JVI Journals.ASM.org

An Epstein-Barr Virus (EBV) Mutant with Enhanced BZLF1 Expression Causes Lymphomas with Abortive Lytic EBV Infection in a Humanized Mouse Model

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Immunosuppressed patients are at risk for developing Epstein-Barr Virus (EBV)-positive lymphomas that express the major EBV oncoprotein, LMP1. Although increasing evidence suggests that a small number of lytically infected cells may promote EBV-positive lymphomas, the impact of enhanced lytic gene expression on the ability of EBV to induce lymphomas is unclear. Here we have used immune-deficient mice, engrafted with human fetal hematopoietic stem cells and thymus and liver tissue, to compare lymphoma formation following infection with wild-type (WT) EBV versus infection with a "superlytic" (SL) mutant with enhanced BZLF1 (Z) expression. The same proportions (2/6) of the WT and SL virus-infected animals developed B-cell lymphomas by day 60 postinfection; the remainder of the animals had persistent tumor-free viral latency. In contrast, all WT and SL virus-infected animals treated with the OKT3 anti-CD3 antibody (which inhibits T-cell function) developed lymphomas by day 29. Lymphomas in OKT3-treated animals (in contrast to lymphomas in the untreated animals) contained many LMP1-expressing cells. The SL virus-infected lymphomas in both OKT3-treated and untreated animals contained many more Z-expressing cells (up to 30%) than the WT virus-infected lymphomas, but did not express late viral proteins and thus had an abortive lytic form of EBV infection. LMP1 and BMRF1 (an early lytic viral protein) were never coexpressed in the same cell, suggesting that LMP1 expression is incompatible with lytic viral reactivation. These results show that the SL mutant induces an "abortive" lytic infection in humanized mice that is compatible with continued cell growth and at least partially resistant to T-cell killing.

Epstein-Barr virus (EBV) is a human herpesvirus that is associated with a variety of different types of human B-cell lymphomas, including endemic Burkitt lymphoma (BL), Hodgkin lymphoma (HL), lymphoproliferative disease (LPD) in immunocompromised hosts, and a subset of diffuse large B cell lymphomas (DLBCLs) (53). Like all herpesviruses, EBV can infect cells in either latent or lytic forms. During latent EBV infection, the virus persists as a nuclear episome and is replicated once per cell cycle using the host cell DNA polymerase and the viral EBNA1 protein via the oriP origin of replication (34). EBV can express up to 9 different viral proteins during latent infection, and at least three different forms of viral latency (which differ in regard to the viral protein expression pattern) have been described (34, 53). EBV-infected cells with type III latency express all 9 latent viral proteins, and this is the only type of EBV infection that is sufficient to transform primary B cells *in vitro* (34, 53). However, cells with type III latency are highly immunogenic, and tumors with this form of latency are only found in patients with profound immunosuppression, such as post-transplant lymphoproliferative disease (PTLD).

Although EBV-infected tumors are composed primarily of cells with one of the latent forms of EBV infection, increasing evidence suggests that a small number of tumor cells with the lytic form of viral protein expression may promote tumor growth (28, 29, 41). We previously showed that B cells harboring a lytically defective EBV mutant (deleted for the BZLF1 immediate-early [IE] gene) grow more slowly than cells transformed with wild-type (WT) virus in a SCID mouse xenograft model (28). More recently, we found that this lytically defective mutant is also impaired for the ability to form lymphomas in a new humanized mouse model engrafted with human hematopoietic $CD34^+$ stem cells and thymic tissue that represents a largely intact human immune system (41). Since fully lytic EBV infection is thought to induce host cell killing, the development of drugs that activate lytic gene expression in tumor cells is being pursued as a potential treatment for EBV-positive malignancies (20, 21, 46, 51). Consistent with this idea, an EBV mutant (ZV ZV′ ZIIR) that has an abnormally high level of lytic gene expression was recently found to be highly defective in transforming primary B cells *in vitro* due to excessive host cell death following viral infection, as noted in the accompanying article by Yu et al. (68).

The switch between latent and lytic forms of infection is initiated by expression of the viral immediate-early (IE) protein BZLF1 (Z). Z is a transcription factor that binds to and activates the EBV early lytic gene promoters, as well as the promoter of the other EBV IE protein, BRLF1 (R) (34, 53). Z and R synergistically induce the expression the early lytic viral genes, which encode the replication machinery required for viral replication mediated by the virally encoded DNA polymerase and the oriLyt origin of replication. Direct Z binding to oriLyt (independent of Z transcriptional function) is required for viral lytic replication. Lytic viral replication is followed by the expression of the late viral genes,

Received 28 March 2012 **Accepted** 9 May 2012
Published ahead of print 23 May 2012
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doi:10.1128/JVI.00770-12

which encode the structural viral proteins required for encapsidation and release of infectious viral particles.

In addition to playing an essential role in activating lytic gene transcription and promoting viral replication, Z exerts numerous effects on the host cell environment. For example, various *in vitro* studies have shown that Z disperses promyelocytic leukemia protein (PML) bodies in the nucleus (1, 8), binds to and inhibits the function of the tumor suppressor protein p53 (11, 42, 57), induces expression of an essential B-cell growth factor, interleukin-6 (IL-6) (29), and inhibits cell cycle progression (11). Z has also been reported to decrease the expression of several different host cell proteins involved in immune-mediated killing of EBV-infected cells, including the gamma interferon receptor (49), the TNFR1 tumor necrosis factor alpha (TNF-α) receptor (48), the CIITA transcription factor (39), and the invariant chain (CD74), which is an essential chaperone for major histocompatibility complex (MHC) class II antigen presentation (71). In addition, Z interacts directly with the p65 component of NF-κB and inhibits its function (25, 47). However, since many of the previously reported conclusions were obtained from *in vitro* studies that used nonphysiological levels of Z and/or were performed outside the context of the intact viral genome, the various effects of Z on the host cell environment in the context of *in vivo* viral infection remain uncertain.

The switch between latent and lytic EBV infection is tightly regulated by cellular and viral factors at multiple different levels. The first step in lytic viral reactivation involves activation of the BZLF1 promoter (Zp) by cellular transcription factors. Lytic EBV reactivation has been associated with plasma cell differentiation in humans (36), and the master regulators of plasma cell differentiation, Blimp1 and XBP1, can activate Zp *in vitro* (5, 10, 62). B-cell receptor (BCR)-mediated signaling also leads to EBV reactivation in EBV-infected Burkitt lymphoma lines with type I latency (63). However, Zp also contains several *cis*-acting "silencing" motifs that inhibit its activity and, hence, promote viral latency. Two of these elements, ZV, and ZV′, are synergistically bound by the ZEB1 and ZEB2 cellular transcription factors (18, 35, 68, 69), and knockdown of ZEB1 and ZEB2 in EBV-infected cell lines leads to enhanced lytic EBV gene expression (18, 19). Another silencing element, ZIIR (which binds to an as-yet-unidentified cellular protein), also inhibits Zp activity (40, 67), and a mutant EBV containing mutations in all three silencing Zp elements (the ZV ZV′ ZIIR triple mutant [tmt]) has more constitutive lytic gene expression *in vitro* than mutant viruses in which only the ZV or ZIIR motifs have been altered (68). Of note, the ZV ZV′ ZIIR "superlytic" mutant (hence referred to here as the "SL" mutant) is highly defective in establishing viral latency in primary peripheral blood B cells *in vitro* due to excessive cell killing. Nevertheless, it can establish long-term viral latency in 293 cells, although it spontaneously reactivates much more efficiently than WT virus in these cells, especially in response to phorbol ester treatment (68). Thus, activation of Zp requires both the loss of repressors and the presence of *trans*-activating factors.

Viral reactivation is also regulated at the level of Z protein transcriptional and replicative functions. For example, we recently showed that the B-cell-specific cellular Oct-2 protein interacts directly with Z and inhibits its DNA-binding activity, thus promoting viral latency in B cells (54). The p65 component of NF-κB, as well as the Myc protein, also interacts directly with Z and inhibits its transcriptional activity (25, 55). Z binding to (and activation of) many early lytic viral genes requires promoter DNA methylation (6, 16), and EBV infection of primary B cells does not result in lytic viral replication until the viral genome has become methylated (30, 31). Furthermore, phosphorylation of Z residue 173 is required for Z replicative function (and, hence, late gene expression) but is not important for early gene activation (17), and direct interaction between Z and p53-BP1 is, likewise, required for viral replication but not early gene expression (3). In addition, two different EBV latency proteins (LMP2A and LMP1) have been reported to inhibit lytic gene reactivation *in vitro* (2, 43, 44, 52). Thus, accumulating evidence derived from *in vitro* studies suggests that Z expression *per se* in EBV-infected cells will not always result in early gene expression and/or lytic viral replication. However, whether "abortive" lytic EBV gene expression (i.e., Z expression in the absence of viral lytic replication) occurs *in vivo*, and under what circumstances, is not clear.

Mice reconstituted with a functional human immune system may provide a vastly improved model (in comparison to previously available models) for studying the roles of specific EBV proteins in viral pathogenesis and the development of B-cell lymphoma in the context of a largely intact human immune response. In contrast to previous models (such as the nonobese diabetic/severe combined immunodeficient [NOD/SCID] mouse), engrafted human B cells can undergo class switching, and T cells can undergo receptor gene rearrangement. Since the human blood cells are derived from hematopoietic ($CD34^+$) stem cells, multiple different stages in the development of B cells and T cells are present. In addition, EBV-infected B cells can engage in interactions with T cells that might alter the B-cell environment; for example, EBV-infected B cells may undergo CD40-mediated signaling in response to contact with CD40 ligand-expressing T cells. EBV-infected B cells can also migrate to different organs and environments (including the bone marrow, spleen, lymph nodes, and engrafted human thymic tissue).

In a recent study, we showed that WT (B95.8 strain) EBV infection in humanized NOD/LtSz-*scid/IL2R*γ^{null} (NSG) mice reconstituted with both human fetal CD34 cells and cotransplanted human thymic tissue [hereafter referred to as "hNSG(thy) mice"] results in EBV-positive diffuse large B-cell lymphomas (DLBCLs) in a subset of infected mice, while other mice successfully control their EBV infection and develop long-term tumor-free viral latency (41). Furthermore, we found that a BZLF1-deleted EBV mutant produced fewer DLBCLs than the WT virus, although it was able to establish persistent latent infection in all infected mice (41). In this study, we investigated the phenotype of the superlytic (SL) EBV mutant in hNSG(thy) mice to examine the effects of increased lytic EBV gene expression on viral pathogenesis and lymphoma formation. Furthermore, we examined the effect of an anti-CD3 (OKT3) antibody (which inhibits T-cell function) on the outcome of EBV infection in the hNSG(thy) mouse model. Our results indicate that the SL mutant is similar to the WT virus in its ability to induce lymphomas in this model, the SL virus-infected lymphomas contain BZLF1-expressing cells with an abortive form of lytic infection, and anti-CD3 OKT3 treatment dramatically impairs host control of EBV infection in this model.

MATERIALS AND METHODS

EBV viruses. A WT (B95-8 strain) EBV bacmid, p2089, which expresses the green fluorescent protein (GFP) and a hygromycin B resistance gene, was constructed using bacterial artificial chromosome technology as de-

scribed previously (15) and was a gift from Henri-Jacques Delecluse. The ZV ZV′ ZIIR tmt (SL mutant) was derived by site-specific base pair substitution mutagenesis of the p2089 bacmid as described previously (68). This mutant contains alterations in three different regions of the BZLF1 promoter (inactivating the ZV and ZV′ ZEB-binding motifs as well as the ZIIR element), and has greatly enhanced BZLF1 expression in comparison to the WT EBV *in vitro*. A WT revertant of p2089-ZV ZV′ ZIIR tmt, p2089-ZV ZV′ ZIIRtmt Rev, was likewise constructed by site-specific mutagenesis of the p2089-ZV ZV′ ZIIR tmt.

Humanized NOD/LtSz-*scid/IL2R*γ^{null} mice. Immunodeficient nonobese diabetic/severe combined immunodeficient (NOD/LtSz-*scid/IL2R*γ^{null}) mice were purchased from Jackson Laboratory (catalogue no. 005557) and used at 6 to 10 weeks of age. Human fetal thymus and liver tissues of gestational ages of 17 to 20 weeks were obtained from Advanced Bioscience Resource (Miltenyi Biotec, Alameda, CA). The recipient mice were conditioned with sublethal (2 to 3 Gy) whole-body irradiation and implanted with fetal thymus and liver fragments under the recipient kidney capsule after irradiation. The mice also received an intravenous (i.v.) injection of purified CD34$^+$ cells isolated from the same fetal liver by the magnetically activated cell sorter (MACS) separation system (Miltenyi Biotec, Auburn, CA). The purity of the injected CD34$^+$ cells was at least 80 to 90%. At 10 weeks post-immune reconstitution, the levels of human hematopoietic cells in mice were determined by multicolor flow cytometric (FCM) analysis using various combinations of the following antibodies: pan-CD45 (clone HI30), CD4 (clone RPA-T4 or OKT4), and CD8α (clone RPA-T8). Antibodies were purchased from commercial sources directly conjugated to fluorescent dyes. Fluorescence-activated cell sorter (FACS) analysis was performed on a FACScalibur (Becton, Dickinson, Mountain View, CA).

Production of infectious virus. Infectious viral particles were produced from 293 cell lines stably infected with the WT or SL mutant viruses following transfection with EBV BZLF1 and GP110 expression vectors as previously described (41). Supernatants were harvested at 72 h posttransfection and filtered through a 0.45-μm-pore-size filter. The virus was concentrated by centrifugation at 18,000 rpm for 3 h using an SW27 rotor, resuspended in phosphate-buffered saline (PBS) overnight at 4°C, and then stored at −80°C. To determine the titer of the EBV stock, Raji cells were infected with serial 10-fold dilutions of virus. After 48 h, cells were treated with 50 ng/ml phorbol-12-myristate-3-acetate (PMA; Sigma) and 3 mM sodium butyrate (Sigma), and the number of GFP-expressing Raji cells was counted 24 h later by fluorescence microscopy. Infection of primary B cells with equal amounts of WT and SL virus (with the titer determined by the green Raji cell assay) resulted in similar levels of latent viral transcripts (68), indicating that the accuracy of the green Raji cell titer assay is not significantly affected by excessive lytic viral gene expression in SL mutant-infected Raji cells.

EBV infection of mice. Mice were injected intraperitoneally (i.p.) with 30,000 green Raji units (GRU) of WT or SL mutant EBV in 250 μl PBS approximately 10 weeks after engraftment of human cells; successful engraftment of human cells was confirmed in each animal prior to EBV infection. Three different groups of animals were used, with the mice in each group each being reconstituted from the same donor cells. In the first group of animals, 3 mice were infected with WT EBV and 3 mice were infected with the SL mutant. In the second group of animals, 4 mice were infected with the SL revertant virus (in which the SL alterations were switched back to the WT virus sequences) and 4 mice were infected with the SL mutant. Since the WT and SL revertant viruses gave similar phenotypes, both here and in prior *in vitro* studies (68), results from group 1 and group 2 animals were combined, and the WT and SL revertant viruses are both referred to here as "WT virus" in results. In the group 2 experiment, one mouse infected with each virus was sacrificed at days 3 and 12 postinfection (p.i.) to examine the viral protein expression at early time points; these mice were not considered when calculating the lymphoma incidence. In the third group of animals, 3 mice were infected with WT virus and 3 mice were infected with the SL mutant virus. In this experiment, mice were treated with an anti-CD3 antibody (OKT3) starting 1 day after EBV infection and continuing daily (except Sunday) thereafter until the time of euthanasia. Mice were sacrificed at day 60 postinfection, unless they became clinically ill earlier. Following euthanasia, multiple different organs (including the lungs, transplanted thymus, spleen, pancreas, liver, and abdominal lymph nodes) were formalin fixed and then examined using a variety of different techniques to determine if animals had persistent EBV infection and/or EBV-positive lymphomas and to assess the viral protein expression pattern. Analysis performed for all animals included hematoxylin and eosin (H&E) staining, EBV-encoded RNA (EBER) *in situ* hybridization, and immunohistochemical (IHC) staining using antibodies directed against CD20 (B-cell marker), CD3 (T-cell marker), CD74, MHC class I, and the EBV proteins EBNA1, EBNA2, LMP1, LMP2A and BZLF1, gp125, and gp350.

TABLE 1 Antibodies used for immunohistochemistry in this study

Antibody	Clone	Source or manufacturer	Dilution
CD20	H1	BD Pharmingen	1:600
CD3	Polyclonal	DakoCytomation	1:200
CD8	SP16	Biocare Medical, LLC.	1:50
LMP1	CS.1-4	DakoCytomation	1:600
LMP2A	15F9	Santa Cruz Biotechnology, Inc.	1:100
EBNA1	EB14	Gift from Richard R. Burgess	1:1,000
EBNA2	PE2	Leica Microsystems	1:100
BZLF1	BZ1	Santa Cruz Biotechnology, Inc.	1:200
BMRF1	G3-E31	Vector Laboratories	1:200
BALF2	OT13D	Gift from Jaap M. Middeldorp	1:1,000
gp350	72A1	Gift from Eric C. Johannsen	1:100
VCA-gp125	L2	Chemicon International	1:100
CD74	LN2	BD Biosciences	1:100
MHC class I	Polyclonal	Santa Cruz Biotechnology, Inc.	1:50

Anti-CD3 antibody treatment. OKT3 antibody was purchased from Imgenex (San Diego, CA). Mice received 2 μg of antibody intravenously (i.v.) per day, starting 1 day after EBV infection and continuing for 6 days a week until the time of euthanasia.

Detection of EBERs by *in situ* hybridization. EBER *in situ* hybridization studies were performed using the PNA *in situ* hybridization (ISH) detection kit (DakoCytomation) according to the manufacturer's protocol as previously described (41).

IHC. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and then examined by immunohistochemistry (IHC) as previously described (41). The antibodies used are listed in Table 1.

Real-time qPCR to measure EBV viral load in plasma. Quantitative PCR (qPCR) targeting the BamHIW region of the EBV genome was used to measure EBV viral load in plasma samples using TaqMan technology as previously described (56). Prior to DNA extraction, plasma was spiked with exogenous internal positive control DNA (Applied Biosystems, Foster City, CA) to control for the effectiveness of extraction and amplification. A standard curve was generated using serial 10-fold dilutions of Namalwa DNA. To determine whether the viral DNA in plasma was from lysed infected cells or from viral particles, in samples that had very high EBV loads, the assay was repeated with or without pretreatment of the sample with DNase I (Roche Diagnostics, GmbH, Mannheim, Germany) at 37°C for 30 min according to the manufacturer's instructions.

Viral genome methylation analysis. Total genomic DNA was isolated from livers of infected animals using the DNeasy tissue kit (Qiagen). Bisulfite modification of genomic DNA was performed using the EpiTect bisulfite kit (Qiagen) in accordance with the manufacturer's instructions. The modified DNA was amplified by PCR using the following EBV *BMRF1* promoter primers: 5′-GATTAAGGGTTATTTGGATTTAGGAG T-3′ and 5′-TCAAAAATCATCTACCACCAACATA-3′. The thermal cycler conditions for the PCR were as follows: 1 cycle at 95°C for 15 min, followed by 14 cycles of 95°C for 30 s and 57°C to >50°C for 30 s with a

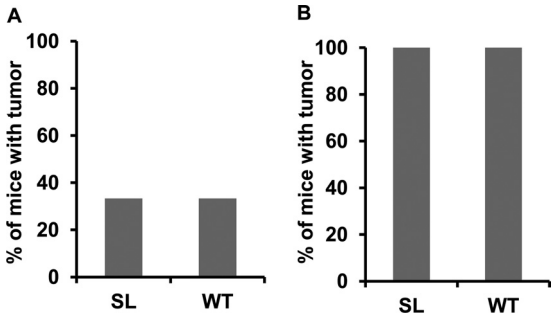

FIG 1 SL EBV mutant can induce lymphoma in hNSG(thy) mice. The percentage of mice developing EBV-positive tumors in SL versus WT virus-infected animals is shown. (A) Animals without OKT3 treatment; (B) animals treated with OKT3.

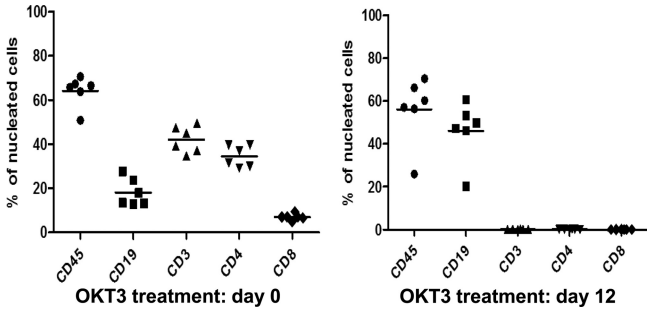

FIG 2 OKT3 treatment depleted peripheral blood human T cells in all treated mice. Peripheral blood was collected from hNSG(thy) mice at days 0 and 12 post-OKT3 treatment. Human CD45, CD19, CD3, CD4, and CD8 cell populations were detected by flow cytometry. Dead cells were excluded from the analysis. Results are presented as the percentage of positively staining cells with each antibody in comparison to the total leukocyte population.

gradual decrease of melting temperature (T_m) of 0.5°C, followed by 72°C for 1 min, and then 36 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 7 min. PCR products were agarose gel purified with a Qiagen gel extraction kit and then cloned into the pGEM-T Easy vector system (Promega) following the manufacturer's instructions. Ten clones were sequenced using both T7 and SP6 primers.

RESULTS

The SL EBV mutant can induce lymphomas in hNSG(thy) mice in the presence and absence of a T-cell-depleting (OKT3) antibody. In our previous study (41), we found that while some WT EBV-infected hNSG(thy) animals successfully control their infection and develop long-term tumor-free viral latency, others develop EBV-positive diffuse large B-cell lymphoma (41). To determine if enhanced lytic viral gene expression affects the outcome of EBV infection in the hNSG(thy) mouse model, mice were infected (following successful engraftment of human cells) with equal amounts of the WT or SL viruses (30,000 green Raji cell units i.p.). As shown in Fig. 1A, similar numbers of lymphomas were found in the WT- and SL virus-infected mice at the time of euthanasia (day 60 postinfection). Two of six of the WT virus-infected animals and 2/6 of the SL virus-infected animals developed lymphomas.

To examine the WT virus and SL mutant virus phenotypes when T-cell function is compromised, hNSG(thy) mice were infected with the WT or SL viruses and then treated daily with the anti-CD3 monoclonal antibody OKT3 (2 μg/animal/day i.v.) starting 1 day after infection and continuing until the time of euthanasia. Treatment with this antibody greatly decreased the number of T cells in peripheral blood of mice, as shown in Fig. 2. As shown in Fig. 1B, OKT3 antibody remarkably inhibited the ability of hNSG(thy) mice to control either WT or SL virus infection. Three of three WT virus-infected animals as well as 3/3 SL virus-infected animals all developed large B-cell lymphomas by day 29 postinfection when treated with the antibody.

The SL mutant and WT virus-infected animals have similar viral loads in the blood, but OKT3 treatment increases viral load following either SL or WT virus infection. To examine more quantitatively the number of virally infected cells in the WT versus SL virus-infected animals, blood was collected at various time points after infection and a quantitative PCR assay was performed to determine the amount of EBV DNA present in the plasma. As shown in Fig. 3A, in the absence of OKT3 antibody treatment, EBV infection was generally very well controlled in this humanized mouse model, and most of the WT virus-infected animals, as well as the SL virus-infected animals, had either undetectable or very low plasma viral loads (less than 50 copies of EBV per ml). The two animals with the highest viral loads (one WT virus-infected animal and one SL virus-infected animal) each had lymphomas at the time the plasma was collected, and thus the higher circulating EBV DNA levels in these animals were likely due to lysed tumor cell DNA. However, even the animals that were found to have lymphomas had relatively low viral loads in the plasma (no greater than 200 copies per ml), consistent with the relatively small size of most EBV-induced lymphomas in the hNSG(thy) model in the absence of OKT3 treatment.

In contrast, OKT3-treated animals often had much higher circulating viral loads (up to 60,000 copies of viral DNA per ml of plasma) (Fig. 3B), consistent with the finding that OKT3-treated animals developed large lymphomas at early time points. Somewhat surprisingly, the WT and SL viruses resulted in similar viral loads in infected animals. To determine whether the viral DNA in plasma was from lysed EBV-infected lymphoma cells or from viral particles, the assay was repeated in the four animals with the highest viral loads (two WT virus-infected animals and two SL virus-infected animals), with or without pretreatment of the samples with DNase I. In each case, DNase I treatment eliminated at least 98.5% of the measured viral DNA load (data not shown), indicating that the viral DNA load was derived from lysed tumor cells.

SL virus induces DLBCLs that contain many Z-expressing cells. Similar to the lymphomas induced by WT EBV, the SL virus-infected lymphomas had histological features of diffuse large B-cell lymphomas heavily infiltrated with T cells in the absence of anti-CD3 treatment (Fig. 4A). As expected, tumors had fewer infiltrating T cells in the OKT3-treated animals (Fig. 4B). To determine if the SL virus-induced lymphomas have more cells expressing Z than the WT virus-induced lymphomas, EBNA2 and Z IHC staining was performed on adjacent tumor slides. As predicted by its *in vitro* phenotype, the SL virus-infected lymphomas contained many more Z-expressing cells (up to 30% of cells in some lymphomas) than did the WT virus-infected lymphomas (Fig. 4C and D). Nevertheless, since EBNA2-expressing cells were still much more common than Z-expressing cells in SL virus-infected lymphomas, clearly not all SL virus-infected lymphoma cells express Z.

Z^+ lymphoma cells can divide. Z has been reported to inhibit

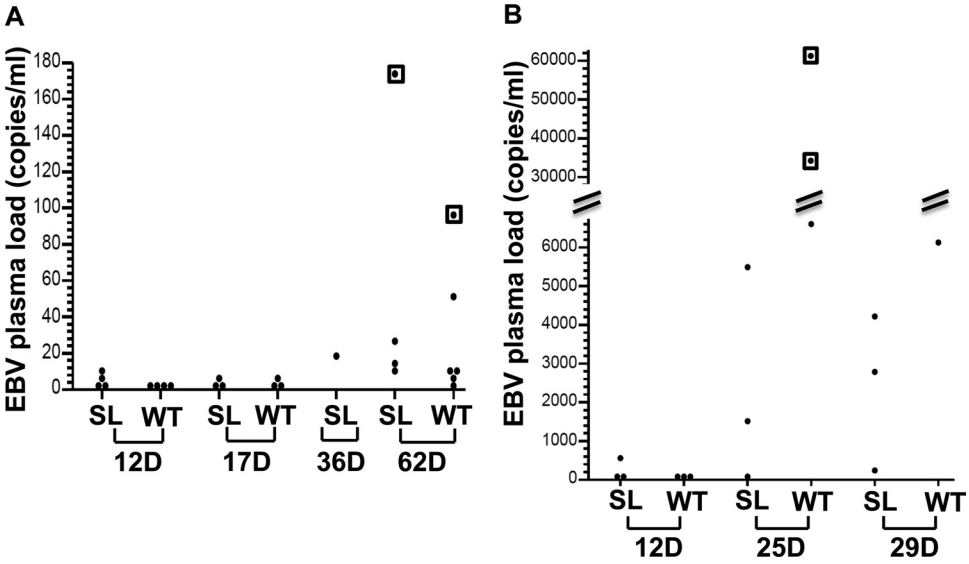

FIG 3 The SL mutant and WT EBV-infected animals have similar viral loads in plasma, but OKT3 treatment increases viral load after EBV infection. qPCR was performed (using primers to detect the BamHIW repeat region of the EBV genome) on purified plasma DNA collected from SL mutant and WT EBV-infected animals at various days postinfection as indicated. (A) Viral load from animals without OKT3 treatment. The boxed dots indicate the two animals that developed tumors. (B) Viral load from animals treated with OKT3. The boxed dots indicate animals that were sacrificed at day 25 due to significant weight loss. All six animals had tumors.

cell cycle progression *in vitro* (11), and lytic EBV infection can kill at least some EBV-infected cell lines (22, 32). Thus, the finding that up to 30% of the SL virus-infected lymphoma cells express Z, yet the SL virus still efficiently induces lymphoma, was unexpected. The appearance of the Z-positive lymphoma cells versus nearby Z-negative lymphoma cells was not found to be correlated with any particular cytologic alteration (data not shown). However, in contrast to the reported inhibitory effect of Z on cell cycle progression *in vitro*, we observed that 2.3% of Z-expressing lymphoma cells were actively dividing (Fig. 5A), even in the presence of many surrounding $CD8^+$ T cells (Fig. 5B), in comparison to 1.2% of the total EBNA2-expressing lymphoma cells. Thus, Z-expressing cells retain the ability to proliferate in this *in vivo* lymphoma model.

The SL mutant establishes an abortive form of lytic EBV infection in lymphoma cells. The findings that WT and SL virus-infected animals have similar viral loads and that Z^+ lymphoma cells can divide suggested that SL virus-infected lymphoma cells might not support fully lytic viral infection. To examine this possibility further, lymphoma specimens were stained with antibodies directed against the immediate-early Z protein, the early lytic viral proteins BMRF1 and BALF2, and the late lytic viral proteins gp125 and gp350/220. Each of these antibodies was validated for IHC use in paraffin-fixed tissue using Akata BL cells treated *in vitro* with or without the lytic inducing agent anti-IgG. As shown in Fig. 6, in the absence of OKT3 antibody treatment, Z-positive lymphoma cells did not detectably express any of the four lytic viral proteins examined, although each of these early and late lytic EBV proteins was easily detected in anti-IgG treated Akata cells. The lymphomas of OKT3-treated animals expressed the early lytic viral proteins (BMRF1 and BALF2), but still did not detectably express late lytic viral proteins (Fig. 6). The findings that late lytic viral proteins are not detected in either the presence or absence of OKT3 treatment and that the viral loads of the SL and WT virus-infected animals are similar suggest that the majority of Z-expressing lymphoma cells in the humanized mouse model are not permissive for the lytic form of viral DNA replication even when T-cell function is compromised.

Since methylation of the EBV genome has been shown to be required for Z activation of many early lytic promoters, as well as the lytic form of viral DNA replication (30), we examined the methylation state of the BMRF1 promoter in an SL virus-infected lymphoma (in an animal not treated with OKT3) that expressed Z but not BMRF1, using bisulfite sequencing analysis. As shown in Fig. 7, the BMRF1 early viral promoter was found to be highly methylated in this lymphoma, making it unlikely that the lack of fully lytic infection was due to inadequate methylation of the viral genome. In addition, since the lytically replicated form of the viral genome is unmethylated, this result confirms that the Z-expressing lymphoma cells contain very little, if any, lytically replicated EBV.

Z^+ lymphoma cells have decreased expression of CD74 and MHC class I. The finding that SL virus-infected lymphomas contain so many Z^+ cells was also surprising (particularly in the animals not treated with OKT3) because cytotoxic T cells directed against the Z protein are highly abundant in mononucleosis patients and are thought to contribute to clearance of virally infected cells (58, 60). Since Z was recently reported to decrease expression of the essential MHC class II peptide chaperone protein, CD74, *in vitro* (71), we performed Z-CD74 costaining studies on SL virus-infected lymphoma cells to determine if Z likewise inhibits CD74 expression in the hNSG(thy) model. These studies confirmed that most Z-expressing cells do not express CD74 (Fig. 8). In addition, since early lytic EBV infection has been reported to decrease MHC class I expression (13), we performed Z-MHC class I costaining studies. Z^+ cells had decreased expression of MHC class I relative to the surrounding cells (Fig. 8). These results suggest that Z expression may be tolerated (even selected for) in lymphomas in the

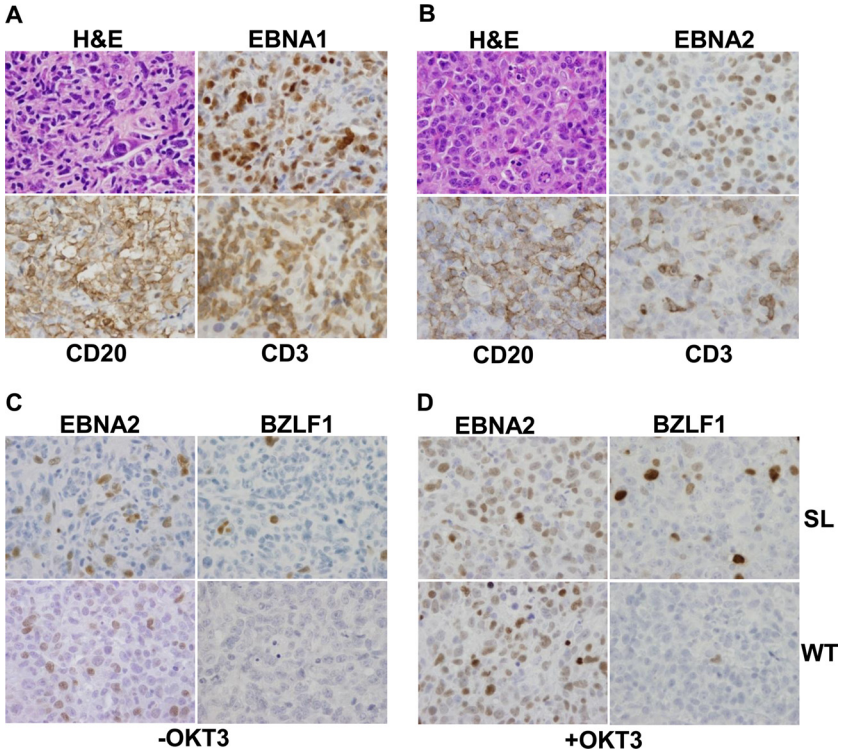

FIG 4 SL virus induces DLBCLs with upregulated BZLF1 expression. (A) SL mutant EBV-induced DLBCL from a non-OKT3-treated animal was stained for H&E, EBV EBNA1 protein, CD20, and CD3 (100× magnification). Large atypical tumor cells showing CD20 membrane staining were infiltrated by numerous small CD3-staining T cells. (B) SL EBV-induced DLBCL from an OKT3-treated animal was stained for H&E, EBV EBNA1 protein, CD20, and CD3 (100× magnification). (C) EBNA2 and BZLF1 staining of DLBCLs in SL mutant EBV-infected (upper panel) and WT EBV-infected (lower panel) (100× magnification) animals not treated with OKT3. (D) EBNA2 and BZLF1 staining of DLBCLs in SL EBV-infected (upper panel) and WT EBV-infected (lower panel) (100× magnification) animals treated with OKT3.

hNSG(thy) model at least partly because Z expression reduces the ability of T cells to recognize infected lymphoma cells.

Lymphomas in OKT3-treated animals have many more LMP1/LMP2A-expressing cells. We previously showed that EBV-induced lymphomas in the hNSG(thy) mouse model usually have surprisingly few cells expressing the LMP1 viral oncoprotein relative to the number of cells expressing EBNA2 (41). A recent LMP1 transgenic mouse model study found that LMP1 induces B-cell lymphomas in the presence, but not absence, of anti-T-cell-depleting antibodies and reported that functional T cells eliminate LMP1-expressing B cells in transgenic mice (70). To determine if OKT3 treatment affects the number of LMP1-positive lymphoma cells in EBV-infected hNSG(thy) mice, EBNA2-LMP1 costaining studies were performed on lymphoma specimens. Similar to our previous findings, very few EBNA2-positive lymphoma cells (less than 1%) coexpressed LMP1 in mice that were not treated with OKT3 (Fig. 9A). In contrast, EBV-infected mice treated with OKT3 developed lymphomas that contained numerous cells coexpressing EBNA2 and LMP1 (Fig. 9B). LMP2A expression was also greatly increased in animals treated with anti-CD3 antibody (data not shown). The EBNA2/LMP1 costaining studies also revealed the presence of a small population of lymphoma cells with the type IIA latency form characteristic of Hodgkin lymphomas ($LMP1^+$ $EBNA2^-$) (Fig. 9C). Together, these results indicate that OKT3 treatment is associated with greatly increased expression of LMP1 and LMP2A in EBV-infected B cells in hNSG(thy) humanized mice, consistent with the rapid onset of EBV-induced lymphomas in these animals.

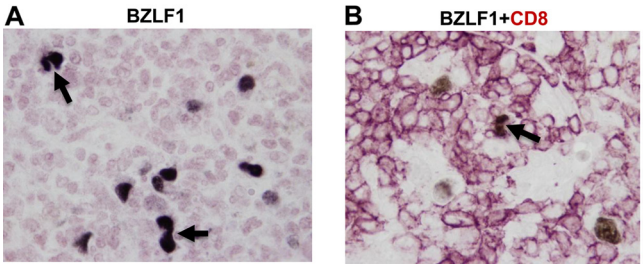

FIG 5 BZLF1-expressing lymphoma cells can divide in hNSG(thy) mice. (A) BZLF1 staining of a DLBCL from an SL mutant EBV-infected animal (without OKT3 treatment). (B) Dual-color immunohistochemistry was performed using anti-BZLF1 (black) and anti-CD8 (pink) antibodies in an SL-EBV-infected animal (without OKT3 treatment). Dividing cells are indicated by black arrows (100× magnification).

Lytic viral protein and LMP1/LMP2A expression are mutually exclusive. Since both LMP1 and LMP2A have been proposed to inhibit lytic viral reactivation in various EBV-infected cell line models *in vitro* (2, 43, 44, 52), we performed LMP1-BZLF1 and LMP1-BMRF1 costaining studies with SL virus-infected lymphoma cells to determine if LMP1 expression is inversely correlated with lytic viral protein expression. Consistent with the results of previous *in vitro* studies, BMRF1 expression was never observed in cells expressing LMP1 and Z expression occurred at only a very low level in cells expressing LMP1 (Fig. 10). Z expres-

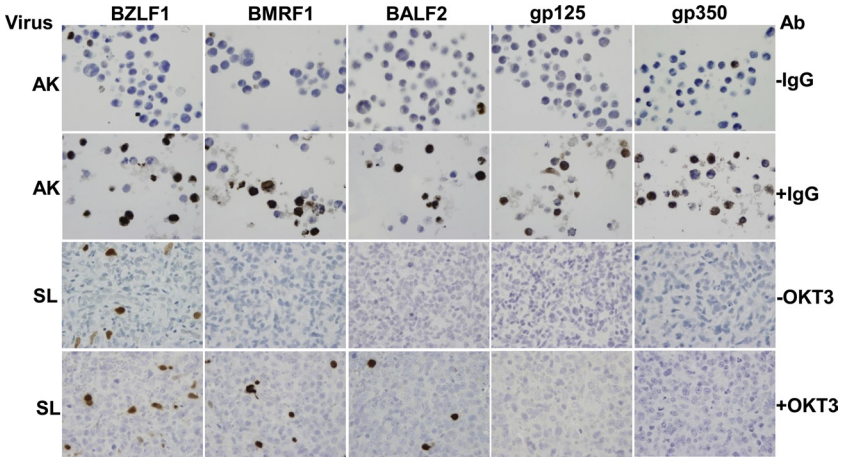

FIG 6 The SL EBV mutant induces an abortive form of lytic EBV infection in lymphoma cells. Anti-BZLF1, anti-BMRF1, anti-BALF2, anti-gp125, and anti-350/220 staining was performed as indicated (100× magnification) to examine expression of immediate-early (BZLF1), early lytic (BMRF1 and BALF2), and late lytic (gp125 and gp350/220) viral proteins in SL EBV-infected animals. Formalin-fixed and paraffin-embedded Akata (AK) BL cells treated *in vitro* without or with anti-IgG pretreatment as indicated in the upper two panels served as the baseline and induced controls for lytic EBV protein expression, respectively. Abortive lytic infection is seen in animals with or without OKT3 treatment, as indicated in the lower two panels.

sion and LMP2A expression were likewise found to be mutually exclusive (Fig. 10). These results suggest that LMP1 and/or LMP2A inhibits BZLF1 expression in lymphoma cells and that the lytically infected population of lymphoma cells in this model is distinct from the subset of cells containing the classic type III form of viral latency (LMP1$^+$ EBNA2$^+$). Our results do not exclude the possibility that BZLF1inhibits LMP1 and LMP2A expression.

The SL mutant establishes asymptomatic viral latency in some hNSG(thy) mice. Since the majority (4/6) of the SL virus-infected animals did not develop lymphoma in the absence of OKT3 treatment, we asked if the SL mutant is impaired for the ability to establish long-term viral latency in the tumor-free animals. Tissues harvested from a variety of different organs of each EBV-infected animal (including spleen, liver, lymph nodes, lungs, kidney, and implanted thymic tissue) were examined for the presence of latently infected cells using EBER *in situ* hybridization and anti-EBNA1 immunostains. Other than two animals sacrificed at day 3 postinfection, each of the WT and SL virus-infected animals had definite EBER-positive and EBNA1-positive cells (most commonly in the spleen) at the time of euthanasia (Fig. 11). Of note, the number of EBV-infected cells present in the tumor-free WT and SL virus-infected animals was usually extremely low (i.e., only a few positive cells found per spleen section), consistent with the ability of these animals to successfully control their EBV infection. No Z-expressing cells or LMP1-expressing cells were detected in any of the tumor-free, SL or WT virus-infected animals euthanized at day 60 postinfection (Fig. 11) (data not shown). These results indicate that the SL virus can establish viral latency in some hNSG(thy)-infected mice and that long-term asymptomatic infection is associated with suppression of Z and LMP1 expression.

To determine if there is an early burst of lytic gene expression prior to the onset of viral latency, we also sacrificed WT and SL virus-infected mice at two earlier time points postinfection to examine the amounts of EBER, Z, and BMRF1 expression. Z$^+$ cells were detected in SL virus-infected animals at days 12 and day 37 postinfection (Fig. 11). Animals examined at day 3 postinfection

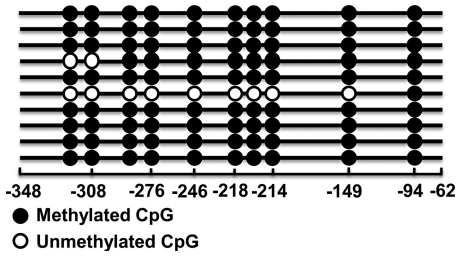

FIG 7 The EBV BMRF1 early lytic promoter is highly methylated in EBV-induced DLBCLs. DNA isolated from an SL mutant EBV-infected DLBCL without OKT3 treatment was bisulfite treated and sequenced. The black dots indicate the methylated CpGs, and white dots indicate the unmethylated CpGs in each sequenced clone. The numbers shown are relative to the BMRF1 transcript start site.

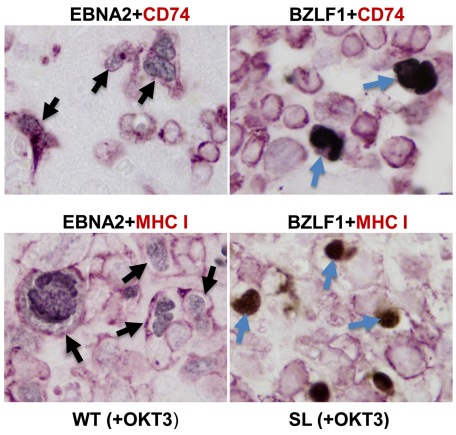

FIG 8 BZLF1-expressing cells have decreased expression of CD74 and MHC I. Dual-color immunohistochemistry was performed using anti-EBNA2 or anti-BZLF1 (black) and anti-CD74 or anti-MHC I (pink) antibodies on DLBCLs from OKT3-treated animals. EBNA2$^+$ costaining was performed on a WT EBV-infected DLBCL, while BZLF1 costaining was performed on an SL mutant EBV-infected DLBCL. Examples of EBNA2 and CD74 or MHCI coexpression are indicated by black arrows; examples of BZLF1-positive cells lacking CD74 or MHCI are indicated by blue arrows.

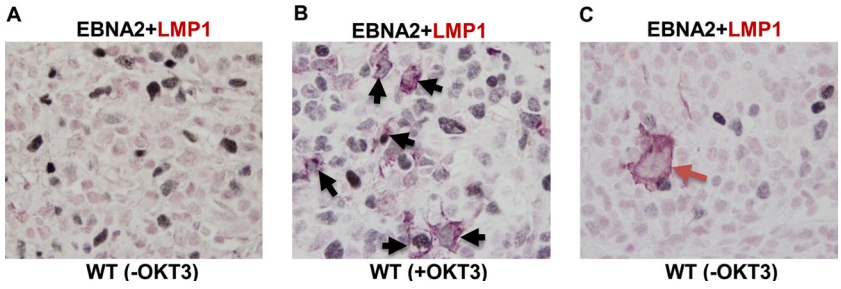

FIG 9 OKT3 treatment greatly increases LMP1 and LMP2A expression in lymphoma cells. Dual-color immunohistochemistry was performed using anti-EBNA2 (black) and anti-LMP1 (pink) antibodies in DLBCLs from WT EBV-infected animals (A and C) and a DLBCL from a WT EBV-infected animal that had been treated with OKT3 antibody (B). Examples of EBNA2-positive cells costaining with LMP1 are indicated by black arrows in panel B. An example of a cell with type IIA latency ($LMP1^+$ $EBNA2^-$) is indicated by a red arrow in panel C. All pictures are at 100× magnification.

had so few $EBER^+$ cells that Z coexpression could not be reliably evaluated. These results suggest that hNSG(thy) mice infected with the SL mutant initially express Z in a subset of virally infected B cells, but that by day 60, the lymphoma-free mice have successfully established viral latency.

DISCUSSION

Although the latent and lytic forms of EBV infection are both essential for long-term success of the virus, it has been difficult to study the various effects of primary lytic viral infection *in vitro* due to the lack of permissive cell lines that support lytic infection. Here we have used a new humanized model, in which both human $CD34^+$ hematopoietic stem cells and human thymic tissue are engrafted into NSG mice, to explore the effect of enhanced lytic EBV gene expression on EBV pathogenesis and lymphoma formation in the presence and absence of an antibody (OKT3) that inhibits T-cell function. These studies were made feasible by the construction of a new superlytic (SL) EBV mutant missing three negatively regulating elements (two ZEB motifs and the ZIIR element) in the BZLF1 IE promoter. Our results show that although B cells infected with the SL mutant are much more likely to express Z than B cells infected with the WT virus, the two viruses are surprisingly similar in their ability to induce lymphomas (in the presence and absence of OKT3 treatment) and to establish persistent tumor-free viral latency. Furthermore, our results show that Z-expressing lymphoma cells in this mouse model contain an abortively lytic form of viral infection that does not appear to inhibit lymphoma cell growth and is associated with decreased expression of MHC class I and CD74. Finally, we find that OKT3 treatment dramatically impairs the ability of hNSG(thy) mice to control either the WT or SL viruses and allows the development of lymphomas with a greatly increased number of LMP1-expressing cells.

The finding that the SL mutant can establish long-term viral latency in some hNSG(thy) mice was not completely unexpected since activation of Z transcription requires not only the inhibition of negatively regulating factors, but also the presence of positively regulating factors. In contrast to studies performed *in vitro*, in the hNSG(thy) model, EBV-infected B cells are often surrounded by T cells and are exposed to a variety of different signals from neighboring cells that may influence the activity of the Z promoter. For example, since CD40 signaling has been reported to inhibit lytic reactivation of EBV-infected B cells *in vitro* (2), the CD40 ligand expressed on the surface of activated CD4 T cells may inhibit Z expression in EBV-infected B cells in hNSG(thy) mice. Furthermore, both LMP1 and LMP2A have been reported to inhibit lytic viral reactivation *in vitro*, and we found that LMP1/LMP2A expression and early lytic viral protein expression are mutually exclusive in SL virus-infected lymphoma cells. Although the exact mechanism or mechanisms by which CD40 signaling and the LMP1 and LMP2A proteins inhibit lytic reactivation are not totally defined, our results here suggest that the mutated SL virus is still responsive to inhibition by these factors. Another interesting possibility to explain the lack of cells coexpressing Z and LMP1 would be that Z inhibits expression of LMP1.

A perhaps more surprising result is our finding that high-level Z expression in a relatively large percentage of EBV-infected lymphoma cells (up to 30% of tumor cells in the OKT3-treated animals) did not appreciably inhibit lymphoma formation or tumor cell division in the hNSG(thy) model. The continued viability of Z-expressing lymphoma cells can be at least partially attributed to the unexpectedly low expression of the late lytic viral proteins in

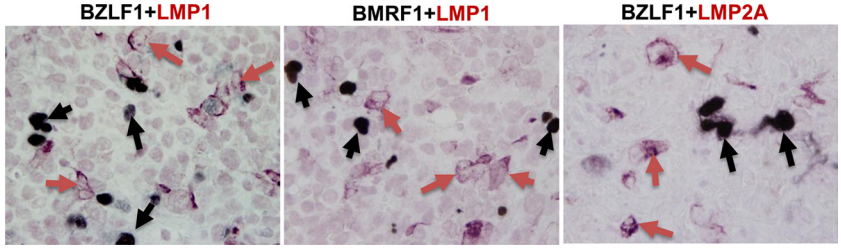

FIG 10 BZLF1/BMRF1 and LMP1/LMP2A expression are mutually exclusive. Dual-color immunohistochemistry was performed using anti-BZLF1 or anti-BMRF1 (black) and anti-LMP1 or anti-LMP2A (pink) antibodies as indicated on SL EBV-infected DLBCLs from OKT3-treated animals. Cells expressing only BZLF1or BMRF1 are indicated by black arrows, while cells expressing only LMP1 or LMP2A are indicated by red arrows.

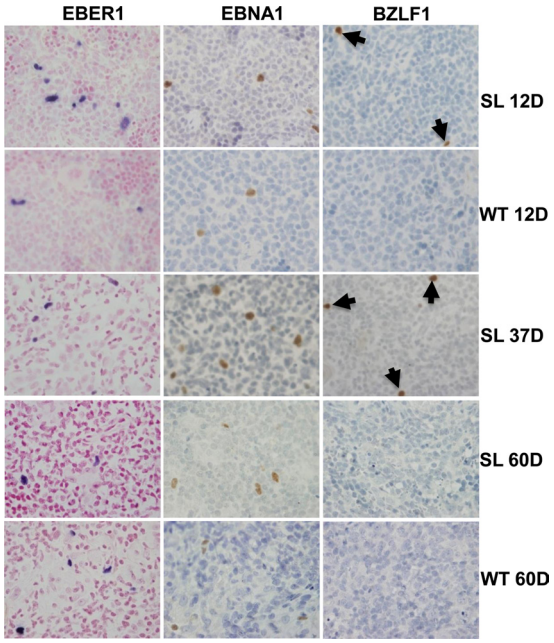

FIG 11 The SL mutant establishes tumor-free viral latency in some hNSG(thy) mice by day 60 postinfection. EBER1, EBNA1, and BZLF1 staining was performed on spleens of WT and SL mutant EBV-infected animals (not treated with OKT3) sacrificed at different days (D) postinfection as indicated (40× magnification). Examples of BZLF1-staining cells are indicated by arrows at the earlier time points, but at day 60, none of these tumor-free animals still had BZLF1-expressing cells.

Z-expressing cells in this model. Consistent with the lack of late viral protein expression in the Z-expressing lymphoma cells, the circulating viral loads of animals infected with the WT and SL viruses were similar, and the high viral loads that occurred in some OKT3-treated animals were eliminated by prior DNase I treatment (indicating that the viral DNA was derived from lysed lymphoma cells rather than packaged viral particles). Thus, most Z-expressing B cells in the hNSG(thy) animal model appear to have an "abortive" form of lytic gene expression that does not result in release of infectious viral particles or killing of host cells.

The high number of Z-positive SL virus-infected lymphoma cells in the hNSG(thy) model (particularly in animals treated with the anti-CD3 antibody) has allowed us to make some new observations with regard to the effects of high-level Z expression in EBV-infected lymphoma cells. For example, in contrast to the predictions of previous *in vitro* studies, we found that Z expression *per se* (in the absence of fully lytic viral infection) does not inhibit proliferation of EBV-infected lymphoma cells. We were also able to confirm that some potentially important previous *in vitro* observations are valid in the hNSG(thy) model. For example, we confirmed that Z-expressing cells often lack CD74 expression, which may provide one explanation for the relative inability of CD4 T cells to eliminate Z-expressing cells in this model. CD74 is essential for presentation of antigens via the MHC class II pathway to CD4 cells (45) and may also play a role in MHC class I presentation to CD8 T cells in dendritic cells (4). Although CD74 expression has been reported to be required for the continued viability of B cells (24), a recent report suggested that expression of the EBV-encoded early lytic BHRF1 protein (a BCL2 homologue) allows Z-expressing cells to survive in the absence of CD74 expression (71). Although we could not confirm whether the SL virus-infected lymphoma cells express the BHRF1 protein (since commercially available anti-BHRF1 antibodies are not suitable for immunohistochemical studies in paraffin-fixed tissues), we speculate that enhanced BHRF1 expression may promote the viability of Z-expressing lymphoma cells. Since a recent study revealed that the EBV EBNA-3B latency protein inhibits DLBCL formation in humanized NSG mice by promoting T-cell infiltration (65), we also attempted to examine EBNA-3B expression in the WT versus SL virus-infected animals, but were unable to do so due to the current lack of a suitable EBNA-3B antibody for IHC.

The finding that most Z-expressing B cells do not express early or late lytic viral proteins in the hNSG(thy) model is consistent with our recent finding that the B-cell-specific transcription factor, Oct-2, inhibits Z transcriptional function (54). The EBV-infected lymphoma cells in this animal model were confirmed to express Oct-2 (data not shown). T-cell-mediated effects may also contribute to the low level of early lytic viral protein expression (in comparison to Z expression) in this model, since early lytic viral protein expression was substantially increased in SL virus-infected animals treated with OKT3. Although this result may be due to enhanced T-cell recognition and killing of cells expressing multiple different early lytic viral antigens, it may also reflect the loss of T-cell-derived signaling (such as CD40L) that promotes viral latency in Z-expressing B cells (2). Since late viral protein expression was not observed even in the presence of the OKT3 antibody treatment, and viral loads were similar in the WT- and SL virus-infected animals, we conclude that most Z-expressing lymphoma cells do not support lytic EBV replication due to intrinsic resistance. Phosphorylation of Z residue S173 is required for viral replication *in vitro* (but not for early lytic gene expression) (17). Thus, an interesting possibility is that Z does not become phosphorylated at residue 173 in the lymphoma cells. However, our studies do not completely exclude the possibility that cells with fully lytic infection are so rapidly killed (by the virus or the host response) that they are difficult to detect.

In any event, our results here clearly show that EBV has encoded multiple different redundant mechanisms for ensuring the efficient establishment of viral latency in B cells, and restricting the fully lytic form of viral infection to appropriate cell types such as plasma cells and oropharyngeal epithelial cells. Consistent with our results here in the hNSG(thy) model, a recent modeling study suggested that almost all of the infectious EBV particles in human saliva are derived from virus replicated within the oropharyngeal epithelial cells, rather than B cells (26). Nevertheless, since lytically infected B cells traveling through the oropharyngeal tissue are thought to be required for infection of the oropharyngeal epithelial cells (7), the ability of EBV to produce infectious viral particles from rare B cells is presumably essential for viral transmission from host to host. Since plasma cell differentiation appears to be relatively infrequent in the hNSG(thy) model (data not shown), and human oropharyngeal epithelial cells are not engrafted in this model, the fully lytic form of EBV infection may be underrepresented. Of note, since one of the three SL virus-infected, OKT3-treated, animals had a high viral load (500 copies of EBV per ml of plasma) at day 12 postinfection, lytic viral replication may occur to at least some extent at early time points in the hNSG(thy) model.

Although the results here suggest that "lytic" induction therapy for EBV-positive tumors may be more difficult to achieve than

previously envisioned, they certainly do not preclude the further development of this therapy. Lytic induction therapy involves a two-tiered approach that includes not only the use of agents to activate BZLF1 expression (for example, histone deacetylase [HDAC] inhibitors), but also the delivery of prodrugs (e.g., ganciclovir) that can be specifically converted to cytotoxic agents by the EBV-encoded protein kinase (14, 20, 21, 64) or thymidine kinase (radioactively labeled 2′-fluoro-2′-deoxy-beta-D-5-iodouracil-arabinofuranoside [FIAU]) (23). Since the EBV-encoded kinases are both encoded by early lytic genes, and most of the tumor cell killing in this approach is mediated by the activated prodrugs, late viral gene expression does not need to be activated in tumor cells for lytic induction therapies to be effective. Furthermore, since we recently showed that HDAC inhibitors dramatically decrease B-cell expression of Oct-2 *in vitro* (54), HDAC inhibitors may be useful not only for activating Z transcription, but also for enhancing the ability of the Z protein to activate expression of the two early lytic viral kinase genes.

In this report, we also demonstrate that functional T cells are absolutely essential for the control of EBV infection in the hNSG(thy) model, since all EBV-infected animals (regardless of whether they were infected with WT or SL virus) rapidly succumbed to EBV-induced lymphomas in the presence of the OKT3 antibody. In addition, we show that the number of EBNA2-LMP1-coexpressing lymphoma cells (i.e., cells with classically defined type III latency) is closely correlated with T-cell function, since EBNA2-LMP1-coexpressing lymphoma cells are rarely observed in the EBV-infected hNSG(thy) model unless the animals are treated with OKT3. *In vitro* studies have shown that LMP1 protein levels in lymphoblastoid cell lines (LCLs) can differ by up to 100-fold from cell to cell and that the LMP1 protein level within each cell varies greatly over time (9, 38). Although LMP1 itself is not efficiently recognized by $CD8^+$ T cells, cells that have high-level LMP1 expression have enhanced MHC class I antigen presentation in comparison to cells that express less LMP1 and are much more efficiently killed by cytotoxic T cells *in vitro* (9). In addition, LMP1 induces CD95 expression on LCLs *in vitro* and renders them susceptible to CD95L-mediated killing by T cells (37). Furthermore, in a recent transgenic mouse model, the ability of LMP1 to cause B-cell lymphomas required inhibition of T-cell function (70).

Thus, LMP1-positive lymphoma cells may be preferentially eliminated by T cells and therefore difficult to detect in EBV-infected hNSG(thy) mice unless they are treated with the OKT3 antibody to inhibit T-cell function. The presence of a high number of LMP1-expressing lymphoma cells in some other humanized mouse models currently being used to study EBV pathogenesis and lymphoma formation (which do not include transplanted human thymic tissue) (12, 59, 61, 66) suggests that the human T cells in these models are not functioning as well as they are in our hNSG(thy) model. Nevertheless, while our finding here that OKT3 treatment greatly increases the number of EBNA2/LMP1-positive cells is consistent with a model whereby LMP1-positive cells are preferentially killed by T cells, it is also possible that T-cell-derived signals or cytokines decrease the level of LMP1 protein in EBNA2-expressing B cells.

In humans, $EBNA2^+$ $LMP1^-$ B cells (known as "type IIB" latency) have been described in mononucleosis patients, as well in AIDS-associated lymphomas and PTLDs (27, 50). Since such cells represent only a portion of the cells in such tumors (which also contain $EBNA2^+$ $LMP1^+$ cells with type III latency), it has been generally assumed that only the cells with type III latency are driving the growth of such tumors. Interestingly, a Burkitt lymphoma was recently described in which a small portion of the primary tumor cells (5 to 10%) expressed EBNA2 but not LMP1 (33). Furthermore, a cell line derived from this tumor which retained the $EBNA2^+$ $LMP1^-$ phenotype was less susceptible to various apoptotic stimuli than a cell line derived from the same tumor that had type I (EBNA1 only) infection. Thus, EBNA2 expression (in the absence of LMP1) may protect tumor cells from various forms of apoptosis and may be selected for until additional cellular mutations have occurred that allow for lymphoma growth in the absence of EBNA2. Our results here also raise questions with regard to the precise definition of type III latency in human lymphoma specimens. In particular, what percentage of $EBNA2^+$ cells must coexpress LMP1 for a tumor to be labeled as having type III latency? Finally, our findings here suggest that high-level LMP1 expression may target EBV-infected lymphoma cells for killing in the presence of functional T cells and that EBV-infected B cells expressing very little (if any) LMP1 can persist as B-cell lymphomas. Thus, high-level LMP1 expression may inhibit lymphoma formation in the context of an intact host cell immune system but promote lymphoma maintenance when T-cell function is compromised.

ACKNOWLEDGMENTS

We thank Henri-Jacques Delecluse for providing the EBV bacmid, Jaap Middeldorp for providing the anti-BALF2 antibody, Eric C. Johannsen for providing the anti-gp350 antibody, and Richard Burgess for kindly providing the anti-EBNA1 antibody (EB14).

This research was supported by grants P01CA22443, R01-CA58853, and R01-CA66519 from the National Institutes of Health and University of Wisconsin Cancer Center support grant P30 CA014520.

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