The Epstein-Barr virus lytic protein BZLF1 as a candidate target antigen for vaccine development

Alex S. Hartlage*,2, Tom Liu*,2, John T. Patton†, Sabrina L. Garman*, Xiaoli Zhang‡, Habibe Kurt§, Gerard Lozanski§, Mark E. Lustberg§, Michael A. Caligiuri*†,2,3 and Robert A. Baiocchi*†,2,3

*The Comprehensive Cancer Center and Arthur G. James Cancer Hospital and Solove Research Institute; †Division of Hematology, Department of Internal Medicine, College of Medicine; ‡Center for Biostatistics; §Department of Pathology; ⋆Division of Infectious Disease; The Ohio State University, Columbus, Ohio 43210.

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2These authors contributed equally to this work.

3Address correspondence and reprint requests to Drs. Robert A. Baiocchi at Robert.baiocchi@osumc.edu or Michael A. Caligiuri at Michael.caliguri@osumc.edu; The Comprehensive Cancer Center and Arthur G. James Cancer Hospital and Solove Research Institute, The Ohio State University, 300 W. 10th Ave, Suite 521B, Columbus, OH 43210. Phone: (614)-685-5667; Fax: (614)-292-3312.
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Abstract

The Epstein-Barr virus (EBV) is an oncogenic, γ-herpesvirus associated with a broad spectrum of disease. While most immune-competent individuals can effectively develop efficient adaptive immune responses to EBV, immunocompromised individuals are at serious risk for developing life threatening diseases such as Hodgkin’s lymphoma and post-transplant lymphoproliferative disorder (PTLD). Given the significant morbidity associated with EBV infection in high-risk populations, there is a need to develop vaccine strategies that restore or enhance EBV-specific immune responses. Here, we identify the EBV immediate-early protein BZLF1 as a potential target antigen for vaccine development. Primary tumors from patients with PTLD and a chimeric human-murine model of EBV-driven lymphoproliferative disorder (EBV-LPD) express BZLF1 protein. Pulsing human dendritic cells (DC) with recombinant BZLF1 followed by incubation with autologous mononuclear cells led to expansion of BZLF1-specific CD8\(^{(+)}\) T cells \textit{in vitro} and primed BZLF1-specific T-cell responses \textit{in vivo}. In addition, vaccination of hu-PBL-SCID mice with BZLF1-transduced DCs induced specific cellular immunity and significantly prolonged survival from fatal EBV-LPD. These findings identify BZLF1 as a candidate target protein in the immunosurveillance of EBV and provide rationale for considering BZLF1 in vaccine strategies to enhance primary and recall immune responses and potentially prevent EBV-associated diseases.
Introduction

The Epstein-Barr virus (EBV) is a B-cell-lymphotropic, γ-herpesvirus that infects greater than 90% of the world’s population. EBV causes infectious mononucleosis (IM) and has a clear association with several lymphoid and epithelial-derived malignancies including Burkitt’s lymphomas, Hodgkin’s disease, and gastric and nasopharyngeal carcinomas (1). Following primary infection, the virus establishes lifelong persistence within the host memory B-cell compartment utilizing restricted latent gene expression programs that can manifest as either resting state (Lat I) or cellular immortalization and transformation programs (Lat II, III) (2-5). In immune-competent hosts, the outgrowth of EBV-immortalized B cells is prevented through a robust expansion of EBV-specific memory T cells directed toward both lytic and latent antigens (6). EBV-positive individuals that possess either congenital or acquired immune deficiencies are thus highly susceptible to viral reactivation and malignant transformation. Furthermore, EBV-sero-positive patients treated with immune suppressive medications following organ/stem-cell transplantation are at significant risk for developing post-transplant lymphoproliferative disorder (PTLD), an often fatal B-cell lymphoproliferative disease (LPD) (7).

Current treatment options for these patients are limited, and while chemo/immunotherapeutic reagents can induce tumor remission, these therapies often lead to opportunistic infections and short disease-free survival due to ablation of EBV-specific immunity (8). Thus, new therapeutic and preventive approaches are needed that specifically target EBV-infected cells while promoting long-term, protective immunity. The development of adoptive cellular therapeutic strategies has proven to be effective (9-11); however, these options have limited availability, are not yet universally applicable, and may be impractical for preventing EBV-associated diseases in
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solid organ transplant patients. Alternatively, the development of vaccination strategies to enhance primary and recall memory immune responses are becoming increasingly considered as both therapeutic and preventive approaches to EBV-associated diseases including IM (12-15). However, progress in this area has been markedly slow, partly because biomarkers that correlate with host protection are not fully known.

The immediate-early protein BZLF1 is a transcriptional activator that initiates the disruption of latency in EBV-infected cells. Upon expression, BZLF1 binds to the promoters of early EBV lytic genes, triggering a signaling cascade that results in viral DNA replication and virion production (16-19). BZLF1 has also been shown to inhibit antiviral cytokine signaling (20, 21), disrupt CD4\(^{(+)}\) T-cell recognition of MHC-II molecules (22), and promote B-cell transformation and lymphomagenesis in a humanized NOD/LtSz-scid/IL2R\(_{γnull}\) mouse model of EBV-related disease (23). Thus, BZLF1 expression is critical for viral activation, persistence, and disease pathogenesis, and, from an immunological standpoint, may represent a prime target of adaptive immune responses. Indeed, BZLF1 is highly immunogenic and elicits robust CD4\(^{(+)}\) and CD8\(^{(+)}\) T-cell responses (6, 24, 25) that dominate the early immune responses in IM patients and produce a healthy pool of EBV-specific memory T cells (26). Furthermore, we have shown that prevention of EBV-LPD in vivo is associated with the expansion of CD3\(^{(+)}\)CD8\(^{(+)}\) T cells specific for BZLF1 (27). To the best of our knowledge, the potential therapeutic and preventative effects of BZLF1 vaccination have not been explored.

Here we show that BZLF1 is expressed in EBV-LPD and is capable of eliciting a robust, memory T-cell response in healthy individuals. Furthermore, we demonstrate that presentation of
full length BZLF1 by human dendritic cells (DC) promotes the expansion of antigen-specific T-cell immunity and prolongs survival in a humanized, spontaneous murine model of EBV-LPD. These data suggest that BZLF1 is a critical target protein triggering immunosurveillance of EBV-infected cells and should be considered as a candidate for strategies to develop an EBV vaccine.
Materials and Methods

Examination of EBV-LPD tumor expression: De-identified, pathologist-verified EBV-LPD tumor samples were obtained from The Ohio State University Pathology Core Facility (Columbus, OH). Patient and hu-PBL-SCID EBV-LPD tumors were stained with anti-BZLF1 (1:300) (Santa Cruz) and visualized by immunohistochemistry according to IRB-approved protocol.

ELISPOT assays: Secretion of human interferon-gamma (IFNγ) was detected with the Human IFNγ enzyme-linked immunosorbent spot (ELISPOT) kit (Mabtech) using MultiScreen-HA plates (Millipore, Bedford, MA) as per manufacturer’s instructions. Spots were visualized and counted using an Immunospot Imaging Analyzer (Cellular Technology Ltd., Cleveland, OH). Spot forming units were calculated as the ratio between spots formed in response to antigen stimulation and spots formed in response to anti-CD3 monoclonal antibody (mAb) stimulation.

Mice: Six-week-old C.B.-17 SCID mice were purchased from Taconic Farms (Germantown, Pennsylvania, USA) and housed in a specific pathogen-free environment. Animals showed no evidence of leaky phenotype (28) as determined by murine IgG ELISA (29). Animal work was approved by The Ohio State University Institutional Animal Care and Use Committee.

Flow Cytometric Analyses: Monoclonal antibodies specific for the following antigens were used: anti-human CD3, CD8, and IFNγ (BD Bioscience). Human HLA-B8 tetramers complexed with allele immunodominant peptides for BZLF1 (RAKFKQLL) and EBNA-3A (FLRGRAYGL) and conjugated with APC were provided by the NIAID Tetramer Facility and
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the NIH AIDS Research and Reference Reagent Program (Atlanta, Georgia, USA) (27). Events were collected on an FC500 flow cytometer (Beckman-Coulter) and analyzed with FlowJo v7.6.1 (TreeStar) software.

**Lymphoblastoid cell line generation and co-culture:** EBV-transformed lymphoblastoid cell lines (LCL) were derived *in vivo* by engrafting SCID mice with human peripheral blood mononuclear cells (PBMC) from healthy EBV-positive donors as described (30). Co-cultures for ELISPOT and IFNγ flow assays were created in 96-well U-bottom plates by combining irradiated LCLs (14,000 rad) with equal numbers of autologous PBMCs. Cultures were maintained in RPMI-1640 containing 10% FBS supplemented with antibiotics and 1% Glutamax (Life Technologies) in the presence of 10 U/ml interleukin-2 (IL2; Prometheus, San Diego, CA).

**Cloning, expression, and purification of recombinant (r) BZLF1:** The open reading frame sequence of EBV BZLF1 was amplified by RT-PCR using RNA isolated from an EBV-positive LCLs; forward primer: ATGATGGACCCAAT, reverse primer: TTAGAAAATTAGAG. The amplified product was cloned into Nde I/Not I sites of the pET26b(+) vector (Novagen), and subsequently introduced into E. coli strain BL26 (Invitrogen) by standard transformation. Expression of BZLF1 protein was induced by 1.5 nM IPTG treatment for 3 hrs. In collaboration with ARVYS Proteins Inc. (Stamford, CT), milligram quantities of low endotoxin-rBZLF1 protein (GMP-grade materials) were purified by a method comprising recovery and washing of inclusion bodies, two gel filtration chromatography steps, and filtration through anion-exchange filters. The identity of rBZLF1 protein was confirmed by mass spectrometry (Nano-LC/MS/MS system).
Generation of rAd5F35/BZLF1 virus: The rAd5F35/BZLF1 adenovirus was generated using the Adeno-X expression system 1 (Clontech Laboratories, Inc.). In brief, BZLF1 cDNA was cloned into Not I sites of the mammalian expression cassette pShuttle2. The I-CeuI/PI-SceI fragment containing the BZLF1 expression cassette was gel-purified and subcloned into I-CeuI/PI-SceI sites of the AdF35 genome (a gift from Dr. Malcolm Brenner, the Baylor College of Medicine) in which the fiber gene of adenovirus serotype 35 is incorporated into an Ad5 capsid. Finally, Pac-I digested pAd5F35/BZLF1 DNA was used to transfect HEK 293 cells. The individual viral plaque was isolated and positive viral clones were used for large-scale production of rAd5F35/BZLF1 stock. The viral titer was determined using the Adeno-X rapid titer kit (Clontech Laboratories, Inc).

Generation of rAAV2/BZLF1 virus: The rAAV2/BZLF1 virus consists of rAAV terminal repeats flanking a CMV-IE promoter BZLF1 gene expression cassette. To produce the rAAV2/BZLF1 vector, a Hela-derived cell line containing the above sequences, the AAV rep and cap genes, and a neomycin resistant selectable marker was constructed as described (31). The rAAV2/GFP virus is identical to rAAV2/BZLF1 except the GFP gene is driven by the CMV-IE promoter. Large-scale rAAV2 viral preparation, purification, and quantification were performed as described (32-34).

In vitro culture and infection of 293T cells: The human amphotropic-packaging cell line HEK 293T (ATCC®) was maintained in a 37°C humidified environment supplemented with 5% CO2 in DMEM media containing 5% FBS and grown to 80% confluence prior to infection. Following
incubation, cells were cultured in the presence of rAAV2/BZLF1 (MOI=10) or rAAV2/GFP (MOI=10), or rAd5F35/BZLF1 (MOI=10) or rAd5F35/null (MOI=10) for 3 hr.

**Immunoblotting:** All immunoblotting was performed as described (35) using antibodies directed against BZLF1 (Santa Cruz) and actin (Santa Cruz). Staining of SDS-PAGE gels were performed using Coomassie Brilliant Blue Staining Solution (Bio-Rad).

**In vitro DC generation and culture:** PBMCs were obtained from healthy donors under an OSU Institutional Review Board-approved protocol. Following Ficoll-Hypaque (GE healthcare Bioscience AB) separation and incubation of $5 \times 10^6$ cells/ml in serum-free media for 2 hr at 37°C, the supernatant containing non-adherent cells was removed. Adherent monocytes were cultured for 3 days with human GM-CSF (50 ng/ml; Peprotech) and IFN-α (1000 U/ml; Schering Corporation, Kenilworth, NJ) in the presence of rBZLF1 protein or an equivalent amount of bovine serum albumin (BSA) (control), or rAd5F35/BZLF1 (MOI=10) or rAd5F35/null (MOI=10; Vector Biolabs), or rAAV2/BZLF1 or rAAV2/GFP, respectively (36). DCs were then co-cultured with autologous HLA-B8(+) PBMCs at a ratio of 1:5 in the presence of 10 IU/mL IL2 for 10 days.

**In vivo studies:** The engrafted humanized SCID (hu-PBL-SCID) mouse model of EBV-LPD has been described (37). Six-week-old SCID mice were injected IP with $5 \times 10^7$ PBMCs that were obtained from a healthy HLA-B8(+) EBV-seropositive donor. For depletion of endogenous murine NK cells, SCID mice were injected IP with anti-asialo-ganglioside 1 antiserum (ASGM-1, Wako Chemicals, Richmond, Virginia, USA) one day before injection of human PBMCs and
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every seven days thereafter for the duration of the study. Engraftment was confirmed by human IgG ELISA as described (37). IP vaccination with DCs previously co-cultured with rBZLF1 or control BSA or virally-transduced DCs (described above) began immediately following initial engraftment.

**Statistical analyses:** For the comparisons of IFNγ secretion in Figure 2, Figure 3E, and Figure 5A, two sample t-tests were used. Log-rank tests were used for the survival analyses in Figure 5 and Kaplan-Meier survival curves were generated to display the results. All analyses were performed using SAS/STAT software version 9.2 (SAS Institute Inc., Cary, NC).
Results

**BZLF1 is expressed in human EBV-LPD.** Recognition of EBV-encoded epitopes by effector lymphocytes is a major component of the adaptive immune system’s ability to clear lytic and latently infected B-cells. To determine whether BZLF1 is expressed in EBV(+) malignancies, we performed immunohistochemistry (IHC) assays on primary EBV(+) lymphoma tumor samples using an antibody specific for BZLF1. Of the tumors analyzed, 6/8 PTLD samples stained positive for BZLF1 (Figure 1A and Table I). Other EBV(+) lymphomas staining for BZLF1 included lymphomatoid granulomatosis (1/3), diffuse large B-cell lymphoma (5/10), and double hit lymphoma (1/1). Both EBV(+) Hodgkin’s lymphoma (6 cases) and EBV(+) unclassifiable lymphomas (8 cases) failed to demonstrate any BZLF1 signal. We next determined whether BZLF1 is expressed in the EBV-LPD tumors that spontaneously developed in hu-PBL-SCID mice engrafted from 3 separate donors of various HLA types (30). Consistent with our patient data, all hu-PBL-SCID tumors were positive for the BZLF1 protein (Figure 1B). These results led us to hypothesize that enhanced recognition of BZLF1 by antigen-specific cellular immunity may provide a protective effect against the development of fatal EBV-LPD in hu-PBL-SCID mice.

**BZLF1 recognition triggers a cell-mediated immune response in healthy carriers.** We next evaluated the cell-mediated immune responses against BZLF1 in vitro. Antigen recognition by the adaptive immune system, specifically by T-cell subsets, is essential to providing longterm, protective immunity against EBV (6). PBMCs were isolated from 24 healthy, EBV-seropositive donors of various HLA types and infected with adenovirus encoding a recombinant BZLF1 (rAd5F35/BZLF1) or null control (rAd5F35/null) for 18 hrs without cytokine treatment. To
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quantify the immune responses against BZLF1, an IFNγ ELISPOT assay was used to detect the number of cytokine-secreting T cells. The degree of responsiveness, as determined by level of IFNγ secretion, varied with each donor, highlighting the inherent diversity of antigen exposure and recall responses (Figure 2).

Recombinant BZLF1 vaccination promotes antigen-specific T-cell expansion and function

in vitro and in vivo. We next examined whether DCs loaded with BZLF1 protein could mediate the expansion of BZLF1-specific memory CD8\(^{(+)}\) T cells in vitro. Milligram quantities of rBZLF1 protein were generated as described above and analyzed for purity by SDS-PAGE analysis (Figure 3A). Purified rBZLF1 was then loaded onto monocyte-derived DCs at varying concentrations and co-cultured with autologous PBMCs from an HLA-B8\(^{(+)}\) donor for 10 days (Figure 3B). To analyze the ability of rBZLF1 protein to generate BZLF1-specific CTLs, a flow cytometric tetramer assay was used to detect the immunodominant HLA-B8-restricted RAK epitope of the BZLF1 protein (27). BZLF1-presenting DCs generated RAK-specific CD8\(^{(+)}\) T cells in a dose-dependent fashion (Figure 3C). To assess the functional status of these T-cell subsets, we restimulated the DC-PBMC co-cultures at day 13 with autologous LCLs. Restimulation induced a RAK-specific IFNγ response that was mediated by CD8\(^{(+)}\) subsets (Figure 3D). Thus, BZLF1 antigen exposure by DCs confers specific CD8\(^{(+)}\) T-cell immunity in vitro for PBMCs from an HLA-B8\(^{(+)\)\} individual.

To determine whether vaccination with rBZLF1 could prime BZLF1-specific T-cell immunity in vivo, hu-PBL-SCID mice were generated using HLA-B8\(^{(+)\) PBMCs from a single EBV\(^{(+)\) donor and injected once with either PBS, GM-CSF (5 µg/mouse), rBZLF1 (100 µg/mouse), or rBZLF1
with GM-CSF as an immune adjuvant (27). Four weeks post-vaccination, \textit{ex vivo} splenocytes were re-exposed to rBZLF1-presenting DCs \textit{in vitro} and assayed for IFN\(\gamma\) secretion by ELISPOT. In comparison to vehicle, vaccination with rBZLF1 alone significantly enhanced BZLF1-specific IFN\(\gamma\) responses \((p=0.0007, n=3, \text{Figure 3E})\), indicating an efficient expansion of functional, BZLF1-specific T cells. Co-administration of rBZLF1 and GM-CSF did not significantly enhance the effect to BZLF1 re-exposure.

**BZLF1 virally-transduced DCs mediate the expansion of BZLF1-specific CTLs \textit{in vitro}**. We next investigated if viral-mediated delivery of BZLF1 to DCs could serve as an effective vaccine strategy against EBV. We compared the ability of adenoviral and adeno-associated viral gene transfer to generate functional, BZLF1-presenting DCs \textit{in vitro}. Monocyte-derived DCs were infected with either an adeno-associated virus (rAAV2/BZLF1) or adenovirus (rAdv5F35/BZLF1) encoding recombinant BZLF1 (or control); infection and overexpression of BZLF1 was confirmed in HEK 293T cells (\textit{Figure 4A and 4C, respectively}). Infected DCs were then co-cultured with autologous HLA-B\(8^{(+)}\) PBMCs for 7 days and analyzed for RAK-specific CD\(8^{(+)}\) T-cell expansion by flow cytometry. DCs transduced with rAAV2/BZLF1 had a minimal effect on RAK-specific CTL expansion compared to DCs incubated with naked rBZLF1 protein (\textit{Figure 4B}); however, rAd5F35/BZLF1-transduced DCs induced a robust expansion of RAK-specific CD\(8^{(+)}\) T cells (\textit{Figure 4D}). Importantly, DCs transduced with rAd5F35/BZLF1 showed a greater expansion of antigen-specific T cells compared to that by DCs transduced with rAd5F35-null control or by rBZLF1-presenting DCs (\textit{compare Figure 4B and Figure 4D}).
In vivo vaccination of hu-PBL-SCID chimeric mice with rAd5F35/BZLF1-transduced DCs induces specific cellular immunity and delays EBV-LPD. The hu-PBL-SCID model of spontaneous EBV-LPD has been used to study potential experimental therapeutic strategies for EBV-LPD (37, 38). We examined whether vaccination of hu-PBL-SCID mice with rAd5F35/BZLF1-transduced DCs (or control) would positively enhance cellular immunity and improve survival against fatal EBV-LPD. HLA-B8(+) PBMCs from a healthy EBV-seropositive donor and 5 x 10^6 autologous DCs transduced with either rAd5F35/BZLF1 or empty vector (rAd5F35/null) were injected IP into SCID mice (n=10/group). Evidence of human cell engraftment was confirmed by assessing human IgG concentrations in serum. Two weeks post-injection, mice in the “booster group” received a second IP injection of either rAd5F35/BZLF1-transduced or rAd5F35/null-transduced DCs as a booster. Five weeks after initial vaccination, 3 mice from the booster group were sacrificed and analyzed for immunosurveillance capacity against EBV-LPD. Splenocytes were co-cultured ex vivo with autologous LCLs and assayed for IFNγ secretion by ELISPOT. Mice vaccinated with rAd5F35/BZLF1-transduced DCs showed significantly higher responsiveness to LCLs relative to that in vector-control vaccinated mice (p<0.0001, n=3, Figure 5A). Single vaccination with rAd5F35/BZLF1-transduced DCs showed a trend toward improved survival (p=0.085, n=10) at a later stage (Figure 5B); however, vaccination followed by single booster delivery significantly delayed fatal EBV-LPD (p=0.014, n=10, Figure 5C), with a median survival of 62 days for the rAd5F35/BZLF1 group and 48 days for the rAd5F35/null group.
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Discussion

EBV is the etiological agent of IM and one of few known human oncoviruses. While most carriers possess the immune components needed to limit the outgrowth of latently infected B cells, immunocompromised patients, such as those with congenital or acquired immunodeficiencies or those having undergone organ transplantation, are at increased risk for malignant transformation due to diminished EBV-specific immunity. EBV is also associated with a broad spectrum of benign (multiple sclerosis, lupus) and malignant diseases (nasopharyngeal, gastric carcinomas) in various immune competent populations; thus, strategies to enhance immunity to EBV represent an unmet need. In the current study, we demonstrate that vaccination of hu-PBL-SCID mice against the EBV immediate early protein BZLF1 can enhance specific cellular immunity and significantly delay the development of lethal EBV-LPD. These protective effects are associated with the expansion and activation of CD8(+) T-cell subsets that recognize the immunodominant HLA-B8-restricted RAK peptide of BZLF1.

Traditionally, EBV-associated malignancies are thought to be driven largely by the cooperative functions of latent viral gene products. Our data support the emerging hypothesis that BZLF1 gene expression is a prominent factor in the development of EBV-associated malignancy (23). We found that both patient and hu-PBL-SCID EBV-LPD tumors show BZLF1-expressing tumor cells that, when targeted in hu-PBL-SCID mice, delay the development of fatal EBV-LPD. Consistent with this observation, Ma and colleagues recently demonstrated that humanized NOD/LtSz-scid/IL2Rγnull mice infected with a replication-defective BZLF1-deleted EBV mutant developed B-cell lymphomas less frequently than control virus-infected mice (23). Since BZLF1 expression has not currently been demonstrated to be sufficient to drive oncogenesis, its
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expression in malignant cells may provide a mechanism for evading tumor surveillance perhaps by downregulating proinflammatory cytokine signaling (20, 21) or by inhibiting the LMP1-induced upregulation of MHC class I molecules in infected cells (39). Horizontal EBV transmission to bystander EBV-negative B lymphocytes may also confer a distinct survival advantage to EBV(+) lymphomas by replinishing latently-infected B-cell populations (23).

Regardless, given the capacity for BZLF1 vaccination to promote EBV-specific immunity and, in turn, delay the progression of fatal EBV-LPD in hu-PBL-SCID mice, it is reasonable to hypothesize that immunosurveillance of BZLF1 by T-cell subsets is a critical factor in the pathogenesis of EBV-LPD and perhaps other associated diseases. Indeed, we have previously shown that prevention of EBV-LPD in hu-PBL-SCID mice treated with combination cytokine therapy is associated with a marked expansion of BZLF1-specific CD8(+) lymphocytes (27). Furthermore, we have demonstrated that successful treatment of post-transplant LPD following renal allografting is associated with high frequencies of RAK-specific CD8(+) T cells (40). Taken together, these findings point to the critical nature of BZLF1 immunosurveillance and the importance of restoring BZLF1-specific immunity in patients at risk or suffering from EBV-associated diseases.

Our vaccination experiment with BZLF1-transduced DCs clearly establishes that BZLF1 vaccination can induce specific cellular immunity capable of recognizing and eliminating EBV-transformed cells in hu-PBL-SCID mice. Indeed, the significant expansion of BZLF1-specific T cells (Figure 3E and 5A) coupled with the increase in survival following repeated vaccination with BZLF1, suggest that BZLF1-specific T cells are expanding and clearing BZLF1-expressing
tumor cells, and is doing so in a chimeric mouse model that is dominated by a dampened human immune response (41). Indeed, all vaccinated animals in our study ultimately developed fatal EBV-LPD. This effect is likely the result of hypofunctioning human T cells in the xenogeneic environment, the selection of viral antigen-loss mutants that have “hidden” BZLF1 from effector lymphocytes (42), and/or the presence of natural viral gene polymorphisms (43) that allow for immune escape. The latter possibility would support the rationale to develop a multivalent vaccine that includes additional target antigens covering a broad spectrum of EBV’s genetic programs including proteins uniquely associated with malignancy. However, the single valent vaccine utilized in this study might be sufficient to prevent clinically fulminant IM that can occur in genetically susceptible individuals (44). We are currently exploring multivalent vaccine approaches, including BZLF1 as one of the vaccine immunogens.

Although recombinant BZLF1 vaccination primed BZLF1-specific T-cell responses in vivo, it is particularly interesting that we did not observe an enhanced effect when GM-CSF was administered as an immune adjuvant (Figure 3E), especially given our prior findings that combination GM-CSF and IL2 therapy protected hu-PBL-SCID mice from fatal EBV-LPD (27). Indeed, others have demonstrated that GM-CSF therapy enhances vaccine-mediated immune responses and confers protective immunity in vivo via the induction of professional antigen-presenting cells (45, 46). In contrast to these observations, however, Serafini and colleagues have shown that high-dose GM-CSF therapy can impair antigen-specific T-cell expansion through the recruitment of myeloid-derived suppressor cells (47). Thus, in future vaccine studies with BZLF1 it may be useful to assess the delivery of low doses of immune adjuvants that promote specific cellular immunity while limiting the growth and recruitment of suppressor cell subsets.
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While our immunization strategy described here significantly prolonged survival from fatal EBV-LPD in hu-PBL-SCID mice engrafted with PBMCs from a HLA-B8(+) individual, we cannot exclude the possibility that BZLF1 vaccination will not be as effective in individuals with other HLA types. Given that recognition of the immunodominant RAK peptide of BZLF1 is HLA-B8-restricted, it remains possible that BZLF1 exposure would produce a comparatively diminished immune response in individuals with other HLA types, especially if additional vaccine components compete with BZLF1 for T-cell receptor binding. While this study does not directly compare cell-mediated immune responses to BZLF1 vaccination in HLA-B8(+) vs HLA-B8(-) individuals, we did observe IFNγ production in donors with a variety of HLA tissue types (Figure 2). Of note, HLA-B8 individuals tested in this experiment produced modest to low levels of IFNγ suggesting that non-HLA-related mechanism(s) are responsible for mediating the immune response to this target protein. Future work will need to directly address distinct differences in EBV antigen responsiveness between individuals with various HLA types.

In summary, we have demonstrated that vaccination with the EBV immediate-early protein BZLF1, a transcription factor that initiates the transition to lytic productive program, can induce a protective effect against the development of fatal EBV-LPD in SCID mice engrafted with PBMCs from an HLA-B8(+) individual. As the hu-PBL-SCID model described here appears to recapitulate the pathogenesis of human EBV-LPD, our findings support consideration of this vaccination strategy in patients, especially HLA-B8(+) patients, who are at risk for EBV-LPD (48) and other EBV-associated diseases. Our findings also support the consideration of BZLF1 as a component in a multivalent vaccine that consists of multiple EBV gene products. Efforts to
identify other latent and lytic antigens that can be included in the development of a multivalent vaccine are currently underway.
Authorship Contributions

T.L. contributed to the design of experiments, data acquisition, analysis and interpretation of data; A.S.H contributed to data acquisition, analysis and interpretation of data, and writing/revision of the manuscript; J.T.P., S.L.G., H.K., and G.L. performed experimental work and interpreted data; X.Z. provided statistical analyses; M.E.L contributed to the conception and design of the study and interpretation of data; M.A.C. and R.A.B. contributed to the design of the study, analysis and interpretation of data, writing/revision of the manuscript, and provided administrative support.
References

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Table 1: BZLF1 expression in EBV(+) primary

<table>
<thead>
<tr>
<th>EBV(+) Lymphomas</th>
<th>BZLF1(+)</th>
<th>% Tumor BZLF1(+)**</th>
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<tr>
<td>Post-transplant lymphoproliferative disorder</td>
<td>6/8 (75%)</td>
<td>1-10%</td>
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<td>Lymphomatoid granulomatosis</td>
<td>1/3 (33%)</td>
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<td>Diffuse large B cell lymphoma</td>
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<td>Hodgkin’s lymphoma</td>
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<tr>
<td>EBV(+) unclassifiable lymphoma</td>
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*Positive expression for BZLF1 was documented for any tumor that demonstrated anti-BZLF1 signal by immunohistochemical analysis.

**Percentage of tumor cellularity that is BZLF1(+).
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Figure Legends

Figure 1. **BZLF1 gene expression in human EBV(+)** lymphoma samples. (A) Immunohistochemical staining of a pathologist-verified EBV(+)-PTLD patient sample with anti-BZLF1. The brown staining indicates the presence of BZLF1 protein; left panel, 20X, right panel, 40X magnification. The figures are representative of six patient samples. (B) Immunohistochemical staining of EBV-LPD tumor sample obtained from hu-PBL-SCID mouse. Brown staining indicates presence of BZLF1 protein; left panel, 20X, right panel, 40X magnification. Data are representative of five tumor-bearing hu-PBL-SCID mice.

Figure 2. **Donor immune responses to BZLF1 antigen exposure.** PBMCs from 24 donors of various HLA types were infected with adenovirus carrying either BZLF1 cDNA (rAd5F35/BZLF1) or an empty-vector control (rAd5F35/null) for 18 hrs without cytokine treatment. Human IFNγ ELISPOT assays were performed immediately following infection with virus. Spot forming units were calculated as described above. Bars show mean ± SD. *p< 0.05 as determined by two sample t-test.

Figure 3. **Recombinant BZLF1 presentation by human DCs promotes BZLF1-specific CD8(+) T-cell expansion and function.** (A) SDS-PAGE protein gel stained with Coomassie Brilliant Blue. Gel contains low range molecular weight markers and rBZLF1 protein at the indicated quantities. (B) Schematic representation of experimental approach. (C) Flow cytometric RAK HLA-B8 tetramer assay of PBMC co-cultures expanded with rBZLF1-loaded DCs. (D) IFNγ intracellular flow cytometric analysis of DC-expanded PBMC co-cultures restimulated with autologous LCLs for 18 hrs. An HLA-B8-restricted FLR tetramer derived from
the EBV latency protein EBNA-3A was used as a negative control. (E) IFNγ ELISPOT assay of ex vivo splenocytes collected from hu-PBL-SCID mice vaccinated with rBZLF1 protein and restimulated with BZLF1-loaded DCs in vitro (p=0.0007, n=3). *p< 0.05 as determined by two sample t-test.

Figure 4. BZLF1 virally transduced-DCs induce BZLF1-specific T-cell immunity in vitro. (A) Immunoblot of BZLF1 protein expression in HEK 293T cells (lysate from 1x10^6 cells/lane) infected with adeno-associated virus encoding GFP alone (rAAV2/GFP) or GFP and BZLF1 (rAAV/BZLF1). (B) Flow cytometric HLA-B8 RAK tetramer assay of PBMC co-cultures expanded with rAAV/GFP or rAAV/BZLF1-infected DCs; 1 ug/mL rBZLF1-loaded DCs were used as a positive control. (C) Immunoblot of BZLF1 protein expression in HEK 293T cells infected with rAd5F35/null or rAd5F35/BZLF1. (D) Flow cytometric HLA-B8 RAK tetramer assay of PBMC co-cultures expanded with rAd5F35/null or rAd5F35/BZLF1-infected DCs. Non-reactive HLA-B8 FLR tetramer was used as a negative control.

Figure 5. Evaluation of in vivo activity of BZLF1 virally transduced-DC vaccination in hu-PBL-SCID mice. SCID mice depleted of murine NK cells were injected IP with 5 x 10^7 HLA-B8(+)EBV(+) PBMCs from a single, healthy donor and 5 x 10^6 autologous monocyte-derived DCs transduced with either rAd5F35/null or rAd5F35/BZLF1. Two weeks post-initial vaccination, mice in the “booster group” received a second IP injection with either rAd5F35/BZLF1 or rAd5F35/null transduced-DCs (5x10^6 DCs per mouse). (A) IFNγ ELISPOT assay of ex vivo splenocytes from booster group restimulated with autologous LCLs in vitro (p<0.0001, n=3). Bars show mean +/- SD. (B) Kaplan-Meier analysis of overall survival for mice that received a single dose vaccination (p=0.085, n=10 each). (C) Kaplan-Meier analysis of overall survival for
mice that received a single booster dose injection two weeks after primary vaccination. \( (p=0.014, \ n=10 \ \text{each}) \). *\( p \leq 0.05 \) as determined by log-rank analysis.
Figure 3

A. A gel showing protein expression at different concentrations of 4 µg, 2 µg, and 1 µg.

B. A diagram illustrating the steps for antigen-specific T-cell expansion:
- GM-CSF
- 3 days in antigen-loaded DCs
- 10 days culture 1:5 with PBMCs
- CD8 T-cell expansion

C. Flow cytometry analysis of BSA and BZLF1 loaded DCs with RAK tetramer.

D. Flow cytometry analysis of BZLF1, BSA, and BZLF1 loaded DCs with CD8 and FLR tetramer.

E. Bar graph showing interferon-γ levels with PBS, GM-CSF, BZLF1, and GM-CSF + BZLF1 treatments.

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The Epstein-Barr virus lytic protein BZLF1 as a candidate target antigen for vaccine development

Alex S Hartlage, Tom Liu, John T Patton, et al.


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