INTRODUCTION

Epstein-Barr virus (EBV) is a double-stranded DNA virus belonging to the family Herpesviridae, with widespread seroprevalence in humans.\(^1\)\(^,\)\(^2\) Epstein-Barr virus normally infects B cells through CD21, resulting in a variety of conditions, including infectious mononucleosis, as well as neoplastic diseases such as Burkitt lymphoma and B-cell lymphoproliferative disorders (LPDs). Among LPDs, immunodeficiency-associated LPDs are also of concern, including post-transplant LPD and HIV-associated LPDs.\(^3\)

The first choice of therapy against EBV-LPDs is rituximab\(^4\); however, repeated treatment could lead to resistance due to the selective expansion of CD20\(^{-}\) cells. In cases where rituximab alone is ineffective for EBV-LPDs, cotreatment with other chemotherapeutic...
agents is required, although the toxicity of these multidrug regimens is a concern, particularly in transplant patients receiving immunosuppressive therapy. Infusion of donor lymphocytes is viable for EBV-LPD patients as a nonchemotherapeutic approach; however, this approach confers a greater risk of developing graft-versus-host disease. Therefore, there remains a critical need to develop novel therapies against EBV-LPD.

Once infected, EBV is able to establish a latent, asymptomatic, lifelong infection in most individuals. The transition from latent to lytic infection in individuals previously infected with EBV is known as reactivation. In lytic infections, BZLF1 and BRLF1, the immediate early genes of the herpesvirus life cycle, are expressed and induce expression of the early (E) genes necessary for viral DNA synthesis, as well as the late genes (L) encoding viral structural proteins. Accumulating evidence shows that upregulation of the lytic cycle promotes lymphomagenesis. In a recent drug screening study, we identified a group of cyclin-dependent kinase (CDK) inhibitors that specifically suppress the expression of EBV late genes encoding for viral structural proteins. From a mechanistic standpoint, the CDK2 complex has been shown to play an important role in the phosphorylation of BDLF4, a key regulator of late gene expression. However, it is not yet clear whether CDK inhibition would be useful for treating EBV-LPDs.

Cyclin-dependent kinase inhibitors have been shown to be effective for the treatment of several diseases, including cancers, neurodegenerative diseases, and viral infections. Cyclin-dependent kinase inhibitors have been shown to exert a wide range of effects, including changes in the cell cycle, apoptosis, and cell differentiation. Alsterpaullone is an ATP-competitive inhibitor comprised of several CDKs, as well as glycogen synthase kinase (GSK)-3β. Alsterpaullone has been shown to suppress CDK1/cyclin B, CDK2/cyclin A, CDK2/cyclin E, CDK5/p25, and GSK3α/β, with IC50 values of 35, 15, 200, and 4 nmol/L, respectively. Previous research has also reported the antiviral effects of alsterpaullone against HIV type-1.

In this study, we examined the therapeutic effect of alsterpaullone against EBV-LPDs in vivo. Using a mouse model, CDK inhibition was shown to decrease tumor size, suppress tumor infiltration, and increase survival rates in mice. Cells treated with alsterpaullone showed lower levels of late gene expression, along with suppression of cell growth. Moreover, the CDK inhibitor suppressed cell cycle progression and induced apoptosis in EBV-infected B cells.

## MATERIALS AND METHODS

### 2.1 Cell culture and reagents

A lymphoblastoid cell line (LCL) was established by infecting B cells derived from a healthy donor with WT or BDLF4-knockout EBV in vitro. An EBV-positive HEK293 cell line (HEK293EBV) was established by transfection of BAC DNA (coding for GFP and hygromycin resistance) carrying the entire EBV genome. Then LCL and EBV-negative human B cell line Akata cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS. HEK293EBV cells were cultured in DMEM (Sigma) supplemented with 10% FBS and 150 µg/mL hygromycin. Alsterpaullone was purchased from Cayman Chemical Company and dissolved in DMSO. Alsterpaullone 2-cyanoethyl (A2CE), and Cdk2/9 inhibitor (CDK2/9i) were purchased from Calbiochem and dissolved in DMSO.

Antibodies against BZLF1, BALF2, BMRF1, BRRF2, BALKF4 (gB), and BKRF4 have been reported previously. Antibodies against CDK1 (S12; BD Biosciences), CDK2 (AC-74; Sigma), p27 (D69C12; Cell Signaling Technology), retinoblastoma (Rb) (4H1; Cell Signaling Technology), pRB Ser780 (C84F6; Cell Signaling Technology), cleaved PARP (D64E10; Cell Signaling Technology), and cleaved caspase (5A1E; Cell Signaling Technology) were prepared for detection of cell cycle- or apoptosis-related molecules. Anti-GAPDH Ab (14C10; Cell Signaling Technology) was used as the endogenous control. Forte (Merck) was used as the HRP substrate, and EZ-Analyzer (ATTO) was used for luminescence detection. As the secondary Ab, HRP-conjugated goat anti-mouse IgG (BioSource) or HRP-conjugated goat anti-rabbit IgG (H + L) (Invitrogen) were used.

### 2.2 Transfection and western blot analysis

The BZLF1 expression vector has been described previously. BZLF1 was transfected into HEK293EBV using Lipofectamine 2000 (Thermo). Alsterpaullone (0.5 µmol/L) was added to the culture medium at 24 hours posttransfection. Cells treated with alsterpaullone for 24 hours were dissolved in sample buffer (50 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 6% 2-mercaptoethanol, 0.0025% bromophenol blue), resolved by SDS-PAGE and transferred to a PVDF membrane. The primary and secondary Abs were prepared by dilution according to previous reports.

### 2.3 Reporter assay

A luciferase expression reporter plasmid, pTATT-oriLyt-Luc, encoding the promoter of the late gene was kindly provided by Eric Johannsen (University of Wisconsin School of Medicine). PSV40-Rluc (Promega) was used as a control reporter plasmid. HEK293EBV cells were transfected with BZLF1 and reporter plasmids. At 24 hours posttransfection, cells were treated with alsterpaullone. Luminescence in cell lysates was determined by PowerScan 4 (BioTek) at 24 hours after treatment.

### 2.4 Measurement of viral DNA by real-time PCR

The amount of viral DNA in cells was quantified by real-time PCR. HEK293EBV cells transfected with BZLF1 were cultured for 24 hours in the presence of alsterpaullone. Cells were then solubilized with lysis buffer (10 mmol/L Tris-HCl, pH 8, 1 mmol/L EDTA) and treated with...
protease K. Real-time PCR was carried out using Fast Start Universal Probe Master (ROX) (Roche Applied Science), eukaryotic 18S rRNA endogenous control (Applied Biosystems), BALF2-specific primer, and FAM/TAMRA fusion BALF2 probe. The sequences of the BALF2 primers were 5′-GCCCGTCCGTTGCTA-3′ (forward primer) and 5′-AAATCTGGTTGTGGTTCTGA-3′ (reverse primer); the probe was 5′-FAM-CTGCCAGTGCATCAAAAGTACACGG-TAMRA-3′.

2.5 | Quantification of virus infectivity by FACS

HEK293EBV cells were transfected with BZLF1 and incubated for 72 hours, after which the supernatant was collected for use as viral fluid. The EBV-negative Akata cells were cocultured with viral fluid for 2.5 hours, incubated for 2 days, and then fixed with 4% paraformaldehyde. Finally, the GFP-positive cell ratio was measured by FACS (FACSCalibur G5 system; Becton Dickinson).

2.6 | Cell cycle and apoptosis analysis

Lymphoblastoid cell lines were cultured in medium containing alsterpaullone, CDK2/9i, and A2CE for 24 hours. Western blotting was then carried out to identify cell cycle- or apoptosis-related molecules. In analyzing cell cycles by FACS, LCLs treated with alsterpaullone were fixed with cold 70% ethanol and stained with Hoechst 33342 (Wako) for 1 hour. For analysis of apoptosis, fixed cells were costained with phycoerythrin (PE) and 7-AAD using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences). Cell cycle and apoptotic cells were detected using FACS and analyzed using ModFit LT and FlowJo software.

2.7 | Cell proliferation assay

For cell proliferation, 2 × 10⁵ LCL cells were seeded in 12-well plates in 1 mL medium containing 0.5 or 1.0 µmol/L alsterpaullone or DMSO. Cells were counted by Trypan blue exclusion test using a Countess automated cell counter (Invitrogen) every 24 hours. All experiments were carried out in triplicate.

2.8 | Epstein-Barr virus LPD mouse model

Six-week-old severely immunodeficient NOG mice (NOD/Shi-scid, IL-2RγKO), were obtained from the Central Institute for Experimental Animals. Human cord blood mononuclear cells, provided by RIKEN, were cocultured with viral fluid obtained from HEK293EBV cells in vitro, and then given i.p. Either DMSO or alsterpaullone (5 mg/kg) was given i.p. every other day for 3 weeks (total of 12 treatments). Tumors formed i.p. were observed under bright field and fluorescence microscopy using an M205 FA (Leica). All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Nagoya University, Japan. The mice were killed when they showed signs of clinical illness (20% weight loss, ruffled coat, and hunching behavior).

2.9 | Immunohistochemistry

After death, the pancreas was removed after re flux fixation with 10% neutral buffered formalin (Wako). Samples were then dehydrated, defatted, and paraffin-infiltrated using a Tissue-Tek VIP 6 tissue processing system (Sakura Finetek). Formalin-fixed paraffin-embedded tissue specimens were prepared with a TEC-P-S (Sakura Finetek). Hematoxylin-eosin staining, immunohistochemical (IHC) staining with an anti-CD20 Ab, and EBV-encoded small RNA (EBER) by in situ hybridization (ISH) were carried out as reported previously.²⁴ For the ISH, FITC-conjugated EBER probe YS200 (Agilent) and an ISH Detection Kit (Agilent) were used according to the manufacturer’s protocol. For the IHC, the antigen retrieval method was undertaken at 98°C for 30 minutes using Target Retrieval Solution pH 9 (Agilent). After blocking with Protein Block (Agilent), a primary Ab reaction was carried out for 1-2 hours with an anti-CD20 Ab (L26; Agilent). Endogenous peroxidase blocking was carried out by dipping in methanol containing 0.3% hydrogen peroxide for 30 minutes. Secondary Ab reaction was carried out with EnVision Dual Link System-HRP (rabbit/mouse) (Agilent) for 30 minutes and reacted with diaminobenzidine (Sigma) for approximately 5 minutes. The stained sections were dehydrated with 100% ethanol and sealed with Entellan New (Millipore). Imaging of stained sections was undertaken with a virtual slide system (VS120; Olympus).

3 | RESULTS

3.1 | Alsterpaullone suppresses late gene expression and virus production

We identified several CDK inhibitors (eg, CDK2/9i, A2CE) as compounds that specifically suppress viral late gene expression; however, there have been no reports of their use in mouse experiments.²⁵,²⁶ Therefore, we decided to use alsterpaullone, the precursor of A2CE, which has a history of in vivo testing.²³ First, we confirmed whether alsterpaullone had the same pharmaceutical efficacy as A2CE with respect to suppression of viral late gene expression during lytic infection. To examine viral gene expression, we transduced BZLF1 into HEK293 cells carrying WT EBV DNA to induce lytic infection. At 24 hours posttransfection, we added alsterpaullone (0.5 µmol/L) directly to the culture medium. Western blot analysis revealed that alsterpaullone treatment suppressed the expression of the late genes BRRF2, gB, and BKRF4 (Figure 1A). A reporter assay using a luciferase expression plasmid driven by a late gene promoter showed that the CDK inhibitor decreased luciferase activity in a dose-dependent manner (Figure 1B). In addition, infectious virus production decreased in response to inhibitor treatment (Figure 1C). In contrast, the amount of viral DNA in cells treated with the inhibitor was the
same as that of untreated cells (Figure 1D). These results indicated that the CDK inhibitor effectively blocked virus production by suppressing late gene expression at the transcriptional level.

3.2 Effect of CDK inhibitor on cell growth in EBV-positive B cells

To analyze the effect of CDK inhibition on cell proliferation, we examined the growth of an EBV-transformed LCL corresponding to EBV-LPD in the presence of alsterpaullone. A previous report showed that alsterpaullone concentrations up to 5 μmol/L did not confer any cytotoxicity in human PBMCs. Here, alsterpaullone treatment decreased the proliferation of the LCL in a dose-dependent manner (Figure 2A).

3.3 Impact of BDLF4 knockout on cell proliferation

Epstein-Barr virus late genes are transcriptionally regulated by the viral preinitiation complex (vPIC). To examine the influence of late gene expression on cell growth, we established an LCL cell line infected with EBV for 24 h, after which the expression of the late gene was measured by reporter assay. Expression of BZLF1 and GAPDH were detected by western blotting. C, Viral DNA was quantified by real-time PCR in reactivated cells in the presence or absence of alsterpaullone. D, HEK293EBV cells in the lytic phase were treated with alsterpaullone or DMSO for 72 h, and the supernatant was cocultured with Akata cells. The GFP-positive rate was measured by FACS. Results are shown as the mean ± SD of 3 independent biological replicates. *P < .05, **P < .01, Student’s t test. E, early; IE, immediate-early; L, late; n.s., no significant difference.

3.4 Cyclin-dependent kinase inhibitor induces apoptosis in EBV-infected B cells

The CDK inhibitor alsterpaullone has been shown to induce G1 cell cycle arrest and apoptosis. Therefore, we evaluated the effect of alsterpaullone on the cell cycle in an EBV-positive B cell line. The LCLs were treated with alsterpaullone at concentrations of 0.1-1.0 μmol/L for 24 hours, after which cell cycle- and apoptosis-related molecules were detected by western blot analysis. Alsterpaullone treatment decreased the expression of CDK2 in a dose-dependent manner (Figure 3A). As Rb, a key cell cycle suppressor gene, is phosphorylated...
by CDK2, it is not surprising that phosphorylation of Rb Ser780 was suppressed following inhibition of CDK2 expression (Figure 3A). Even when treated with other CDK inhibitors during routine drug screens, phosphorylation of Rb was suppressed, and expression of apoptosis-related molecules induced, in these cells (Figure 3B).

Following these results, cells were next treated with different concentrations of alsterpaullone for 24 hours and stained with Hoechst 33342; the cell cycle was analyzed by FACS. The populations of G0/G1 and S phase changed dramatically in response to treatment, and the proportion of sub-G1 phase cells expressing markers for apoptosis increased in a dose-dependent manner (Figure 3C). In cells costained with PE and 7-AAD, differences in apoptosis were detected based on drug treatment (Figure 3D). These results suggest that the CDK inhibitor efficiently induces apoptosis in EBV-infected B cells.

3.5 | Alsterpaullone has an antitumor effect in EBV-LPD mouse model

To analyze the in vivo effects of alsterpaullone, we used a mouse model of EBV-LPD. Cord blood-derived mononuclear cells were cocultured with EBV in vitro and then injected i.p. to severely immunodeficient NOG mice. Beginning the next day, alsterpaullone (1 mg/kg) was given i.p. every other day for 3 weeks. Using these data, we determined the optimal dose and route of the inhibitor with reference to a xenograft model of the ovarian tumor (Figure 4A). Viral DNA levels in the peripheral blood of the treatment group decreased compared with nontreated controls (Figure 4B). Moreover, the survival rate significantly increased following treatment with the inhibitor (Figure 4C). Tumor formation in the pancreas was suppressed by inhibitor treatment (Figure 4D). The H&E staining of tumor tissue sections showed a clear reduction in lymphocyte infiltration in inhibitor-treated mice (Figure 4D). Immunostaining with an anti-CD20 Ab for detecting B cells, and ISH using a nucleic acid probe that specifically binds to EBER, was undertaken to identify EBV-positive B cells. The EBV-positive B cell infiltration was decreased in mice treated with the inhibitor (Figure 4D). BZLF1, a representative marker of lytic infection and the master key gene for promoting the lytic cycle, was detected in the tumor tissue of nontreated control mice (Figure 4D). These results indicated that the CDK inhibitor has an antitumor effect in an EBV-LPD mouse model. We have undertaken an additional mouse experiment to compare the effect of the CDK inhibitor in mice treated with WT EBV and BDLF4 knockout virus (Figure S1). Treatment with the CDK inhibitor showed similar therapeutic effects in the WT-treated and BDLF4 knockout EBV-treated mouse group.

4 | DISCUSSION

In this study, we examined the antitumor effect of the CDK inhibitor alsterpaullone in vivo. Use of alsterpaullone in a mouse model of EBV-LPD resulted in decreased infiltration of EBV-positive B cells and lower levels of EBV DNA in the peripheral blood. These changes were accompanied by an increase in survival rate in treated mice, indicating that CDK inhibition conferred an antitumor effect. In vitro studies using cultured cells suggest that the antitumor effect was mediated by more efficient induction of apoptosis, along with suppression of EBV late gene expression.
These findings suggest that alsterpaullone is a potent suppressor of cell cycle activity, through which it shows its antitumor effect on EBV-LPDs.

A number of recent studies have shown that the expression of viral late genes is correlated with viral DNA replication. The vPIC component, containing BDLF4, promotes late gene transcription by utilizing newly synthesized viral DNA as a template. Similar reports have also shown that alsterpaullone (9-nitro-7,12-dihydroindolo-[3,2-d][1]benzazepin-6(5H)-one) and kenpaullone (9-bromo-7,12-dihydroindolo-[3,2-d][1]benzazepin-6(5H)-one) suppress EBV DNA replication, whereas other studies found that the CDK inhibitors purvalanol and roscovitine, which target cyclin A- and cyclin E-related CDKs, including CDK2, block viral immediate-early and early gene expression. Our findings support the idea that CDKs play an important role in broadly regulating the expression of viral genes during lytic infections.
FIGURE 4  Antitumor effect of cyclin-dependent kinase inhibitor in an Epstein-Barr virus (EBV) lymphoproliferative disorder mouse model. A, Human cord blood-derived mononuclear cells were cocultured with infectious virus obtained from HEK293EBV cells. Cells cocultured with virus were injected i.p. into NOG mice. On day 1 after cell inoculation, mice were treated i.p. with alsterpaullone (Alp) (n = 6; 5 mg/kg) or DMSO (n = 6) every other day for 3 weeks (total of 12 treatments). B, Viral copy numbers in the peripheral blood of treated mice were examined 21 days after cell inoculation and compared with untreated mice. Viral DNA levels were quantified by real-time PCR. *P < .05, Student’s t test. C, Kaplan-Meier survival curves. n = 6 mice per group, P = .043, log-rank test. D, At 30 days after inoculation, the pancreas was removed and observed under bright field and excitation light. Tissue sections of pancreas were prepared, followed by H&E staining, immunohistochemical staining for CD20, and in situ hybridization for EBV-encoded small RNA.
ViRal deubiquitinase (BPLF1), terminase (BALF3), and interleukin-10 (IL-10) (BRLF1) are all expressed during the late phase of EBV replication and contribute to oncogenicity. A recent study from our laboratory found that intragenic deletions are frequently present in the EBV genome of EBV-associated lymphoid malignancies, resulting in aberrant reactivation and increased viral gene expression, including late genes, thereby promoting lymphomagenesis. The CDK inhibitor showed antitumor effects in our mouse experiment, suggesting that the expression of late genes in the viral growth cycle might be involved in the pathogenesis of EBV-LPDs. However, the growth of B cells carrying the BDLF4 knockout virus was comparable to that of WT EBV in vitro, suggesting that the contribution of late genes to tumorigenicity might be limited in vitro, as these cells have already acquired neoplastic growth potential. As lytic cells represent only a small fraction of the tumor tissue, clarifying the contribution of late gene expression to tumorigenic potential is difficult. In fact, western blot analysis of established LCLs revealed that the expression of late genes was below the limit of detection (data not shown). Ongoing studies are focused on identifying how late gene expression is involved in tumor formation and progression using a recombinant virus lacking late gene expression.

In summary, we have shown that a CDK inhibitor, alsterpaullone, suppresses EBV-LPDs in a mouse model. In vitro analysis showed that alsterpaullone alters the expression of cell cycle-related molecules and induces apoptosis, thereby suppressing cell proliferation. Although treatment with a CDK inhibitor showed clear therapeutic effects in the mouse model, we were unable to obtain a complete cure, including evidence of invasive EBV-positive B cells in the treatment group (Figure 4C,4). Further consideration will also be required to determine the appropriate treatment conditions, such as dose/treatment period and combination therapy.

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DISCLOSURE

The authors have no conflicts of interest directly relevant to the content of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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