Lenalidomide, Thalidomide, and Pomalidomide Reactivate the Epstein–Barr Virus Lytic Cycle through Phosphoinositide 3-Kinase Signaling and Ikaros Expression


Abstract

Purpose: Lenalidomide, thalidomide, and pomalidomide (LTP) are immunomodulatory agents approved for use in multiple myeloma, but in some settings, especially with alkylating agents, an increase in Hodgkin lymphoma and other secondary primary malignancies (SPM) has been noted. Some of these malignancies have been linked to Epstein–Barr virus (EBV), raising the possibility that immunomodulatory drugs disrupt latent EBV infection.

Experimental Design: We studied the ability of LTP to reactivate latently infected EBV-positive cell lines in vitro and in vivo, and evaluated the EBV viral load in archived serum samples from patients who received a lenalidomide, thalidomide, and dexamethasone (LTD) combination.

Results: Treatment of EBV-infected B-cell lines with LTP at physiologically relevant concentrations induced the immediate early gene BZLF1, the early gene BMRF1, and the late proteins VCA and BCFR1. This occurred in the potency order pomalidomide > lenalidomide > thalidomide, and the nucleoside analogue ganciclovir enhanced the cytotoxic effects of lenalidomide and pomalidomide in Burkitt lymphoma cells in vitro and in vivo. EBV reactivation was related to PI3K stimulation and Ikaros suppression, and blocked by the PI3K inhibitor idelalisib. Combinations of lenalidomide with dexamethasone or rituximab increased EBV reactivation compared with lenalidomide alone and, importantly, lenalidomide with melphanal produced even greater reactivation.

Conclusions: We conclude LTP may reactivate EBV-positive resting memory B cells thereby enhancing EBV lytic cycle and host immune suppression.

Introduction

The immunomodulatory drugs lenalidomide, thalidomide, and pomalidomide (LTP) have contributed to an improvement in survival in patients with multiple myeloma (1). Consequently, they have become part of the backbone of many therapeutic regimens in the upfront, relapsed, and relapsed/refractory settings. Moreover, lenalidomide is now included in maintenance therapy (MT) in both transplant-eligible and transplant-ineligible populations (2–4).

Although the efficacy of LTP is without question, concern has arisen about a possible increase in secondary primary malignancies (SPM). This has been seen with maintenance after melphalan-based high-dose chemotherapy and autologous stem cell transplant (SCT; refs. 2, 3), or following melphalan-based induction therapy (4). Interestingly, lenalidomide maintenance increased the incidence of Hodgkin lymphoma in two studies, an uncommon SPM seen in patients with multiple myeloma. Indeed, Atal and colleagues reported four Hodgkin lymphoma cases in patients with multiple myeloma who had undergone SCT and none in the placebo group (2), whereas McCarthy reported one case (3). Moreover, a retrospective analysis of three phase III trials in relapsed patients with multiple myeloma demonstrated an SPM incidence of 4% in patients who received lenalidomide versus 1.4% in those who received placebo (5).

One possible reason for the development of Hodgkin lymphoma as an SPM is reactivation of Epstein–Barr virus (EBV). This oncogenic gamma herpes virus is associated with, and contributes to the development and maintenance of a variety of human malignancies, including Hodgkin lymphoma, Burkitt lymphoma, and posttransplant lymphoproliferative disease (PTLD; ref. 6). Primary infection occurs during childhood, and lifelong infection is maintained latently in resting memory B cells, with occasional...
viral reactivation. In the absence of immune control, such as in patients who undergo SCT, loss of EBV control can give rise to PTLD (7). The use of HYPER-CVAD regimens in leukemia and lymphoma patients, and methotrexate use in patients with autoimmune disease, increases the incidence of EBV-positive lymphomas (8, 9). It is also interesting to note that lenalidomide-MT in relapsed/refractory multiple myeloma patients following allo-graft SCT induced herpes virus reactivation (EBV and herpes simplex virus) requiring acyclovir prophylaxis (10). In contrast, chemotherapeutics such as doxorubicin or gemcitabine have been shown to reactivate EBV from a latent infection in EBV-positive malignancies to a lytic infection, which has led to clinically significant regression of EBV-positive tumors (11). Hence, the ability of chemotherapy agents to induce lytic EBV infection can be a therapeutic modality for EBV-positive malignancies in combination with the nucleoside analogue ganciclovir, or it could give rise to secondary EBV-positive malignancies in immunocompromised hosts. These possibilities drove us to determine whether lenalidomide reactivates the EBV lytic cycle, which could sensitize tumor cells to the effects of ganciclovir and/or contribute to the development of Hodgkin lymphoma as an SPM.

Translational Relevance

The expanding use of immunomodulatory drugs in multiple myeloma, other hematologic malignancies, and diseases other than cancer demonstrates the potential effectiveness of this class of agents. Their long-term use, such as in the maintenance therapy setting, however, may reveal previously unidentified downstream effects and toxicities. Our findings show the reactivation of the Epstein–Barr virus (EBV) into its lytic life cycle from a previously latent state occurs primarily through the stimulation of PI3K signaling. This phenomenon has the potential to be a novel therapeutic strategy against EBV-positive malignancies in combination with ganciclovir. However, these findings also raise concern about the immunosuppressive effects of these drugs, and provides a possible link to EBV-related second primary malignancies.

Materials and Methods

Reagents

Lenalidomide was from Celgene, whereas thalidomide, pomalidomide, idelalisib, LY294002, SB202190, PD98059, and dexamethasone were purchased from Selleck Chemicals. Doxorubicin, melphalan, and ganciclovir were purchased from Sigma-Aldrich as were methotrexate and bortezomib. Rituximab was from The MD Anderson Cancer Center Pharmacy. All drugs were dissolved in DMSO, except for melphalan, which was dissolved in ethanol, and rituximab, which was in 0.9% sodium chloride.

Cell culture and patient samples

MUTU-I and KEM-I (gifts from Alan Rickinson [Birmingham Cancer Research UK Cancer Centre, School of Cancer Sciences, University of Birmingham, Birmingham, United Kingdom] and Jeff Sample [Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine, Pennsylvania]), are EBV–Burkitt lymphoma cell lines. The EBV–Burkitt lymphoma cell line DaudiI and the lymphoblastoid cell line (LCL) B95.8 were from the ATCC, whereas the donor 4 (D4-WT) LCL and BZLF-1-deleted LCL (D4-ZKO) were described previously (12). All lines were validated through The MD Anderson Cancer Center Characterized Cell Line Core Facility. Cells were grown in RPMI 1640 (Life Technologies) with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich). Archived serum samples were sourced from patients with myeloma enrolled in a phase I/II clinical trial (13), which was in compliance with the Declaration of Helsinki according to an Institutional Review Board–approved protocol.

Immunoblotting

Protein expression was measured by immunoblot analysis as described previously (14). The antibody to BZLF-1 was from Santa Cruz Biotechnology, whereas the anti-AKT, phospho (p)-GSK3α/βSer21/9, p-AKTSer473, FoxO1, and Ikaros antibodies were from Cell Signaling Technology. Antibody to BMRF1 was obtained from Millipore, anti-β-actin from Sigma-Aldrich, and anti-VCA p18 antibodies were from Thermo Scientific. Total AKT and pAKTΔSer473 levels were also measured using sandwich ELISA Kit as recommended by the manufacturer Cell Signaling Technology.

Cell proliferation assay

The WST-1 tetrazolium reagent from Roche was used to determine the effects on cell proliferation. Viable cell numbers (Annexin-V- and TO-PRO-3-negative) were measured using Annexin-V-Pacific Blue and TO-PRO-3 (Life Technologies) in combination with Count Bright beads on a Fortessa flow cytometer (Becton Dickson) using FlowJo, version 10 (Tree Star, Inc.).

Blockade of PI3K signaling

DAHDI cells were pretreated for 1 hour with DMSO alone or with LY294002, PD98059, SB202190, idelalisib, and in combination with LTP for 48 hours.

Lentiviral overexpression of Ikaros

MUTU-I cells were infected with a lentivirus encoding Ikaros or a control virus as described previously (15).

In vivo xenograft

Experiments were performed in accordance with protocols approved by the institutional Animal Care and Use Facility. CB-17 SCID mice (Harlan Laboratories, Inc.) were inoculated in the right flank subcutaneously with 5 × 10⁶ MUTU-I cells. When tumor burdens reached approximately 100 mm² in volume, mice were randomized into groups of five mice to receive intraperitoneal vehicle (PBS) daily, lenalidomide daily, ganciclovir thrice weekly, or both agents.

Quantitative real-time PCR and semi quantitative reverse-transcription PCR analysis

Total RNA harvested from cells was reverse transcribed as described previously (16). cDNA was subjected to PCR using
primers and conditions for BZLF1, BCRF1, and βM as published previously (16, 17). Quantitative real-time PCR (qPCR) for BGLF4 was performed on a StepOne PCR analyzer (Applied Biosystems) using SYBR Green Master Mix (Life Technologies) with sense primer 5’-TGACGGAGCTGTATCACGAG-3’ and antisense primer 5’-CCAGGGGCCAATACACTACCA-3’ based on the GenBank EBV sequence: AJ507799.2. EBV viral load in serum samples was measured using the EBV R-gene Kit (Argene), according to the manufacturer’s instructions.

Drug synergy assays
To detect the presence of synergistic interactions, the methods of Chou and Talalay were used (18). Data were analyzed using CalcuSyn Version 2 software (Biosoft), and combination indices (CI) calculated.

Statistical analyses
Data were subjected to statistical analyses using the SEM. The significance of drug–effect relationships was determined by one-tailed unpaired t tests using Excel software (Microsoft Corporation), and results were considered significant when P < 0.05. For in vitro studies, an analysis of cooperative effects of lenalidomide and ganciclovir on tumor growth was performed using a Bayesian bootstrapping approach (19). Linear mixed-effect models were used to study the change of EBV viral load over time in patient serum samples and the effects of valacyclovir treatment. An unstructured covariance model was used to account for interpatient variability and the longitudinal nature of the data. SAS version 9.2 and S-Plus version 8.04 (SAS Institute) was used in the analyses to satisfy the normality assumption of the transformation of logarithm to the base 10 of the EBV viral load per patient variability and the longitudinal nature of the data. The unstructured covariance model was used to account for interpatient variability and the longitudinal nature of the data. SAS version 9.2 and S-Plus version 8.04 (SAS Institute) was used.

Immunomodulatory agents reactivate latent EBV infection
We sought to determine whether LTP-induced EBV reactivation in latent infected LCL and Burkitt lymphoma cell lines. Clinically relevant concentrations (20–22) of LTP weakly enhanced expression of the immediate early gene product BZLF1, and the early viral gene product BMRF1 in B95.8 and D4 LCL cells (Fig. 1A). Reverse-transcription PCR (RT-PCR) analysis also showed a dose-dependent increase in BZLF1 transcription and induction of BCRF1 (viral IL-10), a marker of the late stages of EBV replication with lenalidomide treatment (Fig. 1B). In contrast, the Burkitt lymphoma cell lines DAUDI, KEM-I, and MUTU-I showed robust BMRF1 and BZLF1 induction, along with the expression of the late protein, VCA (Fig. 1C). BMRF1 induction was similar with 1 and 5 μmol/L lenalidomide and pomalidomide, and equivalent to methotrexate, a known EBV reactivation inducer (9). Pomalidomide was particularly effective in DAUDI and KEM-I, followed by lenalidomide and thalidomide (Fig. 1C). This potency in reactivating EBV parallels the known clinical efficacy of these agents in multiple myeloma (23).

EBV lytic cycle induction by lenalidomide and pomalidomide enhances their activity in Burkitt lymphoma and LCL cells
To determine the contribution of the EBV lytic cycle to the cytotoxic effects of immunomodulatory drugs, we evaluated D4 cells bearing wild-type (WT) EBV or D4 cells transformed using a BZLF1 gene–deleted (D4-ZKO) EBV, rendering them incapable of entering lytic cycle. Treatment of WT LCLs with LTP reduced the viable cell number to 60% with lenalidomide, 90% with thalidomide, and 50% with pomalidomide (Fig. 2A), but the ZKO LCL cells displayed little to no change in viability with lenalidomide and thalidomide, whereas pomalidomide only reduced viability

Figure 1.
Immunomodulatory agents reactivate lytic EBV infection. A, B95.8 and D4 LCL cell lines were treated for 48 hours with vehicle, LTP, or methotrexate as a positive control, and extracts were immunoblotted with the indicated antibodies. B, RT-PCR on D4 LCLs following treatment with lenalidomide (LEN) for 48 hours with primers for BZLF1, BCRF1, and a loading control using β2M or a 1:10 dilution of the cDNA. C, the EBV− Burkitt lymphoma cell lines DAUDI, KEM-I, and MUTU-I were treated as above. Protein levels of BZLF1, BMRF1, and VCA, along with β-actin as a loading control, were determined. Representative images are shown from one of three independent experiments.
We next evaluated the effect of LTP on DAUDI, MUTU-I, and KEM-I. Pomalidomide was the most effective in suppressing DAUDI and MUTU-I proliferation, with IC$_{50}$ so of 0.3 and 0.25 μmol/L, respectively, whereas the KEM-I IC$_{50}$ plateaued at approximately 1 to 3 μmol/L (Fig. 2B). Lenalidomide in the three lines did not achieve an IC$_{50}$, but did reduce proliferation to 75% in DAUDI at 0.6 μmol/L and 53% in MUTU-I cells at 1 μmol/L (Fig. 2B). Lenalidomide had no significant effect in KEM-I, whereas thalidomide had no significant effect in any of them (Fig. 2B).

As we cannot introduce a BZLF1-deleted EBV into Burkitt lymphoma cells because these cells harbor an endogenous WT EBV, we next evaluated whether lenalidomide and pomalidomide stimulated the lytic cycle enough to result in conversion of ganciclovir to its active triphosphate form. This can then compete with deoxyguanosine triphosphate (dGTP) and be inserted into the cellular DNA, leading to apoptosis of the infected cells (24). DAUDI and MUTU-I cells were treated for 1 week with either lenalidomide (1 μmol/L) or pomalidomide (0.25 μmol/L) alone, or in combination with ganciclovir (50 μmol/L). Annexin-V/TO-PRO-3 and Count Bright bead flow cytometry were used to determine the viable cell numbers. Values represent the mean ± SEM from three independent experiments. An unpaired t-test was performed to evaluate for significance and "**" denotes $P$ values of $<0.01$.

**Figure 2.**
EBV lytic cycle enhances growth inhibition in response to lenalidomide (LEN) and pomalidomide (POM) and synergize with ganciclovir (GCV) in SCID mice. A, LCL cells bearing a wild-type EBV (D4 WT) or a BZLF1-deleted EBV (D4 ZKO) were treated with LTP (1 μmol/L) or vehicle for 1 week. Flow cytometric analysis was then performed after staining with Annexin-V/TO-PRO-3 and Count Bright beads, from which the viable cell number was calculated and normalized to the vehicle control group. B, Burkitt lymphoma (BL) cell lines were treated for 4 days with lenalidomide, thalidomide (THAL), pomalidomide, or vehicle, cell viability was determined using the WST-1 reagent, and results were expressed as the percentage viability relative to the vehicle control, which was arbitrarily set at 100%. C, DAUDI and MUTU-I cells were treated for 1 week with either lenalidomide (1 μmol/L) or pomalidomide (0.25 μmol/L) alone, or in combination with ganciclovir (50 μmol/L). Annexin-V/TO-PRO-3 and Count Bright bead flow cytometry were used to determine the viable cell numbers. Values represent the mean ± SEM from three independent experiments. An unpaired t-test was performed to evaluate for significance and "**" denotes $P$ values of $<0.01$. D, SCID mice were inoculated with MUTU-I cells subcutaneously and monitored until tumors were established. Five mice per group were injected intraperitoneally with vehicle, lenalidomide (50 mg/kg) daily, ganciclovir (50 mg/kg) three times per week, or the combination. Tumor volumes were measured and are plotted as a function of time for each group. Statistically significant differences comparing the combination to the single agents were determined using an unpaired t-test, and a $P$ value of $<0.02$ is indicated by "**". MUTU-I was treated with DMSO, LTP at 1 or 5 or 1 μmol/L methotrexate as a positive control for 48 hours. RNA was harvested and cDNA synthesized and qPCR performed for BGLF-4 with RQ values normalized to the DMSO control. An unpaired t-test was performed to evaluate for significance and "**" denotes $P$ values of $<0.05$ relative to the DMSO control.
DAUDI cells reduced the live cell fraction to 12% versus 30% with pomalidomide alone. MUTU-I cells were even more sensitive to the combination, with a reduction of the live cells to 8% versus 21% for pomalidomide alone (Fig. 2C, right).

To determine the presence of any synergistic interactions with varying LTP and ganciclovir concentrations, we performed synergy assays with DAUDI, MUTU-I, and KEM-I treated with LTP or ganciclovir alone, or in combination with ganciclovir. DAUDI cells demonstrated an enhanced suppression of cell growth with LTP in combination with ganciclovir with concentrations as low as 0.4 μmol/L lenalidomide or pomalidomide and 4 μmol/L ganciclovir. In comparison, 10 μmol/L thalidomide with 100 μmol/L ganciclovir actually overcame the stimulation of growth observed with thalidomide (Supplementary Fig. S1A). KEM-I cells demonstrated less growth suppression with lenalidomide and pomalidomide both requiring 10 μmol/L in combination with 100 μmol/L ganciclovir, whereas no activity was present with thalidomide and ganciclovir (Supplementary Fig. S1B). MUTU-I cells, in contrast, showed an enhanced suppression of cell growth with LTP and ganciclovir at a range of concentrations, with pomalidomide and ganciclovir being particularly effective, whereas thalidomide and ganciclovir overcame the growth stimulation of thalidomide (Supplementary Fig. S1C). Isobologram analysis was performed to determine whether these interactions were synergistic as defined by Chou and Talalay (18). MUTU-I treated with lenalidomide and ganciclovir demonstrated synergy at all the concentrations used, with CI values ranging from 0.031 to 0.314. Similarly, pomalidomide and ganciclovir were also highly synergistic across the concentrations, with CIs of 0.02 to 0.55. In comparison, only 0.4 μmol/L thalidomide and 4 μmol/L ganciclovir showed synergy, with a CI of 0.141 (Supplementary Table S1). Pomalidomide and ganciclovir were also synergistic against KEM-I across the concentration ranges used, with CIs of 0.03 to 0.2 (Supplementary Table S1). In DAUDI cells, pomalidomide with ganciclovir at the lowest concentrations were synergistic, with CIs of 0.027 to 0.670, whereas lenalidomide and ganciclovir were synergistic to additive, with CIs of 0.417 to 1.154. Note that no CIs are shown for lenalidomide and thalidomide with KEM-I, or thalidomide with DAUDI, as the single agents had no change in viability or stimulated growth (i.e., cell viability over 100%), which precludes isobologram analysis (Supplementary Table S1). These data show that EBV lytic cycle induction by LTP also coincided with suppression of phospho-glycogen synthase kinase (GSK) 3 α/βSer21/9 (Fig. 3B) and protein kinase B/AKTSer473 (Fig. 3B).

Suppression of EBV reactivation using SB202190 or LY294002 may be an off-target effect as these inhibitors have a broad range of mechanisms, including suppression of casein kinase 1 and NF-kB, which may influence EBV reactivation. We therefore evaluated ideasilib, a highly specific PI3K-p110δ inhibitor (29). Treatment of DAUDI cells with lenalidomide and pomalidomide induced significant BMRF1 expression, and enhanced pGSK3α/βSer21/9 and pAKTSer473 levels (Fig. 3C). In contrast, concomitant addition of a clinically relevant concentration of ideasilib completely suppressed stimulation of BMRF1, pGSK3α/βSer21/9 and pAKTSer473 (Fig. 3C). LTP may also regulate EBV reactivation through PI3K-mediated suppression of the transcription factor Forkhead-box-O1 (FoxO1), which, if suppressed, leads to loss of Ikaros (30), an EBV latency regulator (15). Treatment of DAUDI cells with lenalidomide slightly decreased FoxO1 and depleted Ikaros, leading to the enhancement of BZLF1 and BMRF1 and the induction of pAKTSer473 (Fig. 3D). The addition of ideasilib with lenalidomide led to a further depletion of Ikaros and greater suppression of FoxO1. This was in contrast to treatment with ideasilib alone, which actually enhanced FoxO1 and did not have any effect on Ikaros. These data confirm the role of PI3K stimulation by LTP in EBV reactivation.

**Blockade of PI3K signaling suppresses lenalidomide-induced EBV reactivation**

EBV reactivation by chemotherapy agents is mediated through stimulation of PI3K, MAPK kinase (MEK), and p38-MAPK signaling (26–28). We therefore assessed the effects of MEK, PI3K, and p38-MAPK inhibitors in combination with LTP on EBV reactivation. LTP were unable to consistently induce BMRF1 or BZLF1 in the presence of LY294002 or SB202190, whereas the MEK inhibitor had a limited effect on reactivation (Fig. 3A). The induction of the EBV lytic cycle by LTP also coincided with stimulation of PI3K signaling, as evidenced by enhanced expression of phospho-glycogen synthase kinase (GSK) 3 α/βSer21/9 and protein kinase B/AKTSer473 (Fig. 3B). Isobologram analysis showed that LTP stimulate the BGLF-4 lytic cycle. Isobologram analysis showed that LTP stimulate the BGLF-4 lytic cycle.
stable at concentrations of up to 5 μmol/L (Fig. 4A). In contrast, thalidomide did not increase pAKT\textsuperscript{SER473} significantly at low concentrations. At 1 μmol/L, thalidomide resulted in a twofold increase, which increased to threefold at 5 μmol/L. This pattern of induction of pAKT\textsuperscript{SER473} followed by a plateau directly reflected the induction of BMRF1 observed in Fig. 1B.

As shown in Fig. 3, idelalisib suppressed pAKT\textsuperscript{SER473} at the clinically relevant concentration of 1 μmol/L. We next determined the optimal concentration in vitro at which idelalisib could suppress pAKT\textsuperscript{SER473}. Treatment of DAUDI cells with concentrations of idelalisib as low as 50 nmol/L led to a 95% decrease in the basal levels of pAKT\textsuperscript{SER473}, with complete suppression at concentrations up to 1 μmol/L (Fig. 4B). We also determined that a concentration of idelalisib as low as 25 nmol/L was in the effective range to suppress basal pAKT\textsuperscript{SER473} levels and had no effect on cell viability (Fig. 4B, right). We therefore used idelalisib (25 nmol/L) in combination with varying LTP concentrations to determine whether it could reverse the LTP-induced decrease in proliferation.

Single-agent lenalidomide suppressed the growth of DAUDI cells to 70% compared with the vehicle control, and this remained constant across the concentration range used; the addition of idelalisib completely reversed this growth suppression at all concentrations used (P < 0.05 compared with the control; Fig. 4C). Pomalidomide as a single agent decreased the cell viability to 90% at 0.08 μmol/L and to 80% at 0.4 μmol/L, and this growth suppression was reversed by idelalisib (P < 0.05). At pomalidomide, concentrations of 2 and 10 μmol/L, however, idelalisib was not able to reverse the cell growth suppression (Fig. 4C, right). Thalidomide did not induce any decrease in cell viability, as previously observed, and the addition of idelalisib resulted in no significant change to the cell viability (Fig. 4C, middle).

Overexpression of Ikaros attenuates lenalidomide-induced EBV reactivation

Lenalidomide induces proteasome-mediated degradation of the Ikaros family of proteins (31), and Ikaros suppresses EBV reactivation. Inhibition of PI3K suppresses lenalidomide (LEN)-induced EBV reactivation. A, DAUDI cells were incubated with vehicle, LTP (5 μmol/L), or in combination with inhibitors of MEK (PD98059; 50 μmol/L), PI3K (LY294002; 15 μmol/L), or p38 (SB202190; 20 μmol/L) for 48 hours, and immunoblotted with the indicated sera. B, immunoblotting of DAUDI cells treated with LTP (5 μmol/L) was performed for markers of PI3K activation and EBV reactivation. C, DAUDI cells were treated with LTP (5 μmol/L) alone or in combination with the PI3K subunit inhibitor idelalisib (1 μmol/L) for 48 hours, and lysates probed for PI3K activation markers and EBV reactivation markers. D, DAUDI cells were treated with lenalidomide (5 μmol/L), idelalisib (1 μmol/L), or the combination for 24 hours and immunoblotted for the markers of PI3K activation, EBV reactivation, FoxO1, and Ikaros. Representative images are shown from one of three independent experiments.

Figure 3.
reactivation in Burkitt lymphoma cells (15). We therefore evaluated whether LTP-mediated Ikaros degradation is associated with EBV reactivation. Although exposure of DAUDI and KEM-I cells to 1 μmol/L of lenalidomide or pomalidomide strongly induced Ikaros degradation, coinciding with strong BMRF1 and BZLF1 induction, no change was seen with thalidomide (Fig. 5A). Overexpression of Ikaros in MUTU-I cells attenuated both BZLF1 and BMRF1 induction by lenalidomide at low concentrations (0.1–0.5 μmol/L), compared with the control virus (Fig. 5B). However, this effect was lost at higher concentrations (1–5 μmol/L) of lenalidomide, with complete loss of the overexpressed Ikaros at 5 μmol/L (Fig. 5B). We also evaluated the ability of the proteasome inhibitor bortezomib to block Ikaros-mediated degradation by lenalidomide. Although lenalidomide alone induced loss of Ikaros, with accompanying increases in BMRF1, BZLF1, and pAKT\textsuperscript{Ser473} (Fig. 5C), addition of a low concentration of bortezomib (5 nmol/L) with lenalidomide suppressed the induction of BMRF1, BZLF1, and pAKT\textsuperscript{Ser473} but failed to rescue Ikaros expression (Fig. 5C).

**Combinations of lenalidomide with chemotherapy agents enhance EBV reactivation**

A combination of lenalidomide and thalidomide with dexamethasone in lenalidomide resistant/refractory myeloma patients may to some extent overcome lenalidomide resistance (13). This combination could also result in enhanced EBV reactivation, as corticosteroid use with methotrexate enhanced EBV reactivation, raising the risk of development of EBV lymphomas (9). We...
therefore examined the EBV viral load in 18 archived serum samples from patients who received a lenalidomide, thalidomide, and dexamethasone combination (Supplementary Fig. S2A and Fig. 6A; ref. 13) and for whom a serum sample was available at baseline and after the first and last cycles of therapy (number of cycles, 3–15). qPCR evaluation of the baseline pretreatment serum indicated that most patients had no or low levels of virus, with a median load of 3.5 × 10^5 copies/mL (note: two patients had a high titer of virus at baseline) and a range of 0 to 3.7 × 10^8 copies/mL (Fig. 6A; Supplementary Table S3). After one cycle of the treatment combination, the median viral load increased to 1.0 × 10^7 copies/mL with a range of 0.3 to 3.9 × 10^8 copies/mL, but this increase was not significant when compared with the baseline serum value (P = 0.08). However, analysis after the final cycle for the 18 patients demonstrated an increase in the median viral load to 2.3 × 10^8 copies/mL, with a range of 0 to 4.0 × 10^9, which was highly significant compared with the baseline (P = 0.0016; Fig. 6A; Supplementary Table S3). Six patients received valacyclovir for varicella zoster infection at intermittent times (and not continuously) throughout the trial. To account for any differences in treatment outcome due to valacyclovir, we compared the six patients receiving valacyclovir to the 12 who did not and did not observe any statistical difference in the EBV viral load (P = 0.3628; Supplementary Fig. S2B).

To see if we could model this effect in vitro, we treated DAUDI cells with the agents alone or in dual or triplicate combinations. Lenalidomide alone stimulated strong BMRF1 expression along with BZLF1 and VCA, whereas a similar thalidomide concentration stimulated BMRF1 weakly (Fig. 6B). As expected, a clinically relevant concentration of dexamethasone also stimulated BMRF1, BZLF1, and VCA expression (Fig. 6B). The lenalidomide and thalidomide combination did not increase BMRF1 expression over either agent alone, whereas lenalidomide and dexamethasone or thalidomide and dexamethasone combinations induced significantly higher BZLF1, BMRF1, and VCA expression. A combination of all three agents was no more efficacious at EBV reactivation than the lenalidomide and dexamethasone combination (Fig. 6B).

Finally, we sought to investigate the interplay of lenalidomide with other drugs used to treat myeloma and lymphoma patients. In DAUDI cells, lenalidomide, methotrexate, doxorubicin, and melphalan all induced significant amounts of BMRF1, BZLF1, and VCA, whereas rituximab induced BZLF1, VCA, and BMRF1 weakly, and a low concentration of the proteasome inhibitor, bortezomib, had no effect (Fig. 6C, left). When lenalidomide was combined with doxorubicin or melphalan, enhanced reactivation was observed, whereas bortezomib suppressed the lenalidomide induction of BMRF1 and VCA. In comparison, EBV reactivation was induced in MUTU-I cells by a low dose (0.5 μmol/L) of lenalidomide, and strongly induced by single-agent methotrexate, doxorubicin, melphalan, and, to a lesser extent, by rituximab (Fig. 6C, right), whereas bortezomib again had no effect. However, when lenalidomide was combined with methotrexate, doxorubicin, melphalan, or rituximab, enhanced BMRF1, BZLF1, and VCA induction was observed beyond the single agents alone.

**Discussion**

Incorporation of thalidomide, lenalidomide, and pomalidomide into the myeloma therapeutic armamentarium has contributed substantially to patient survival. Given the contributory role, EBV plays in approximately 50% of Hodgkin lymphoma, and reports of Hodgkin lymphoma in patients on lenalidomide-MT, we hypothesized that lenalidomide, and its analogues thalidomide and pomalidomide, may induce EBV reactivation. This...
hypothesis was further supported by the fact that the immunosuppressant methotrexate in nonmalignant diseases such as rheumatoid arthritis enhanced the risk of EBV-positive lymphomas (9). In this study, we found that LTP reactivated EBV in latently infected B-cell lines in the order of eficacy pomalidomide > lenalidomide > thalidomide. LCL cells were weakly inducible into lytic cycle, whereas Burkitt lymphoma cells were readily induced (Fig. 1). Combinations of ganciclovir with lenalidomide or pomalidomide enhanced the growth inhibitory effect in Burkitt lymphoma cells (Fig. 2C and Supplementary Fig. S1), and this was further borne out in a mouse model (Fig. 2D). The ability of LTP to reactivate EBV and sensitize cells to the bystander effect of ganciclovir indicates a potential use in EBV reactivation therapies for EBV-positive B-cell malignancies. Notably, similar approaches were shown to be effective using in vitro and in vivo models (11, 32, 33), and clinically with combinations of gemcitabine, valproic acid, and ganciclovir in nasopharyngeal carcinoma patients, which produced disease stabilization (34).

One mechanism of action of LTP against multiple myeloma is through induction of proteasomal degradation of Ikaros (31, 35, 36). We observed this effect with lenalidomide and pomalidomide at concentrations as low as 1 μmol/L, which correlated with EBV reactivation (Fig. 5A). Interestingly, the addition of a low concentration of bortezomib (5 nmol/L) failed to rescue Ikaros but did suppress the lytic cycle along with PI3K signaling, as seen by the decrease in pAKTSer473 (Fig. 5C). The inability of bortezomib to prevent proteasomal suppression of Ikaros mediated by lenalidomide has been shown to be both dose and time dependent, requiring pretreatment for 1 hour with 100 nmol/L bortezomib to prevent Ikaros loss (37). The low concentration range of 3 to 5 nmol/L was shown to be less effective in that study, as in our experiments. The ability of low bortezomib concentrations to block induction of the lytic cycle is most likely due to its reported ability to suppress pAKTSer473 by decreasing upstream kinases in the PI3K signaling cascade (38).

Ikaros is known to suppress the EBV lytic cycle through regulation of Octamer transcription factor-2 and Paired Box-5, B-cell transcription factors which promote latency (15, 39, 40), leading to the assumption that LTP-mediated EBV reactivation occurs through this mechanism. Indeed, we found that Ikaros...
overexpression attenuated lenalidomide induction of the lytic cycle, but did not result in complete blockade (Fig. 5B). This is likely due to our additional finding that PI3K played a role in lytic EBV induction. Lenalidomide is known to stimulate PI3K activity (41), and we also showed that LTP stimulated PI3K activity, coinciding with EBV reactivation (Figs. 3 and 4). Importantly, this stimulation was reversed by PI3K inhibition with idelalisib, which led to complete suppression of EBV reactivation. Of note is the fact that lenalidomide and pomalidomide at low concentrations (0.1 μmol/L) can eliminate Ikaros and also stimulate significant PI3K signaling, as shown by significant pAKTSer473 upregulation (Figs. 4A and 5B). This effect plateaued at 0.5 μmol/L and did not increase further for both lenalidomide and pomalidomide, thereby explaining why no significant changes in BHRF1 occurred between 1 and 5 μmol/L in the Burkitt lymphoma and LCL cells (Fig. 1). However, thalidomide did show a dose-dependent increase in pAKTSer473 at 5 μmol/L, but does not suppress Ikaros, which suggests that the ability of LTP to induce EBV reactivation is primarily due to the stimulation of PI3K signaling, which is enhanced by lenalidomide and pomalidomide’s simultaneous suppression of Ikaros.

EBV reactivation by LTP, therefore, is likely due to several mechanisms. First, stimulation of PI3K suppresses FoxO1, whose function is required for proper Ikaros mRNA splicing and whose loss results in Ikaros suppression (30). This effect was apparent with lenalidomide treatment (Fig. 3D). Second, LTP binding to Cereblon induces Ikaros degradation, further suppressing Ikaros expression. Finally, direct PI3K stimulation by LTP results in lytic cycle activation and appears to be a primary mechanism. The weaker induction of lytic cycle in LCLs compared with Burkitt lymphoma cells is likely due to the fact that LCLs have EBV latency state III, with lower Ikaros levels compared with the Burkitt lymphoma cells, which are in latency state I (15).

Finally, we evaluated EBV reactivation with lenalidomide in combination with commonly used chemotherapeutics for myeloma and lymphoma in patient serum samples and cell lines. In a small number of samples from patients with myeloma who received a lenalidomide/thalidomide/dexamethasone combination, we could see that multiple cycles of the combination resulted in a significant increase in the EBV load in the serum (Fig. 6A and Supplementary Table S3), and this was reproduced in vitro using cell lines (Fig. 6B). This was in line with previously reported data showing glucocorticoids stimulate EBV reactivation (42), which we found was enhanced by addition of lenalidomide or thalidomide. Furthermore, we demonstrated expression of the late protein VCA in lenalidomide- and pomalidomide-exposed Burkitt lymphoma cells (Fig. 6B) and by RT-PCR we detected the late protein BHRF1 in LCLs (Fig. 1), indicating full activation of the EBV lytic cycle, which will result in viral release. Melphalan, doxorubicin and methotrexate also reactivated EBV, which was enhanced with the lenalidomide addition (Fig. 6C). Rituximab did not induce EBV reactivation, but as reported for rituximab/dexamethasone combinations (43), rituximab/lenalidomide increased EBV reactivation.

Our findings that LTP reactivates EBV in latently infected B cells raises several interesting questions, particularly in light of lenalidomide being linked to induction of SPMs in patients with myeloma on lenalidomide-MT (44, 45). Although the most common hematologic SPMs were acute myeloid leukemia and myelodysplastic syndromes, two lenalidomide maintenance phase III trials reported Hodgkin lymphoma in the lenalidomide arms, with four cases in Atal and colleagues (2) and one in McCarthy and colleagues (3), with no cases in the placebo arms. In the context of thalidomide, two case reports on patients with myeloma found EBV-positive Hodgkin lymphoma as an SPM after thalidomide-containing regimens. One patient received thalidomide maintenance for 2 years with prior melphalan therapy (46), and another received three cycles of vincristine, doxorubicin and dexamethasone, and four cycles of bortezomib, thalidomide, and dexamethasone salvage with development of Hodgkin lymphoma 5 years later (47). A study examining SPM incidence in patients with myeloma treated in the pre-lenalidomide era (1997–2008) reported only one case of Hodgkin lymphoma out of 589 patients (48). In contrast, four patients developed Hodgkin lymphoma as an SPM on lenalidomide-MT out of 306 patients treated by Attal and colleagues.

Patients with myeloma frequently display varying degrees of immunosuppression due to secondary hypogammaglobulinemia, high-dose chemotherapy, use of LTP, proteasome inhibitors, or corticosteroids. In the face of varying degrees of immunosuppression, lenalidomide could reactivate dormant EBV-positive B cells, which would normally attract both humoral and cytotoxic T-cell responses, resulting in killing of the EBV-positive B cells and elimination of infectious virions. Lack of immune surveillance in the face of continual EBV stimulation may eventually exhaust remaining protective immunity. This would increase the small pool of latently infected EBV-positive B cells, estimated to be around 1 in 10^5 cells, which would then have the potential to become transformed and, potentially, malignant. Combinations of dexamethasone with lenalidomide could result in enhanced EBV reactivation and immune suppression, and the R^2 regimen comprising lenalidomide and rituximab used in lymphoma patients (49) could have a similar effect. It has been reported that lenalidomide-treated patients with myeloma have an increased incidence of varicella zoster and herpes simplex virus infections (50), suggesting LTP may reactivate the herpes virus family per se. The potential relationship between lenalidomide and EBV reactivation should be a consideration for patients treated with lenalidomide long term. Further studies are needed to determine if reactivation contributes to lenalidomide-mediating clinical toxicities, if there is a possible contribution to SPMs, and if the risk of this could be reduced by the addition of antiviral therapy such as acyclovir or valacyclovir in high-risk patient groups.

Disclosure of Potential Conflicts of Interest
R.Z. Orlowski reports receiving commercial research grants from and is a consultant/advisory board member for Celgene Corporation. No potential conflicts of interest were disclosed by the other authors.

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