ORIGINAL ARTICLE

Immunomodulatory drugs target IKZF1-IRF4-MYC axis in primary effusion lymphoma in a cereblon-dependent manner and display synergistic cytotoxicity with BRD4 inhibitors

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Primary effusion lymphoma (PEL) is an aggressive type of non-Hodgkin lymphoma localized predominantly in body cavities. Kaposi's sarcoma-associated herpes virus (KSHV) is the causative agent of PEL. PEL is an incurable malignancy and has extremely poor prognosis when treated with conventional chemotherapy. Immunomodulatory drugs (IMiDs) lenalidomide and pomalidomide are Food and Drug Administration-approved drugs for the treatment of various ailments. IMiDs display pronounced antiproliferative effect against majority of PEL cell lines within their clinically achievable concentrations, by arresting cells at G_0/G_1 phase of cell cycle and without any induction of KSHV lytic cycle reactivation. Although microarray examination of PEL cells treated with lenalidomide revealed activation of interferon (IFN) signaling, blocking the IFN pathway did not block the anti-PEL activity of IMiDs. The anti-PEL effects of IMiDs involved cereblon-dependent suppression of IRF4 and rapid degradation of IKZF1, but not IKZF3. Small hairpin RNA-mediated knockdown of *MYC* enhanced the cytotoxicity of IMiDs. Bromodomain (BRD) and extra-terminal domain (BET) proteins are epigenetic readers, which perform a vital role in chromatin remodeling and transcriptional regulation. BRD4, a widely expressed transcriptional coactivator, belongs to the BET family of proteins, which has been shown to co-occupy the super enhancers associated with MYC. Specific BRD4 inhibitors were developed, which suppress *MYC* transcriptionally. Lenalidomide displayed synergistic cytotoxicity with several structurally distinct BRD4 inhibitors (JQ-1, IBET151 and PFI-1). Furthermore, combined administration of lenalidomide and BRD4 inhibitor JQ-1 significantly increased the survival of PEL bearing NOD–SCID mice in an orthotopic xenograft model as compared with either agent alone. These results provide compelling evidence for clinical testing of IMiDs alone and in combination with BRD4 inhibitors for PEL.

INTRODUCTION

Primary effusion lymphoma (PEL) is an aggressive type of non-Hodgkin lymphoma localized predominantly in body cavities, which is observed primarily in patients with HIV infection[1] and is associated with infection by Kaposi's sarcoma-associated herpesvirus (KSHV).[2] The prognosis of PEL is extremely poor with a median survival of 4 and 6 months in HIV-positive and -negative patients, respectively.[3] Thus, there is an urgent need to develop new treatment regimens for PEL.

Thalidomide and its analogues, lenalidomide and pomalidomide, are collectively known as immunomodulatory drugs (IMiDs).[4] Thalidomide was originally introduced as a sedative but was later withdrawn from the market due to birth defects and neuropathy.[5] Subsequently, thalidomide was found to significantly improve the response rate and survival of patients with multiple myeloma (MM).[6] The second-generation IMiDs, lenalidomide and pomalidomide, possess more potent anti-myeloma, anti-inflammatory and immunomodulatory activities than thalidomide.[5] Thalidomide directly binds to and inhibits the cereblon (CRBN) ubiquitin ligase[7] and CRBN has been shown to be required for the anti-myeloma activity of IMiDs.[8,9] Lenalidomide-bound CRBN acquires the ability to degrade Ikaros family zinc finger proteins 1 and 3 (IKZF1 and IKZF3), two specific B-cell transcription factors. The loss of IKZF1 and IKZF3 was shown to be both necessary and sufficient for the anti-myeloma effect of lenalidomide.[10,11]

Bromodomain (BRD)-containing proteins regulate lysine acetylation,[12] an important mechanism to regulate chromatin structure. BRD and extra-terminal (BET) subfamily has four members, BRD2, BRD3, BRD4 and BRDT; all of them share a common domain structure. In recent times, potent and highly specific BRD4 inhibitors were developed.[13–15] These inhibitors were shown to suppress *MYC* transcriptionally and demonstrate promising preclinical activity against *MYC*-driven cancers.[16–18]

Here we report that PEL cells are highly sensitive to IMiDs, lenalidomide and pomalidomide within their physiologically achievable concentrations. Furthermore, we discovered that low-dose combinations of IMiDs with BRD4 inhibitors displayed synergistic antiproliferative activity against PEL.

RESULTS

IMiDs show selective cytotoxicity towards PEL

To examine the cytotoxicity of IMiDs against PEL, we treated a panel of 35 logarithmically growing hematopoietic cell lines

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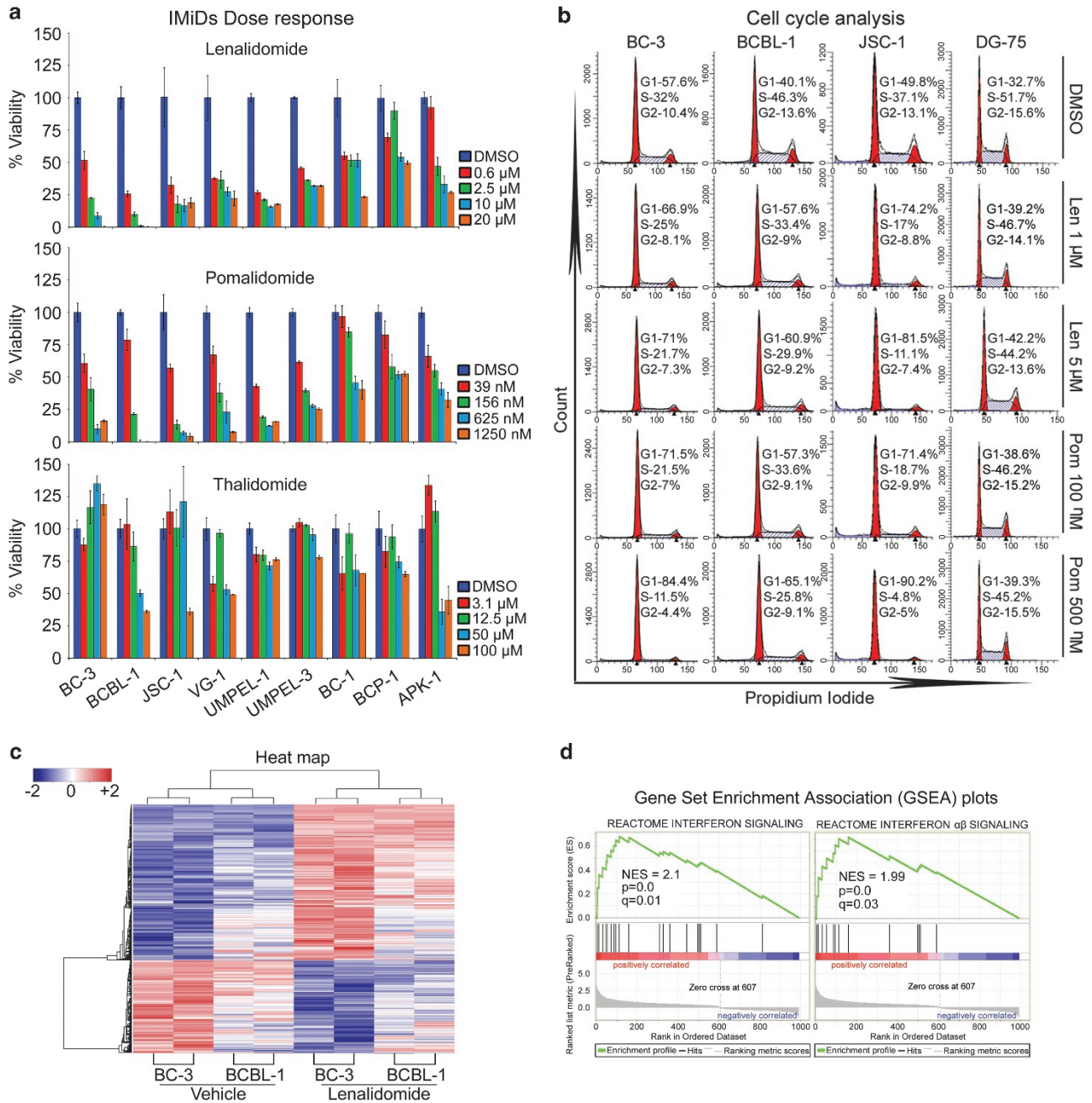
Figure 1. IMiDs are effective against PEL. (**a**) PEL cell lines were treated with increasing concentrations of lenalidomide, pomalidomide and thalidomide for 5 days, and cell viability was measured using an MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The values shown are mean $\pm$ s.e. ($n = 3$) of a representative experiment performed in triplicate for three times. (**b**) Cell cycle analysis of BC-3, BCBL-1, JSC-1 and DG-75 cells treated with indicated doses of lenalidomide (Len) and pomalidomide (Pom) for 48 h. Cells were stained with propidium iodide and analyzed by flow cytometry. Data are representative of more than three individual experiments. (**c**) Heat map representation of 992 genes that are up- or downregulated ($P < 0.05$) in BC-3 and BCBL-1 cells following 24 h treatment with lenalidomide (5 μM). (**d**) Gene Set Enrichment Analysis showing enrichment of gene sets, which are involved in IFN signaling among genes affected by lenalidomide treatment in PEL. NES, normalized enrichment score; q, false discovery rate.

(Supplementary Table 1) for 5 days with increasing concentrations of IMiDs. At concentrations that are achievable clinically (2.2 μM for lenalidomide[19] and 179 nM for pomalidomide[20]), six out of the nine PEL cell lines (BC-3, BCBL-1, JSC-1, VG-1, UMPEL-1 and UMPEL-3) were sensitive to IMiDs with IC$_{50}$ (50% inhibitory concentration) ranging from 0.2 to 1.2 μM and from 32 to 111 nM for lenalidomide and pomalidomide, respectively (Figure 1a and Table 1), whereas BC-1, BCP-1 and APK-1 were sensitive to only higher doses of IMiDs with IC$_{50}$ ranging from 2.6 to 10 μM and

from 226 to 744 nM for lenalidomide and pomalidomide, respectively (Figure 1a and Table 1). MM.1S (Myeloma), Daudi (Burkitt's lymphoma) and TMD8 (activated B-cell diffuse large B-cell lymphoma (ABC-DLBCL)) were also sensitive to both IMiDs with IC$_{50}$ ranging from 0.2 to 2.1 μM and from 38 to 113 nM for lenalidomide and pomalidomide, respectively (Supplementary Figure S1). All the remaining cell lines were either resistant to IMiDs or required higher doses for a moderate effect (Supplementary Figure S1). Consistent with its known

Table 1. List of PEL cell lines, their characteristics and IC_{50}^{a} for IMiDs

Cell line	Year established	Associated virus	Source	HIV status	Lenalidomide ($IC_{50}, \mu M$)	Pomalidomide (IC_{50}, nM)
BC-3	1995[52]	KSHV	Pleural effusion	Negative	0.96	107
BCBL-1	1996[53]	KSHV	NA	Positive	0.20	74
JSC-1	2000[54]	KSHV and EBV	Peritoneal effusion	Positive	0.28	34
VG-1	1998[55]	KSHV	Pleural effusion	Negative	0.87	101
UMPEL-1	2010[56]	KSHV and EBV	Pleural effusion	Negative	0.36	32
UMPEL-3	2013[57]	KSHV and EBV	Peritoneal effusion	Positive	1.2	111
BC-1	1992[58]	KSHV and EBV	Peritoneal effusion	Positive	2.6	744
BCP-1	1995[59]	KSHV	PBMC	Negative	10	396
APK-1	Pre-2003[60]	KSHV	NA	NA	2.8	226

Abbreviations: EBV, Epstein-Barr virus; IC_{50}, 50% inhibitory concentration; IMiD, immunomodulatory drug; KSHV, Kaposi's sarcoma-associated herpes virus; NA, not available; PBMC, peripheral blood mononuclear cell; PEL, primary effusion lymphoma. [a]IC_{50} values were calculated using Graphpad Prism 5 software.
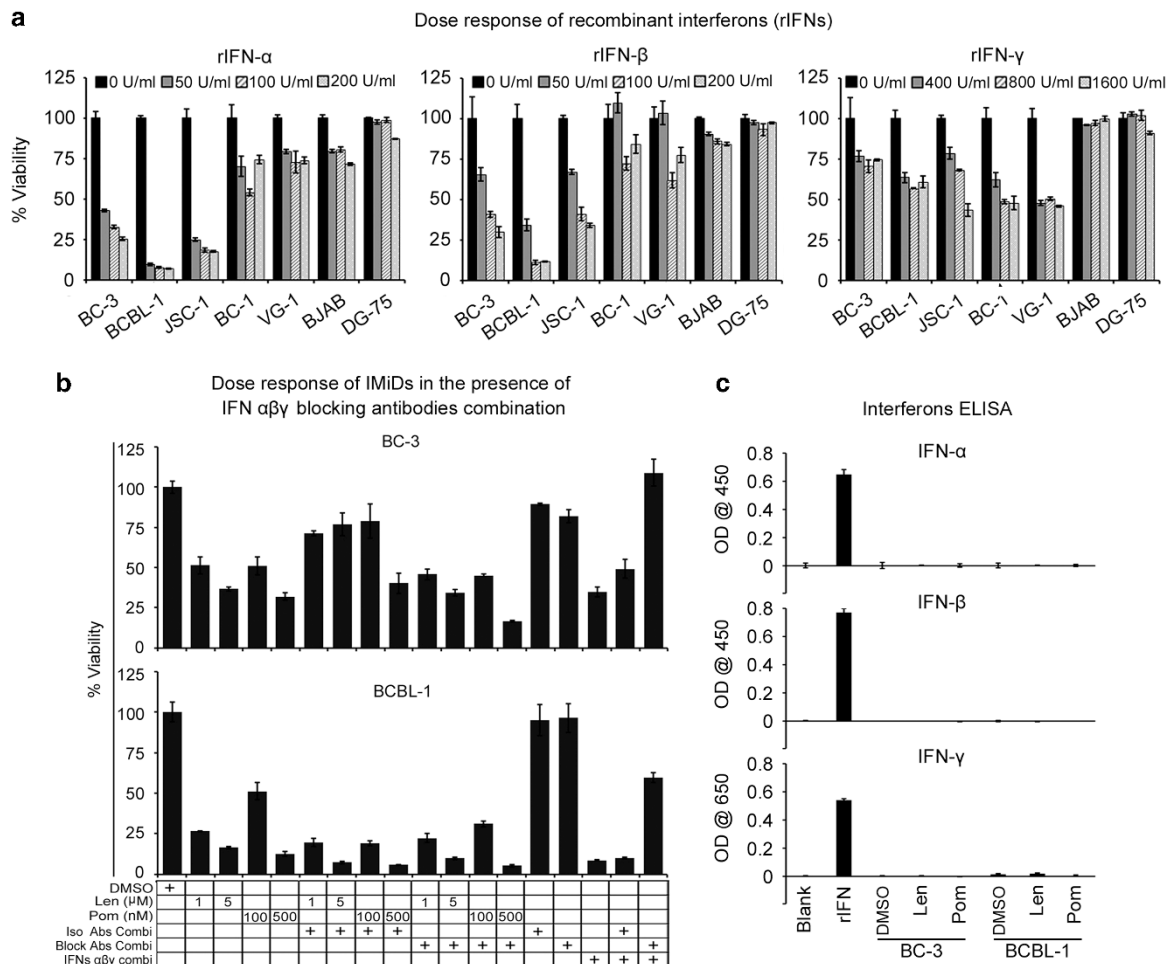

Figure 2. PEL cells are sensitive to IFNs α, β and γ. (**a**) BC-3, BCBL-1, JSC-1, BC-1, VG-1, BJAB and DG-75 cells were treated with indicated concentrations of recombinant IFNs for 5 days and cell viability was measured using an MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The values shown are mean $\pm$ s.e. ($n = 3$). (**b**) Blocking of IFNs α, β and γ (IFNs $\alpha\beta\gamma$) together did not block the antiproliferative activity of IMiDs in PEL. BC-3 and BCBL-1 were treated with indicated concentrations of IMiDs, IFNs $\alpha\beta\gamma$ and IFNs $\alpha\beta\gamma$-blocking antibodies combined (Block Abs Combi) for 4 days. IFN-α-blocking antibody was used at a concentration, which blocks 450 U/ml of IFN-α by 50%; IFN-β-blocking antibody was used at a concentration, which blocks 350 U/ml of IFN-α by 50%; and IFN-γ-blocking antibody was used at a concentration, which blocks 1090 U/ml of IFN-γ by 50%. Isotype antibodies (Iso Ab) corresponding to same species was used as control. The values shown are mean $\pm$ s.e. ($n = 3$). (**c**) IFNs are not secreted into the supernatants of PEL cells on treatment with IMiDs. BC-3 and BCBL-1 cells were treated with dimethyl sulfoxide (DMSO, vehicle control), lenalidomide 5 μM (Len) and pomalidomide 500 nM (Pom) for 48 h. Recombinant IFNs (rIFNs)—α, β and γ—were used at a concentration of 100, 200 and 1000 pg/ml, respectively, as positive controls.

requirement for *in vivo* metabolism,[8] thalidomide did not have any major effect on the growth of any of the cell lines tested or required a high dose for moderate effect (Figure 1a and Supplementary Figure S1). Treatment of PEL cells with IMiDs resulted in G1 cell cyle arrest (Figure 1b and Supplementary

Figure S2A). In contrast, IMiDs had no major effect on cell cycle progression in DG-75 (Burkitt lymphoma) and OCILY-8 (germinal center B-cell diffuse large B-cell lymphoma) cells that were resistant to their antiproliferative effect (Figure 1b and Supplementary Figure S2A).

Gene Set Enrichment Analysis identifies activation of IFN signaling in PEL by lenalidomide

To delineate the gene network affected by lenalidomide, BC-3 and BCBL-1 cells were treated with lenalidomide (5 μM) for 24 h followed by genome-wide microarray analysis. Unsupervised hierarchical clustering separated samples according to their treatment group, indicating a common transcriptional response to treatment with lenalidomide in PEL (Figure 1c). Rather than inducing nonspecific changes in gene expression, lenalidomide induced significant changes in a limited number of genes. Thus, there were 992 genes (390 down- and 602 upregulated genes) whose expressions were changed significantly ($P < 0.05$) in both the cell lines. We used a Gene Set Enrichment Analysis program to identify functional gene sets that were enriched in PEL cells on treatment with lenalidomide.[21] Among the gene signatures identified by this analysis were gene sets containing genes that are known targets of interferon (IFN) and MYC signaling pathways (Figure 1d and Supplementary Figure S2B). We used quantitative reverse transcriptase–PCR to confirm upregulation of IFNs and IFN-specific genes by lenalidomide in PEL (Supplementary Figure S2C).

IFNs α, β and γ are cytotoxic to PEL but are not essential for the antiproliferative effect of IMiDs

In the case, IMiDs block the proliferation of PEL by activating the IFN pathway, then treatment with recombinant IFNs should mimic the effect of IMiDs. To test this hypothesis, we treated a panel of cell lines with increasing concentrations of recombinant IFNs α, β and γ. All the PEL cell lines were sensitive to recombinant IFNs α, β or γ (Figure 2a). In particular, BC-3, BCBL-1 and JSC-1 were highly sensitivity to IFNs α and β. Although BC-1 and VG-1 cells were relatively resistant to IFNs α and β, they were sensitive to IFN-γ. In contrast, DG-75 and BJAB, the two IMiD-resistant cell lines, showed little or no inhibitory effect on treatment with any IFN (Figure 2a).

Interestingly, the neutralizing antibodies against IFNs α, β and γ, when used singly (Supplementary Figure S3) or in combination (Figure 2b), did not block the antiproliferative effect of IMiDs

against PEL, although they effectively blocked the antiproliferative effect of their respective recombinant IFNs. Further none of the IFNs were secreted into the supernatants of the PEL cells treated with IMiDs (Figure 2c). Collectively, these results suggest that activation of the IFN pathway is not solely responsible for the antiproliferative effect of IMiDs against PEL.

IMiDs have no effect on KSHV lytic replication in PEL

It was conceivable that induction of KSHV lytic replication contributed to the cell death and activation of IFN signaling observed following treatment with IMiDs. KSHV replication and transcription activator is a master regulator and marker for lytic reactivation.[22] Treatment of PEL cell lines with IMiDs failed to induce replication and transcription activator expression, as determined by immunoblotting (Supplementary Figure S4A). In addition, we failed to detect infectious virions in the supernatant from IMiD-treated PEL cells when assayed on 293-PAN-Luc reporter cell line (Supplementary Figure S4B).[23] Thus, IMiDs do not induce lytic reactivation of KSHV.

IMiDs downregulate IRF4 expression in PEL

MM cells are addicted to IRF4 (Shaffer et al.[24]) and the antiproliferative activity of lenalidomide and pomalidomide in myeloma and ABC-DLBCL is associated with downregulation of IRF4.[25,26] To delineate the role of IRF4 in the survival of PEL and in their response to IMiDs, we used western blotting to compare its expression in a panel of 35 cell lines comprising 11 hematologic malignancies, including 9 PEL cell lines. Expression of IRF4 was robust in all cell lines derived from PEL, MM, ABC-DLBCL, Waldenstrom macroglobulinemia and Hodgkin lymphoma, but was weak to absent in the cell lines derived from other hematologic malignancies (Figure 3a). Treatment of BC-3, BCBL-1 and JSC-1 with IMiDs resulted in significant decrease in the expression of IRF4 and its downstream target MYC, thus suggesting that IMiDs exert their cytotoxicity toward PEL by
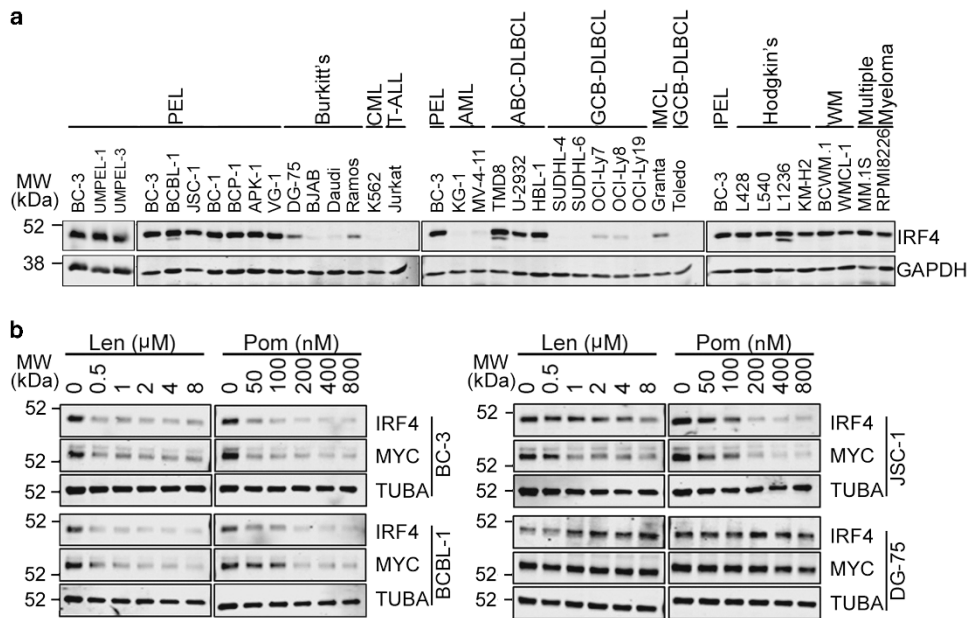

Figure 3. Uniform expression of IRF4 in PEL. (**a**) Expression of IRF4 in a panel of 35 hematological cancer cell lines. Cell lysates were prepared from logarithmically growing cell lines and blotted for IRF4 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Blots are representative of three individual experiments. PEL, primary effusion lymphoma; CML, chronic myelogenous leukemia; T-ALL, T-cell acute lymphoblastic leukemia; AML, acute myelogenous leukemia; ABC-DLBCL, activated B-cell diffuse large B-cell lymphoma; GCB-DLBCL, germinal center B-cell diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; WM, Waldenstrom macroglobulinemia; MW, molecular weight. (**b**) Immunoblot analysis showing the effect of lenalidomide (Len) and pomalidomide (Pom) at the indicated doses for 48 h on the expression of IRF4, MYC and TUBA (Tubulin, loading control) in BC-3, BCBL-1, JSC-1 and DG-75 cells. Blots are representative of three individual experiments.
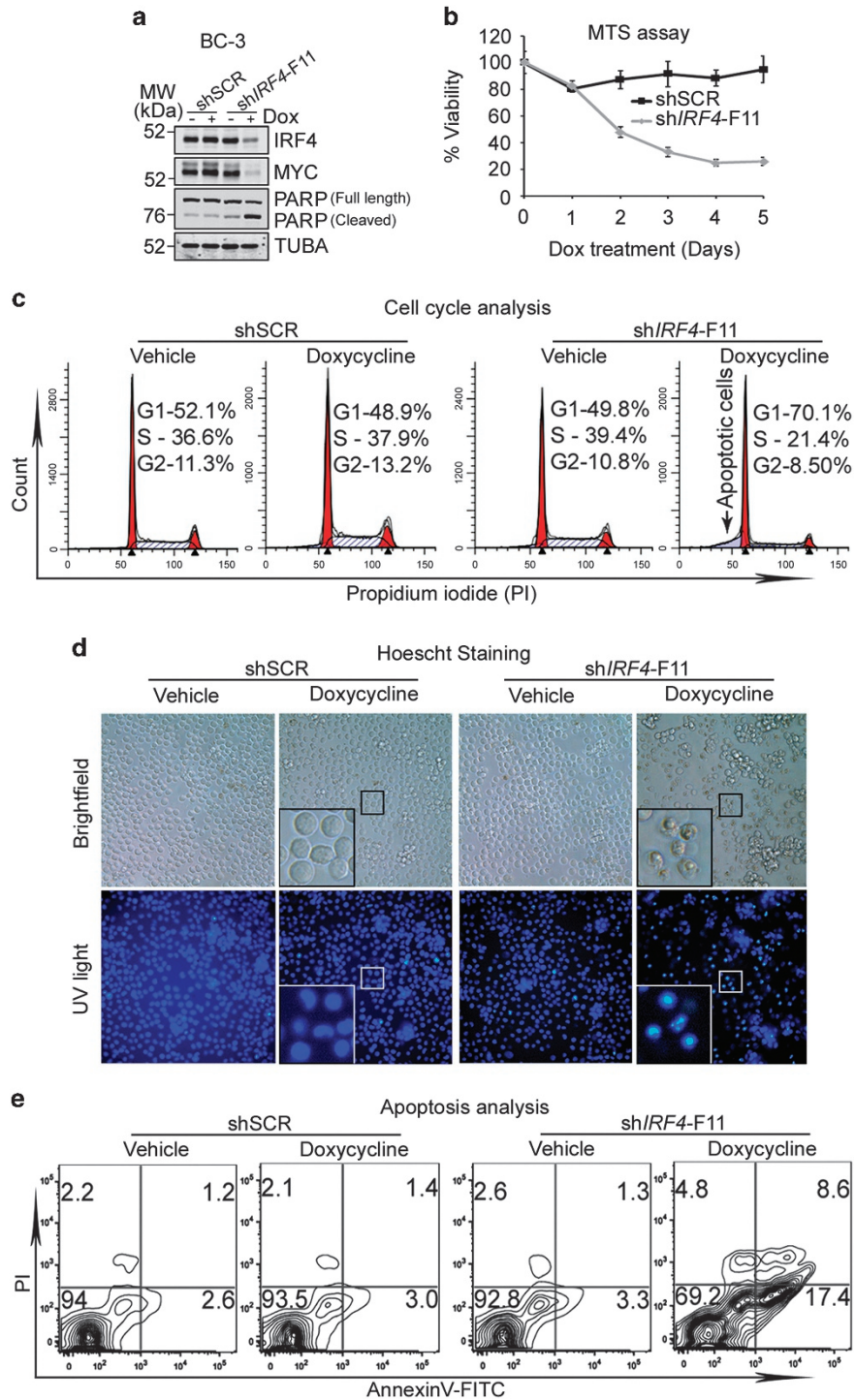
Figure 4. PEL cells are addicted to IRF4. (**a**) BC-3 cells stably expressing tetracycline-inducible H1 promoter (H1/TO)-driven shRNA targeting *IRF4*, clone F11 (sh*IRF4*-F11) and shRNA targeting scrambled sequence (shSCR) were treated with Dox (500 ng/ml) for 3 days and immunoblotted for the expression of IRF4, MYC, poly (ADP-ribose) polymerase (PARP) and TUBA. (**b**) BC-3 cells stably expressing shSCR and sh*IRF4*-F11 were treated with Dox for indicated time points and cell viability was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The values shown are mean ± s.e. of a representative experiment performed in triplicate for three times. (**c**) Cell cycle analysis of BC-3 cells stably expressing shSCR and sh*IRF4*-F11 treated with and without Dox for 48 h. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry. Data are representative of two individual experiments. (**d**) BC-3 cells stably expressing shSCR and sh*IRF4*-F11 were treated with Dox for 72 h. Cells were then stained with Hoescht 33342 (50 μg/ml) and photographed. (**e**) BC-3 cells stably expressing shSCR and sh*IRF4*-F11 were treated with Dox for 48 h, stained with annexinV-FITC/PI and analyzed for apoptosis by flow cytometry. Data are representative of two individual experiments.
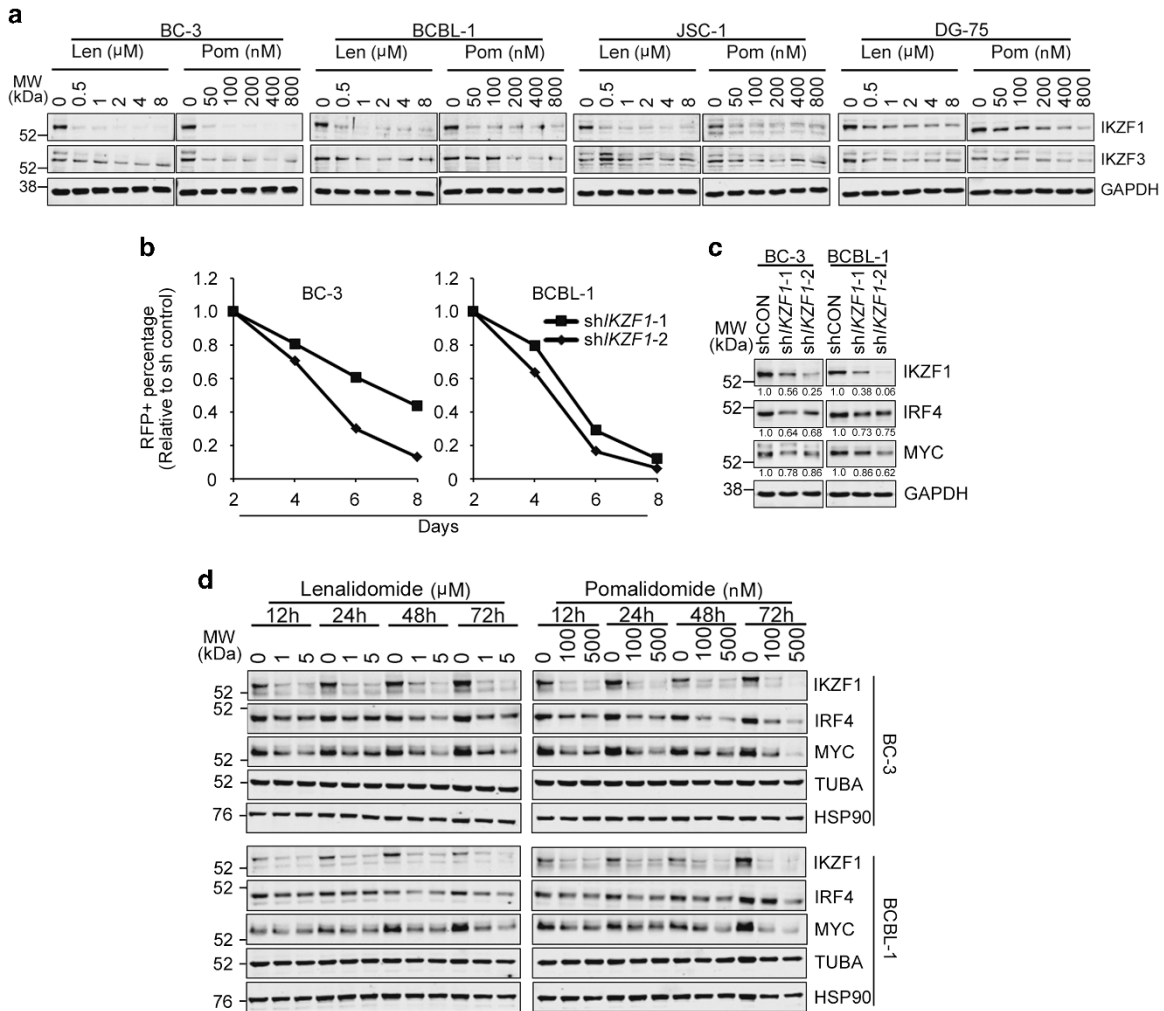
Figure 5. IMiDs rapidly downregulate IKZF1 and silencing of *IKZF1* is toxic to PEL. (**a**) Immunoblot analysis showing the effect of treatment with lenalidomide (Len) and pomalidomide (Pom) at the indicated doses for 48 h on the expression of IKZF1, IKZF3 and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (loading control) in BC-3, BCBL-1, JSC-1 and DG-75 cells. Blots are representative of two individual experiments. (**b**) Change in % red fluorescent protein (RFP) positivity over time in BC-3 and BCBL-1 cells infected with viruses encoding RFP and the indicated shRNAs. The day 2% RFP for each virus was normalized to 1 and subsequent values were expressed relative to cells infected with a virus encoding RFP and a control shRNA. Data are representative of two individual experiments. (**c**) Immunoblot analysis of BC-3 and BCBL-1 cells transiently infected with lentiviruses expressing the indicated shRNAs for 72 h. Immunoblots were quantified (normalized to the expression of GAPDH) using image studio version 5.0 from LI-COR Biosciences (Lincoln, NE, USA). Blots are representative of two individual experiments. (**d**) Immunoblot analysis showing the expression IKZF1, IRF4, MYC, TUBA and HSP90 (loading controls) in BC-3 and BCBL-1 cells treated with indicated concentrations of IMiDs for 12, 24, 48 and 72 h. Blots are representative of two individual experiments.

downregulating IRF4 (Figure 3b). In contrast, IMiDs had no significant effect on the levels of IRF4 and MYC in DG-75 cells (Figure 3b).

PEL cells have constitutive nuclear factor-κB (NF-κB) activity due to the presence of KSHV viral proteins[27–29] and aberrant NF-κB activity has been shown to upregulate the expression of IRF4.[30] To test whether IMiDs repress IRF4 expression by inhibiting NF-κB pathway, BC-3 and BCBL-1 cells stably expressing an NF-κB promoter-driven luciferase reporter construct were treated with increasing concentrations of IMiDs. As shown in Supplementary Figure S5A, IMiDs failed to block NF-κB promoter-driven luciferase activity. Further, no change in the secretion of interleukin-6, a known target of classical NF-κB pathway,[31] processing of p100 into p52 and expression of NF-κB pathway proteins were observed in IMiD-treated PEL cells (Supplementary Figures S5B and C). Taken together, these results demonstrate that IMiDs have no significant effect on the constitutive NF-κB activity present in PEL cells.

PEL cells are addicted to IRF4 for survival

To provide genetic evidence in support of the hypothesis that IMiDs exert their cytotoxic effect against PEL through downregulation of IRF4, we studied the effect of IRF4 knockdown in BC-3 cells. For this purpose, we generated a polyclonal population of BC-3 cells expressing a tetracycline-inducible-H1 (TO/H1) promoter-driven small hairpin RNA (shRNA) targeting *IRF4* (sh*IRF4*) (Supplementary Figures S6A and B) followed by generation of single cell clones (Supplementary Figure S6C). On treatment with doxycycline (Dox), significant downregulation of IRF4 was observed in a number of clones (Supplementary Figure S6C). Downregulation of MYC, a target of IRF4,[24] and cleavage of poly (ADP-ribose) polymerase on Dox treatment was observed only in those clones where IRF4 was downregulated (Figure 4a and Supplementary Figure S6C). Strikingly, cellular proliferation was decreased rapidly only in clones were IRF4 was downregulated on treatment with Dox (Figure 4b and Supplementary Figure S6D). Treatment of a clone, BC-3-sh*IRF4*-F11, with Dox resulted in G1 cell
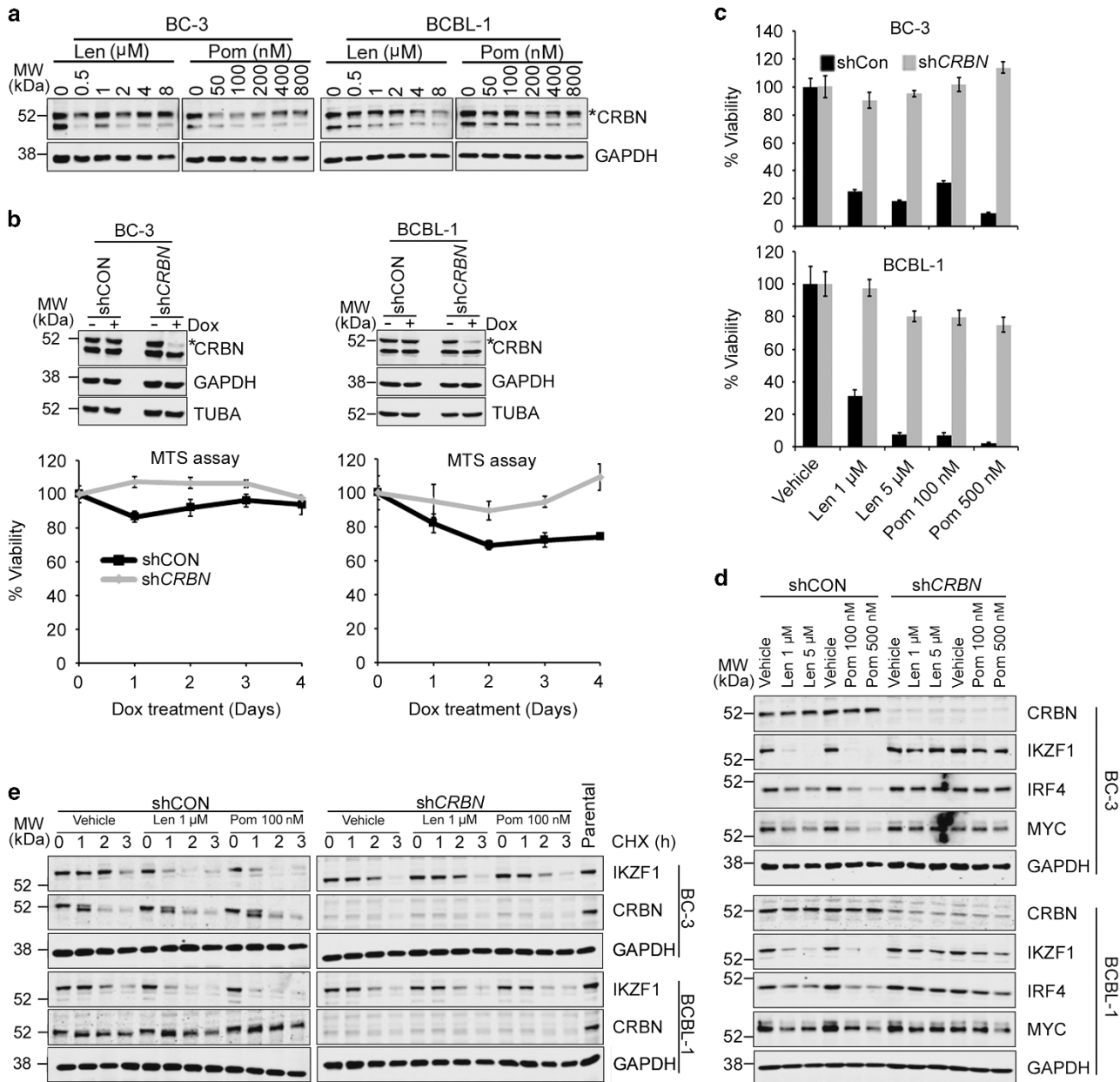
Figure 6. CRBN is dispensable for the survival of PEL cells but is essential for the antiproliferative activity of IMiDs in PEL cells. (**a**) Immunoblot analysis showing the effect of lenalidomide (Len) and pomalidomide (Pom) at the indicated doses for 48 h on the expression of CRBN and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) in BC-3 and BCBL-1 cells. The band corresponding to CRBN is marked with an asterisk. Blots are representative of two individual experiments. (**b**) Upper panel: BC-3 and BCBL-1 cells stably expressing tetracycline-inducible shRNA targeting *CRBN* (sh*CRBN*) and shRNA targeting scrambled sequence (shCON) were treated with Dox (500 ng/ml) for 4 days and immunoblotted for the expression of CRBN, GAPDH and TUBA. Blots are representative of two individual experiments. Lower panel: BC-3 and BCBL-1 cells stably expressing shCON and sh*CRBN* were treated with Dox for indicated time points and cell viability was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The values shown are mean ± s.e. of a representative experiment performed in triplicate for two times. (**c**) BC-3 and BCBL-1 cells stably expressing shCON and sh*CRBN* were pre-treated with Dox for 3 days followed by treatment with vehicle and IMiDs at indicated concentrations for 6 days in the presence of Dox and cell viability was measured by MTS assay. The values shown are mean ± s.e. of a representative experiment performed in triplicate for three times. (**d**) BC-3 and BCBL-1 cells stably expressing shCON and sh*CRBN* were pre-treated with Dox for 3 days followed by treatment with vehicle and IMiDs at indicated concentrations for 48 h in the presence of Dox, and cell lysates were collected and immunoblotted for indicated proteins. Blots are representative of two individual experiments. (**e**) BC-3 and BCBL-1 cells stably expressing shCON and sh*CRBN* were pre-treated with Dox for 3 days followed by treatment with vehicle or IMiDs along with Dox in the presence of 100 μg/ml of cycloheximide (CHX) for 0, 1, 2 and 3 h, respectively. Whole-cell lysates were immunoblotted for IKZF1, CRBN and GAPDH. Blots are representative of two individual experiments. Note: The CRBN antibody gives a nonspecific band when CRBN is probed as first antigen but when the blot is probed for some other antigen, then stripped and probed for CRBN and then the intensity of the nonspecific band is decreased or gone.

cycle arrest (Figure 4c) and the appearance of cells with condensed and fragmented nuclei suggestive of apoptosis (Figure 4d), a finding further confirmed by staining with annexinV/propidium iodide (Figure 4e). In contrast, Dox treatment had no significant effects on cell cycle progression and apoptosis in BC-3-shSCR cells (Figures 4c–e). Collectively, the above results suggest that down-regulation of IRF4 is toxic to BC-3 cells by inhibiting cell cycle progression and through induction of apoptosis.

IMiDs rapidly downregulate IKZF1 and silencing of *IKZF1* is toxic to PEL

Ikaros family proteins IKZF1 and IKZF3 are B-cell transcription factors that have crucial roles in immunity and cell-fate decisions.[32] Recently, it was shown that IMiDs selectively degrade these transcription factors in MM cells.[10,11] In PEL, both IMiDs led to significant and near-complete downregulation of IKZF1 in all the three PEL cell lines even at the lowest concentration (that is, 0.5 μM lenalidomide and 50 nM pomalidomide) tested, but had only a modest effect in the DG-75 cell line (Figure 5a). In contrast, the effect of IMiDs on the level of expression of IKZF3 was modest at best and, in general, required higher doses of the drugs (Figure 5a). Consistent with the results seen with IMiDs, silencing of *IKZF1* by two different shRNAs were selectively toxic to PEL cells (Figure 5b and Supplementary Figure S7A) and was accompanied by partially reduced expressions of IRF4 and MYC (Figure 5c). Additional studies revealed that IMiDs downregulate IKZF1 expression at the posttranslational level (Supplementary Figures S7B and C). Furthermore, time-course experiments revealed rapid and near-complete downregulation of IKZF1 expression as early as 12 h post treatment even at the lowest concentrations of both IMiDs (Figure 5d). In contrast, the levels of IRF4 and MYC were less sensitive to downregulation by IMiDs (Figure 5d). Thus, near-complete down-regulation of these proteins was either not observed or required treatment with longer duration (that is, 48 h) and higher concentrations of the drugs (Figure 5d). Collectively, these results support the hypothesis that IKZF1 is an upstream target of IMiDs in PEL.

We also checked the hypothesis that IKZF1 may be responsible for the high-level expression of IRF4 observed in PEL cells. We found that IRF4 and IKZF1 are both consistently expressed in PEL, myeloma, Waldenstrom macroglobulinemia, ABC-DLBCL and Hodgkin's lymphoma cell lines. However, there was little correlation between IRF4 and IKZF1 expression in cell lines derived from other hematologic malignancies (Supplementary Figure S8). Therefore, although it is possible that IKZF1 may contribute to the overexpression of IRF4 (and MYC) in PEL, it is unlikely to be the sole regulator of their expression.

CRBN is dispensable for the survival of PEL

IMiDs exert their antiproliferative effect by binding to their cellular protein target CRBN.[7–9] However, we failed to observe a significant and consistent effect of IMiDs on the expression of CRBN in BC-3 and BCBL-1 cells (Figure 6a). It has been shown that silencing of *CRBN* by shRNA significantly decreases the proliferation of MM[8] and ABC-DLBCL cells.[25] We generated polyclonal populations of BC-3 and BCBL-1 cells stably expressing a TO/U6 promoter-driven shRNA targeting *CRBN* (sh*CRBN*).[10] Treatment of sh*CRBN*-expressing cells with Dox for 4 days significantly downregulated the expression of CRBN (Figure 6b, upper panel), whereas Dox treatment was without effect in control shRNA-expressing cells (Figure 6b, upper panel). Interestingly, silencing of CRBN expression did not have any significant effect on the proliferation of BC-3 and BCBL-1 cells (Figure 6b, lower panel). Thus, in contrast to myeloma and ABC-DLBCL cells, CRBN is dispensable for the survival of PEL.

CRBN is essential for the antiproliferative effect of IMiDs in PEL

We next asked the question whether CRBN is essential for the activity of IMiDs in PEL. Although IMiDs significantly inhibited the proliferation of shCON-expressing PEL cells, the antiproliferative activity of IMiDs was almost completely blocked in sh*CRBN*-expressing PEL cells (Figure 6c). Furthermore, treatment with IMiDs failed to induce G1 cell cycle arrest in sh*CRBN*-expressing PEL cells, but successfully did so in shCON-expressing PEL cells (Supplementary Figure S9). Further, IMiDs treatment resulted in a near-complete abrogation of IKZF1 expression in shCON-expressing BC-3 and BCBL-1, which was accompanied by a significant decrease in the expressions of IRF4 and MYC but was without any effect on the expression of CRBN (Figure 6d). Remarkably, IMiDs had no significant effect on the expression levels of IKZF1, IRF4 and MYC in the sh*CRBN*-expressing BC-3 and BCBL-1 cells (Figure 6d). In addition, CRBN is essential for the posttranslational degradation of IKZF1 by IMiDs, as observed by a complete block in the degradation of IKZF1 by IMiDs in sh*CRBN*-expressing cells (Figure 6e), whereas IKZF1 was degraded within 1 h by IMiDs in shCON-expressing cells (Figure 6e). These results clearly suggest that CRBN is essential for the antiproliferative potential of IMiDs in PEL. However, we did not observe a significant difference in the level of expression of CRBN between IMiD-sensitive and -resistant cell lines (Supplementary Figure S10), suggesting that the resistance to IMiDs in these cells is not linked to CRBN expression.

Knocking down *MYC* by shRNA enhances the sensitivity of IMiDs to PEL

To test whether the loss of MYC could synergize with IMiDs, we generated polyclonal population of BC-3 cells stably expressing TO/H1-driven shRNAs targeting *MYC* (sh*MYC*) and a scrambled sequence (shSCR). Consistent with our published results,[18] treatment of BC3-sh*MYC* cells with Dox resulted in a significant

Figure 7. Knocking down *MYC* enhances the antiproliferative effect of IMiDs in PEL, BRD4 inhibitors and IMiDs display synergistic antiproliferative activity against PEL. (**a**) BC-3 cells stably expressing tetracycline-inducible H1 promoter (H1/TO)-driven shRNA targeting *MYC* (sh*MYC*) and shRNA targeting scrambled sequence (shSCR) were treated with Dox (500 ng/ml) for 4 days and immunoblotted for the expression of MYC and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Blots are representative of two individual experiments. (**b**) BC-3 cells stably expressing shSCR and sh*MYC* were treated with Dox for indicated time points and cell viability was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The values shown are mean ± s.e. of a representative experiment performed in triplicate for three times. (**c**) BC-3 cells stably expressing shSCR and sh*MYC* were treated in the presence/absence of Dox with indicated concentrations of IMiDs or vehicle for 72 h and cell viability was measured by MTS assay. ***Significance at the level of $P \leqslant 0.001$. The values shown are mean ± s.e. of a representative experiment performed in triplicate for three times. (**d**) Cell cycle analysis of BC-3 cells stably expressing shSCR and sh*MYC* that were treated in the presence/absence of Dox with indicated concentrations of IMiDs or vehicle for 72 h. Data are representative of two individual experiments. (**e**) Apoptosis analysis of BC-3 cells stably expressing shSCR and sh*MYC* that were treated in the presence/absence of Dox with indicated concentrations of IMiDs or vehicle for 48 h. Data are representative of two individual experiments. (**f**) BC-3 and BCBL-1 cells were treated with low doses of lenalidomide (Len) in combination with low doses of three structurally different BRD4 inhibitors (JQ-1, IBET151 and PFI-1) for 5 days and then assessed for viability using MTS assay. Combination index (CI) was calculated using the calcusyn software, which is based on the method of Chou and Talalay.[33] Each BRD4 inhibitor was tested in combination with lenalidomide at 12 different combinations (please see supplementary Tables 2–8 for details). CI values of < 1 denotes synergism and CI values > 1 denotes antagonism. Data presented are representative of three individual experiments performed in triplicate.
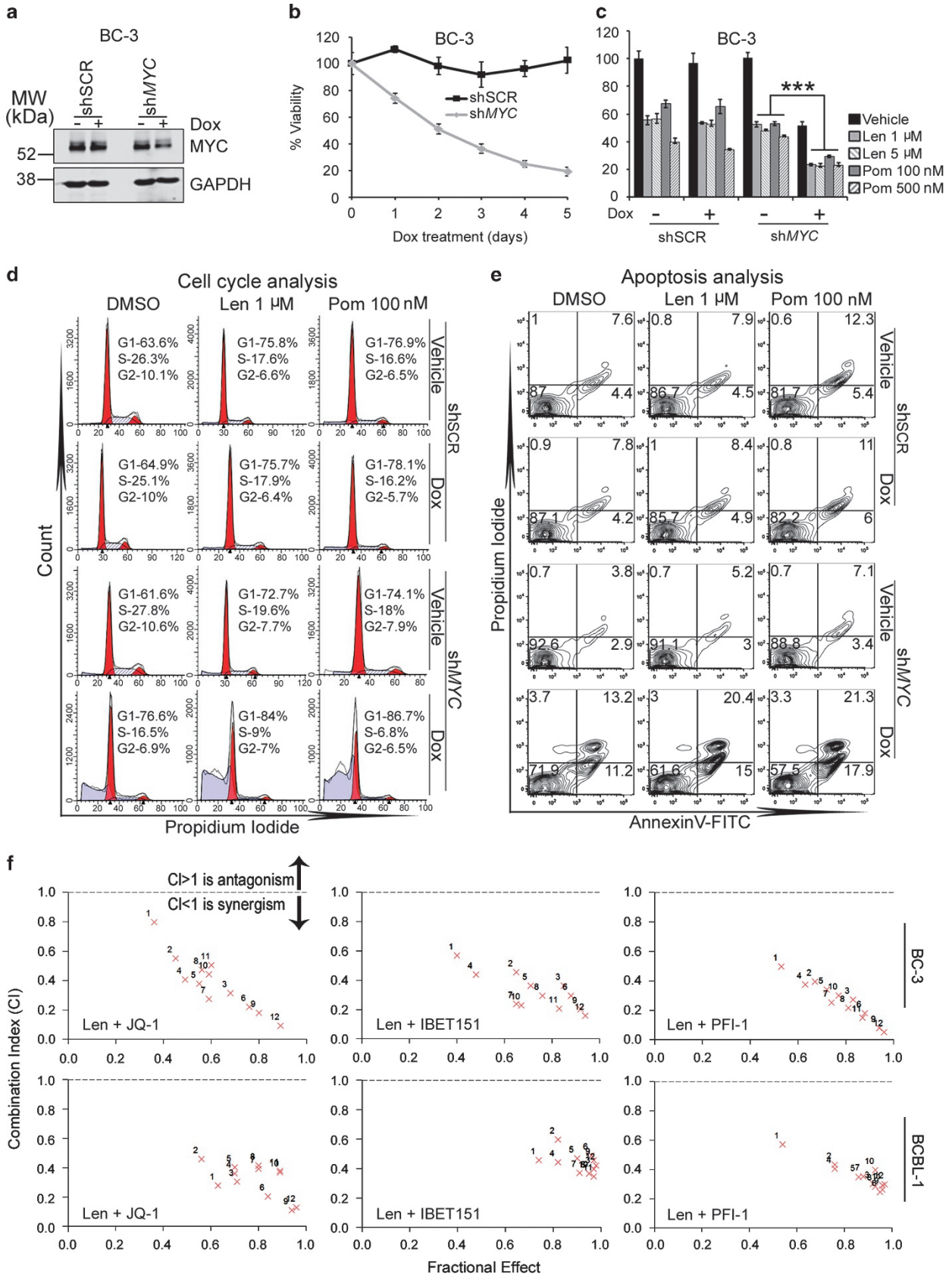
downregulation of MYC (Figure 7a), which was accompanied by a decrease in cell proliferation (Figure 7b), without any effect on BC-3-shSCR cells (Figures 7a and b). Interestingly, knockdown of MYC significantly enhanced the antiproliferative effect of IMiDs (Figure 7c), which was accompanied by cell cycle arrest and apoptosis (Figures 7d and e). In contrast, no significant difference in cell proliferation, cell cycle progression and apoptosis was observed in BC-3 shSCR cells treated with IMiDs in the presence or the absence of Dox (Figures 7c–e). These results pointed to the existence of a potential synergism between IMiDs and inhibition of MYC.

BRD4 inhibitors JQ-1, IBET151 and PFI-1 are synergistic with IMiDs in PEL

Recently, it has been shown that MYC transcription can be targeted using BRD4 inhibitors.[16–18] JQ-1, IBET151 and PFI-1 are three structurally distinct BRD4 inhibitors.[13–15] To test whether IMiDs show synergistic antiproliferative activity when combined with BRD4 inhibitors, BC-3 and BCBL-1 cells were treated with low doses of lenalidomide in combination with low doses of JQ-1, IBET151 and PFI-1, respectively. The combination index was calculated using the calcusyn software, which is based on the method of Chou and Talalay.[33] Lenalidomide was highly synergistic with all BRD4 inhibitors at all the combination doses tested in both BC-3 and BCBL-1 cells (Figure 7f and Supplementary Tables 2). In contrast, lenalidomide is not synergistic with the inactive isomer of JQ-1 in either cell line (Supplementary Tables 9 and 10). Furthermore, combined treatment with lenalidomide and JQ-1 in BC-3 and BCBL-1 cells significantly decreased the expression of MYC and IRF4 at both protein (Figure 8a) and mRNA (Supplementary Figure S11A) levels as compared with treatment with either drug alone. The combination of lenalidomide with JQ-1 also resulted in G1 cell cycle arrest, cleavage of poly (ADP-ribose) polymerase and the appearance of apoptotic cells as compared with treatment with either drug alone (Supplementary Figure S11B and Figures 8a and b, respectively).

Knocking down BRD4 by shRNA enhances the sensitivity of IMiDs to PEL

To confirm whether the observed synergism between lenalidomide and the BRD4 inhibitors is due to inhibition of BRD4, we generated stable clones of BC-3 and BCBL-1 cells expressing TO/H1 promoter-driven shRNA targeting BRD4 (shBRD4). Treatment of BC-3 and BCBL-1 cells expressing shBRD4 with Dox resulted in a significant downregulation of BRD4 (Figure 8c and Supplementary Figure S11C) and decrease in cellular proliferation (Figure 8d and Supplementary Figure S11D), whereas Dox treatment had no effect on BRD4 expression or cellular proliferation in BC-3 and BCBL-1 cells expressing a scrambled shRNA sequence (shSCR) as control (Figures 8c and d and Supplementary Figures S11C and D). More importantly, Dox enhanced the

antiproliferative activity of IMiDs in the shBRD4-expressing BC-3 and BCBL-1 cells but was without effect in shSCR-expressing cells (Figure 8d and Supplementary Figure S11D).

Lenalidomide and JQ-1 are synergistic against PEL *in vivo*

To check the *in vivo* efficacy of lenalidomide, alone and in combination with JQ-1, BC-3 cells were injected into the intraperitoneal cavity of NOD–SCID mice. Five days after the injection, animals were randomly assigned to vehicle control, lenalidomide (50 mg/kg once daily for 28 days), JQ-1 (50 mg/kg once daily for 28 days) and the combination. Intraperitoneal inoculation of BC-3 cells resulted in rapid tumor growth and massive ascites, which resulted in weight gain (Figure 8e). There was a significant reduction ($P \leqslant 0.01$) in body weight gain (a measure of ascites)[34] of animals treated with lenalidomide and JQ-1 when compared with vehicle control (Figure 8e). In addition, the combination of lenalidomide and JQ-1 showed a further reduction in body weight gain over time when compared with mice treated with either agent alone (Figure 8e). Furthermore, the median survival of mice that received combination treatment (51 days) was significantly ($P \leqslant 0.01$) increased as compared with the median survival of mice treated with lenalidomide (35 days) or JQ-1(42 days) alone (Figure 8f).

DISCUSSION

In this study, we demonstrate that a majority of the PEL cells are highly sensitive to lenalidomide and pomalidomide, two Food and Drug Administration-approved drugs for the treatment of MM. Both drugs have predictable and manageable safety profiles and limited cumulative long-term toxicity,[35,36] making them attractive treatment options for PEL.

The antiproliferative action of lenalidomide in PEL cell lines was associated with the activation of the IFN signaling pathway. However, we did not detect IFN in the supernatant of IMiD-treated cells and neutralizing antibodies against IFNs failed to block the activity of IMiDs against PEL. As IRF4 has been recently shown to modulate IFN signaling,[25] these results prompted us to explore the role of IRF4 in the antiproliferative effects of IMiDs in PEL. We observed that IRF4 is not only uniformly expressed in PEL cell lines but is significantly downregulated following treatment with IMiDs. Furthermore, shRNA-mediated silencing of *IRF4* was toxic to PEL cells, thereby supporting the argument that downregulation of IRF4 contributes to the antiproliferative effect of IMiDs in PEL. Expression of IRF4 in myeloma cells has been attributed to their plasmacytic differentiation.[24] As PEL cells resemble plasma cells in the gene expression profile,[37] the uniform expression of IRF4 in these cells may also reflect their plasma cell lineage. IRF4 expression is also a feature of ABC-DLBCL.[25,38] In these cells, oncogenic mutations affecting the B-cell receptor and MYD88 signaling pathways induce NF-κB,[39,40] which is a strong inducer of

Figure 8. (**a**) BC-3 and BCBL-1 cells were treated with vehicle, lenalidomide 1 μM (L1), pomalidomide 100 nM (P100), JQ-1 50 nM (J50), JQ-1 100 nM (J100) and the combinations for 48 h. Whole-cell lysates were immunoblotted for MYC, IRF4, poly (ADP-ribose) polymerase (PARP) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Blots are representative of three independent experiments. (**b**) Apoptosis analysis of BC-3 and BCBL-1 cells treated with vehicle or indicated concentrations of IMiDs and JQ-1, or the combination for 72 h. Data presented are representative of two individual experiments. (**c**) BC-3 cells stably expressing tetracycline-inducible H1 promoter (H1/TO)-driven shRNA targeting BRD4 (shBRD4) and shRNA targeting scrambled sequence (shSCR) were treated with Dox (500 ng/ml) for 4 days and immunoblotted for the expression of BRD4 and GAPDH. The band corresponding to BRD4 is marked with an asterisk. Blots are representative of two individual experiments. (**d**) BC-3 cells stably expressing shSCR and shBRD4 were treated in the presence/absence of Dox with indicated concentrations of IMiDs or vehicle for 4 days, and cell viability was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The values shown are mean ± s.e. of a representative experiment performed in triplicate for two times. (**e**) Body weight gain of mice injected with BC-3 cells followed by indicated treatments (*n* = 7 in each group) over the period of experiment. Statistically significant differences (on day 27 of the treatment) are shown by asterisks (*) and (**) at the levels of $P \leqslant 0.05$ and 0.01, respectively. The black arrows in the *x* axis mark the start (day1) and end (day28) of the treatment. (**f**) Survival curves (Kaplan–Meier) of mice injected with BC-3 cells followed by indicated treatments (*n* = 7 in each group). The survival curve was generated in GraphPad Prism 5 software and statistical values for the curves are calculated by log rank (Mantel–Cox) test. Asterisks (*) and (**) indicate significance at the level of $P \leqslant 0.05$ and 0.01, respectively.

cytotoxic IFNβ.[41] IRF4, however, places a brake on IFNβ production by repressing IRF7, thereby allowing ABC-DLBCL to survive and proliferate.[25] Furthermore, IRF4 is believed to promote ABC-DLBCL survival by transactivating CARD11 and potentiating NF-κB signaling.[25] Although oncogenic mutations affecting the B-cell receptor and MYD88 signaling pathways have not been reported in PEL, they do possess constitutively active NF-κB signaling pathway due to the activity of KSHV-encoded viral FLICE inhibitory

protein K13.[27,28,42] Therefore, similar to ABC-DLBCL, IRF4 may be upregulated in PEL cells to augment the prosurvival aspect of NF-κB signaling while simultaneously protecting against the deleterious effects (for example, IFNβ production) of uncontrolled NF-κB activation. IRF4 is also known to bind to MYC promoter and stimulate MYC gene expression.[24] Even though PEL cells lack structural alterations in the MYC gene,[43] they nevertheless demonstrate elevated MYC expression, which has been shown
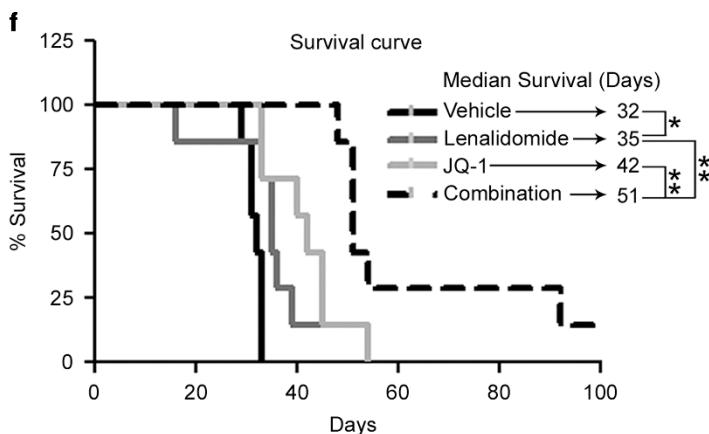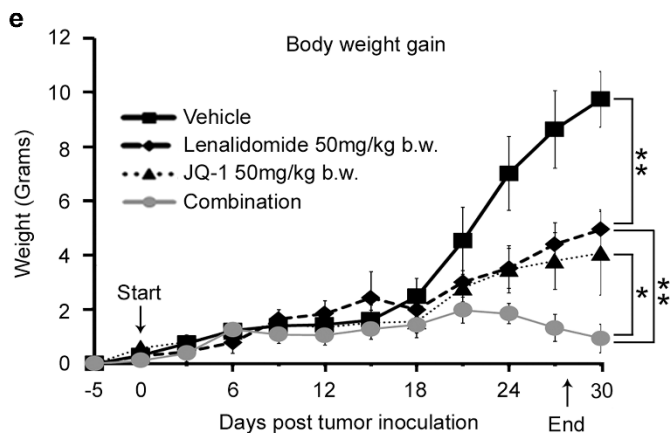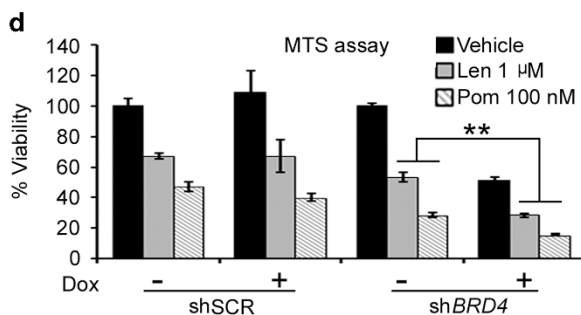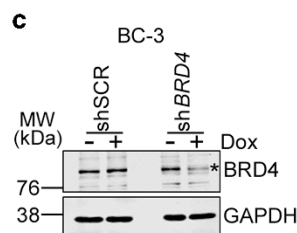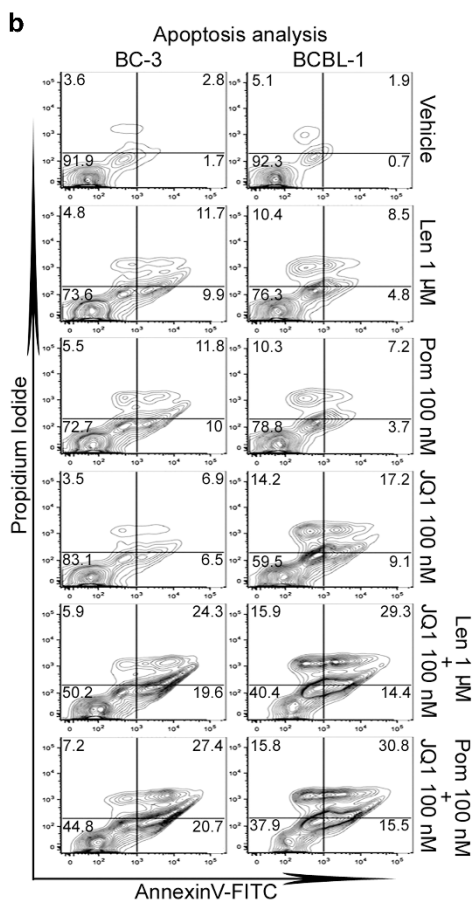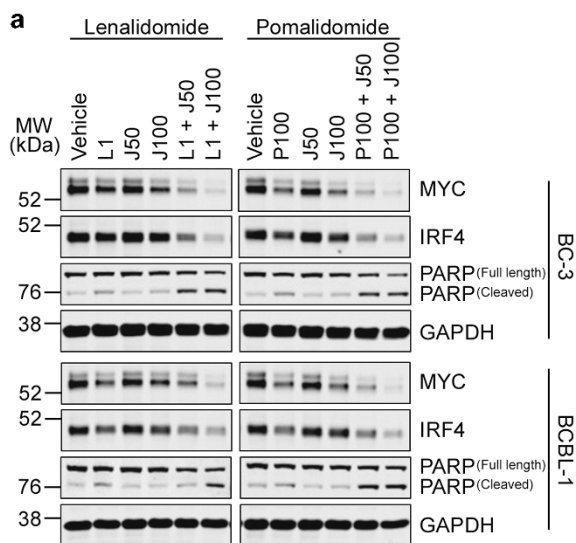
to be essential for their survival and proliferation.[44] The over-expression of IRF4 in PEL might contribute to the elevated MYC expression observed in these cells.

IMiDs were shown to degrade both IKZF1 and IKZF3 in MM.[10,11] In contrast, we observed that IKZF1 was the primary target of IMiDs in PEL cells. The expression of IKZF1 was downregulated earlier than IRF4 and MYC. In addition, *IKZF1*-specific shRNAs was not only toxic to PEL cells but also partially downregulated the expression of IRF4 and MYC. IKZF1 has been previously shown to bind to IRF4 promoter and regulate its expression at the transcriptional level.[10] Furthermore, MYC is a known transcriptional target of IRF4.[24] Taken together with prior studies, our results suggest that degradation of IKZF1 by IMiDs downregulates IRF4 expression at the transcriptional level, which in turn downregulates MYC expression.

CRBN is the direct cellular-binding target of IMiDs[7] and is essential for their immunomodulatory and antiproliferative activities.[8,9] We observed that CRBN is dispensable for the survival of PEL, which is in contrast to the situation in MM and ABC-DLBCL cells where shRNA-mediated knockdown of *CRBN* has been reported to be toxic.[8,25] However, although CRBN is not essential for the survival of PEL, it is essential for the antiproliferative activity of IMiDs in PEL, as all the IMiD-induced anti-PEL effects are blunted in cells expressing an shRNA targeting *CRBN*.

Our study along with work of others[26] suggest that MYC is one of the downstream target of IMiDs in PEL. We found that shRNA-mediated knockdown of *MYC* enhanced the antiproliferative effect of IMiDs on PEL, thus suggesting a potential synergism between IMiDs and inhibition of MYC. BRD4 inhibitors have been shown to block MYC expression.[16–18] In support of this premise, we observed striking synergy between low doses of lenalidomide and BRD4 inhibitors (JQ-1, IBET151 and PFI-1) against PEL. Furthermore, shRNA-mediated *BRD4* knockdown also enhanced the cytotoxicity of IMiDs toward PEL, suggesting that the synergism observed between IMiDs and BRD4 inhibition may not be limited only to the BRD4 inhibitors used in our study. There are several potential explanations for the observed synergism between IMiDs and BRD4 inhibitors. First, as inhibition of MYC is not complete on treatment with lower doses of IMiDs (Figure 5d), addition of low doses of BRD4 inhibitors may eliminate any residual MYC expression seen following IMiD treatment. Second, apart from MYC, BRD4 inhibitors are known to modulate the expression of other genes[45], which may have synergistic cytotoxicity when combined with IMiDs. Finally, in addition to degrading IKZF1/IKZF3 via CRBN, IMiDs may also degrade other proteins, which may result in synergistic cytotoxicity when combined with BRD4 inhibitors.

In summary, we provide strong *in vitro* and *in vivo* data showing that IMiDs are effective against PEL, and combined treatment of IMiDs with BRD4 inhibitors have synergistic activity against this deadly incurable cancer. BRD4 inhibitors have shown promising activity against multiple cancers in pre-clinical studies, and at present there are five BRD4 inhibitors in phase 1–2 clinical trials.[12] Our results suggest that IMiDs, alone and in combination with BRD4 inhibitors, deserve further testing for the treatment of PEL. While this study was in its final stage of completion, a case report was published describing the successful treatment of a PEL patient with lenalidomide,[46] which supports our pre-clinical data.

MATERIALS AND METHODS

Cell lines
BC-3, BCBL-1, JSC-1, BC-1, BCP-1, VG-1 and APK-1 were obtained from Dr Jae Jung (University of Southern California, Los Angeles, CA, USA). UMPEL-1 and UMPEL-3 were provided by Drs Izidore Lossos and Juan Ramos, respectively (both from University of Miami, Miami, FL, USA). DG-75 was obtained from Dr Alan Epstein (University of Southern California). All the cells were grown in conditions as described previously.[47] The cell lines

were expanded, stored in liquid nitrogen and used within 3 months after resuscitation. The identities of the PEL cell lines were routinely authenticated by western blotting detection of KSHV LANA. No further authentication of cell line characteristics was done. The authentication information for the remaining cell lines is not available.

Cell viability, cell cycle, apoptosis, luciferase assays and western blotting
Cell viability, cell cycle, apoptosis, luciferase assays and western blotting were performed as described earlier.[47–51]

Lentiviral shRNA constructs
shRNA oligonucleotides (Supplementary Table 11) directed against human *IRF4*, *MYC* and *BRD4* mRNAs were annealed and cloned into a modified pENT entry vector containing a TO/H1 promoter as described previously.[18] Lentiviral shRNAs for *CRBN*, *IKZF1*-1 and *IKZF1*-2 along with their respective controls were obtained from Dr. Willian Kaelin Jr (Harvard University, Cambridge, MA, USA).[10]

Real-time reverse transcriptase–PCR
Real-time quantitative reverse transcriptase–PCR analysis was performed as described earlier[47] using gene-specific PCR primers listed in Supplementary Table 12.

PEL orthotopic tumor model
A total of 2×10^7 BC-3 cells were injected intraperitoneally into female NOD–SCID mice (NCI Frederick, 6 weeks old). Five days later, the mice were randomly divided in to four groups ($n = 7$ each). Investigators are not blinded. Vehicle control (10% hydroxypropyl-β-cyclodextrin), lenalidomide 50 mg/kg b.w. (once daily), JQ-1 50 mg/kg b.w. (once daily) and the combination were administered intraperitoneally for 28 days. Then, the animals were monitored for survival. Body weight gain was measured once in 3 days as a surrogate measure of tumor progression.[34] The experiments were performed following the approval of the Institutional Animal Ethics Committee of the University of Southern California.

Statistical analysis
Two-tailed unpaired Student's *t*-test was used to test for differences between two groups. Differences with a *P*-value $\leqslant 0.05$ were considered statistically significant. Data were given as mean ± s.e. All the experiments were reproduced atleast twice. No inclusion/exclusion criteria are applied and none of the samples or animals was excluded from the analysis. The vehicle and drug treatments were performed at the same time in the same condition. The investigators are not blinded for any of the experiments.

Detailed information about Materials and Methods is provided in the Supplementary Information.

CONFLICT OF INTEREST

ACCESSION CODES

Microarray gene expression data has been deposited under accession number GSE60618 at the website Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/).

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