab response measured in the blood likely reflects a combination of these as a systemic response but is not a direct measurement of any given compartment. Although blood-based Abs can serve as accessible markers, further tumor-based studies are needed to understand whether any of our observations reflect the viral proteins expressed in the tumor. In summary, our findings revealed a trend of consistently elevated Ab response to EBV antigens in patients who did not respond to EBVST immunotherapy with progressive or stable disease compared with responders.

Although these results suggest a potential association between elevated Ab responses and poor clinical outcomes, none of the findings retained statistical significance after FDR correction, highlighting the exploratory nature of this study.

We propose that the identified set of elevated anti-EBV Abs that distinguished responders/nonprogressors from nonresponders/progressors could serve as predictive serological markers of clinical outcome after EBVST infusion in individuals diagnosed with

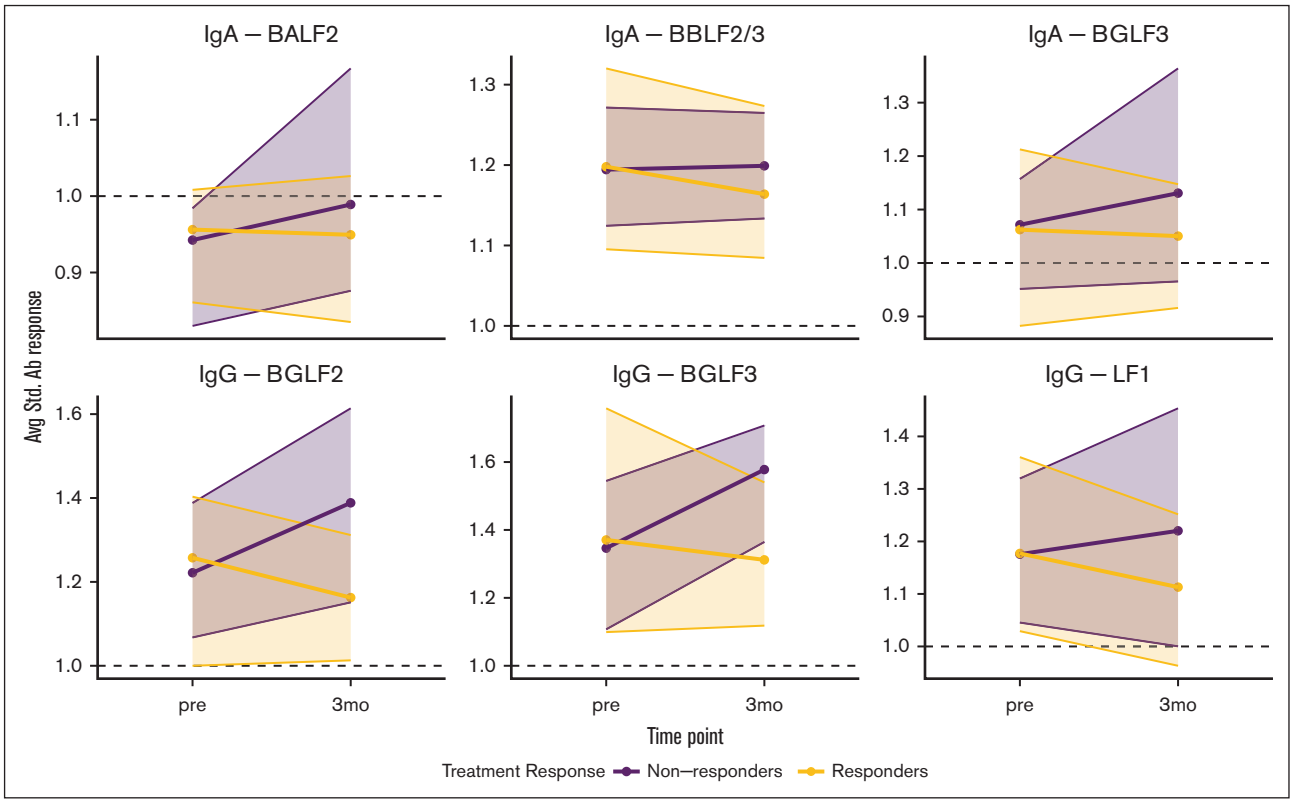

Figure 3. Change in Ab response between preinfusion and 3 months postinfusion. Ribbon plots showing the mean change (with 25th to 75th percentile range) in Ab response (SSI) for EBV-specific antibody markers in responders (n = 36; yellow) and nonresponders (n = 20; purple). Only antibodies with nominal *P* value of < .05 in adjusted logistic regression models are shown.

EBV-associated lymphomas. Further research is required to validate these initial observations in larger patient cohorts and using independent methodologies. Such efforts will be critical for enhancing our understanding of the underlying mechanisms and for optimizing EBVST immunotherapy and other EBV-directed therapeutic strategies.

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Authorship

Contribution: Y.D.S. performed protein microarray studies; Y.D.S., C.P., and N.W.V.B. performed statistical analyses; Y.D.S., N.W.V.B., A.E.C., D.L.D., and C.P. wrote the manuscript; C.M.R., R.H.R., and H.E.H. designed the clinical research study; C.M.R., R.H.R., H.E.H., and A.E.C. selected samples for laboratory study; D.L.D. and A.E.C. designed and supervised this study and acquired funding; D.L.D., C.P., and A.E.C. designed and supervised statistical analyses; Y.D.S., N.W.V.B., Z.L., A.E.C., C.P., and D.L.D. interpreted data and critically reviewed the manuscript; and all authors reviewed and approved the final manuscript.

Conflict-of-interest disclosure: H.E.H. has equity in AlloVir and Marker Therapeutics, and has served on the advisory boards for Tessa Therapeutics, March Biosciences, and Fresh Wind Biotechnologies. C.M.R. has equity in AlloVir and Marker Therapeutics; has served on the advisory boards for Tessa Therapeutics and Marker Therapeutics; has received research support from Tessa Therapeutics; and her spouse has interests in Walking Fish Therapeutics, Abintus, Allogene, Memgen, Turnstone Biologics, Coya Therapeutics, TScan Therapeutics, Oncimmune, and Poseida Therapeutics. Z.L. is currently employed by Merck & Co, Inc. The remaining authors declare no competing financial interests.

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