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ORIGINAL ARTICLE

Kaposi's sarcoma herpesvirus lytic replication compromises apoptotic response to p53 reactivation in virus-induced lymphomas

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Primary effusion lymphomas (PELs) are aggressive Kaposi's sarcoma herpesvirus (KSHV)-induced malignancies with median survival time <6 months post-diagnosis. Mutations in the *TP53* gene seldom occur in PELs, suggesting that genetic alterations in the *TP53* are not selected during PEL progression. We have reported that p53 reactivation by an inhibitor of the p53–MDM2 interaction, Nutlin-3, induces selective and massive apoptosis in PEL cells leading to efficient anti-tumor activity in a subcutaneous xenograft model for PEL. Here, we show compelling anti-tumor activity of Nutlin-3 in the majority of intraperitoneal PEL xenografts *in vivo*. Interestingly, our results demonstrate that spontaneous induction of viral lytic replication in tumors could drastically attenuate the p53-dependent apoptotic response to Nutlin-3. Moreover, viral reactivation compromised p53-dependent apoptosis in PEL cells treated with genotoxic anti-cancer agents doxorubicin and etoposide. We have recently demonstrated that the Ser/Thr kinases Pim 1 and 3 are required to trigger induction of the lytic replication cascade of KSHV. We have now assessed the ability of a novel Pim kinase inhibitor to restore the Nutlin-3-induced cytotoxicity in lytic PEL cells. PEL cells induced to lytic replication by phorbol esters showed 50% inhibition of active viral replication following treatment with the Pim kinase inhibitor. Importantly, co-treatment of these cells with the kinase inhibitor and Nutlin-3 resulted in a robust restoration of the Nutlin-3-induced cell death. These results highlight the potential impact of activation of viral lytic replication on disease progression and response to treatment in KSHV-induced lymphomas.

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INTRODUCTION

Kaposi's sarcoma herpesvirus (KSHV) is the causative agent in three different tumor types; Kaposi's sarcoma, multicentric Castelman's disease (MCD) and primary effusion lymphoma (PEL) (reviewed in Ablashi *et al.*[1]). PEL is a non-Hodgkin type lymphoma characterized by liquid tumor growth in serous body cavities,[2] which occurs predominantly, but not exclusively in HIV-positive patients with advanced AIDS.[3,4] Despite extensive research on KSHV-driven pathogenesis, PEL remains an incurable disease with 2–6 months median survival after diagnosis. The current treatment modalities involve cytostatic drugs with DNA damaging activities (reviewed in Casper[5]). These chemotherapeutics are neither potent nor selective for PEL and regular chemotherapy has been restricted by short-lived responses.[6]

TP53, the major human tumor suppressor gene, is mutated or deleted in close to half of all malignant tumors.[7] Under normal circumstances, p53 levels and activity are controlled by its negative regulator, MDM2.[8] In a subset of tumors with wild-type (wt) p53, MDM2 is present at excess levels, which correlates with rapid tumor progression and resistance to anti-cancer therapies. Intriguingly, MDM2 overexpression is the only mechanism that disables p53 function in these tumors, suggesting that overexpression of MDM2 is an alternative way to inactivate p53 function.[9] Therefore, eradication of tumor cells by releasing the MDM2-bound p53 and thereby reactivating the p53 apoptotic machinery has become an attractive strategy for cancer treatment, especially for cancers with wt p53. Recently reported small-molecule inhibitors of the p53–MDM2 interaction (Nutlins)[10] have demonstrated potency in induction of apoptosis selectively in several human tumor cell lines with wt p53.[11–13]

Interestingly, the majority of PELs do not have alterations in their *TP53* gene,[2,14] and we have previously demonstrated that restoration of the p53 pathway by Nutlin-3 results in regression of subcutaneous PEL xenografts.[15] Here, we study the efficacy of Nutlin-3[10] in an intraperitoneal (IP) mouse model for PEL. Since IP xenografts mimic biological aspects of human lymphoma (for example, tumor microenvironment and ascites formation), they represent a more relevant model for PEL than subcutaneous tumors. In addition, we observe unexpected resistance of some of the IP PEL tumors to the Nutlin-3-induced apoptosis and

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provide evidence that this is due to spontaneous viral reactivation in the tumors.

RESULTS

Nutlin-3 shows anti-tumor activity in an IP PEL model *in vivo*

Our previous study showed that restoration of the p53 pathway by Nutlin-3 led to tumor regression in a subcutaneous murine PEL xenograft model.[15] With the aim of generating an animal model similar to PEL in humans, we inoculated patient-derived KSHV-positive BC-3 cells carrying an nuclear factor (NF)-κB-regulated luciferase reporter gene (BC-3/NF-κB-luc)[16] into the IP cavity of SCID (data not shown) or NOD/SCID mice. Tumor growth could be followed by detection of bioluminescence from the luciferase reporter gene, which allows non-invasive monitoring of the disease progression and rapid detection of the efficacy of anti-tumor therapies.

After inoculation of the tumor cells, we monitored the tumor formation by whole body imaging and started the IP Nutlin-3 administration after tumor establishment (luciferase signal intensity between 1×10^5 to 4×10^5 photons). Tumor regression was evident in 87% (13/15) of mice already after three doses of the drug, and at the end of the treatment (seven doses) regression was observed in 60% (9/15) of the animals (Figure 1a). Accordingly, a significant decrease ($P = 0.0057$; Student's *t*-test) in the luciferase signal was observed when all the Nutlin-treated mice were analyzed (Figure 1b). We also followed the response to Nutlin-3 by monitoring the body weight of the animals. The average mass in the control group increased 21% due to lymphoma progression and ascites formation, whereas the average body weight in the Nutlin-3-treated mice did not change significantly (Figure 1c; $P = 0.0001$; Student's *t*-test).

Viral lytic replication impairs apoptotic potential of Nutlin-3

Our *in vivo* data show that the overall response to Nutlin-3 treatment was excellent when the treatment was started right after the tumor establishment (Figure 1). However, when we treated eight mice with more advanced tumors as indicated by significant bioluminescent signal and evident ascites formation as judged by the dramatic increase in their body weight prior the start of the treatment with seven doses of Nutlin-3, four mice (50%) did not respond (Figure 2a). In order to study the mechanism underlying the resistance, we collected ascites for protein and mRNA analyses from the four non-responder mice, and compared those with the residual ascites collected from two mice that responded to treatment. Stabilization of p53 was reduced threefold and induction of p21CIP1 (indicative of restoration of the p53 pathway) almost twofold in a non-responder ascites (#1) in contrast to the responder one (#5; Figure 2b; Supplementary Figure S1). Consistent with the impaired activation of the p53 pathway, tumor cells from the non-responder mice failed to undergo apoptosis in response to Nutlin-3, since no staining for TUNEL or active caspase-3 was detected, whereas a clear apoptotic response was detected in the responder (Figure 2c).

As viral gene expression is intimately linked to PEL survival, we next interrogated the KSHV gene expression pattern in the non-responder mice. To this end, we first confirmed the presence of KSHV in the ascitic cells of both the non-responder and responder mice by immunoblotting for the latency-associated nuclear antigen (LANA). Although some variation in the latency-associated nuclear antigen expression level was observed between tumor cells collected from different mice (Figure 2d, upper panel), this did not correlate with the response to Nutlin-3. Thereafter, we analyzed the protein levels of viral replication, and transcription activator protein (RTA) encoded by the immediate early lytic gene ORF50,[17] and the capsid glycoprotein encoded by the late K8.1

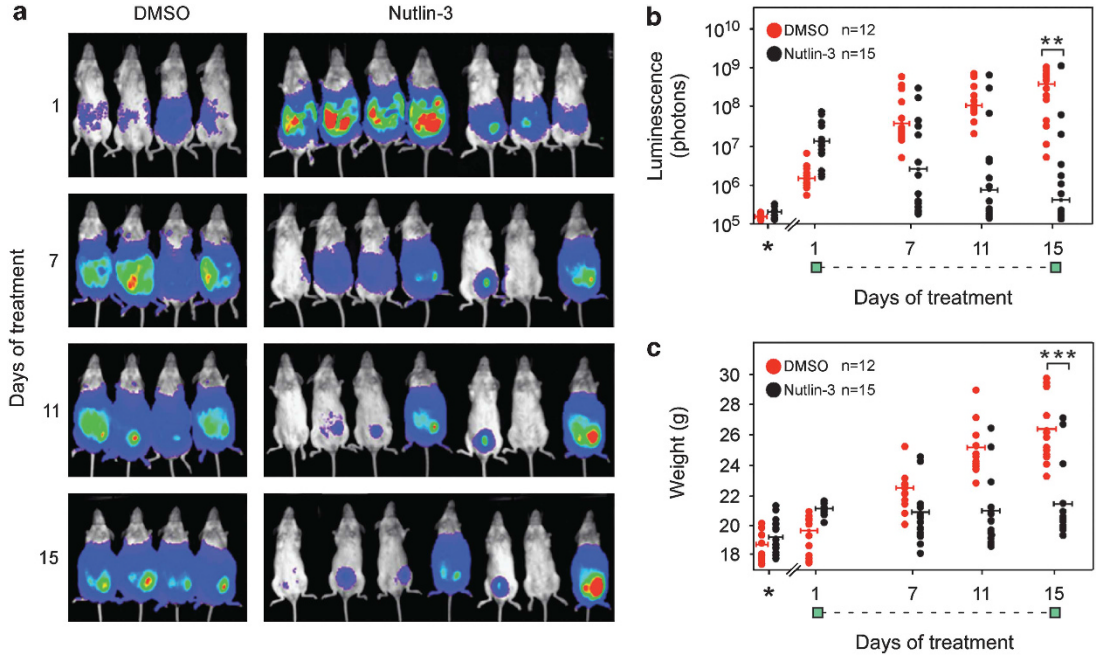

Figure 1. Nutlin-3 showed anti-tumor activity in an IP PEL model. (**a**) NOD/SCID mice were injected with 1×10^7 BC-3/NF-κB-luc cells and the intensity of bioluminescence signal was monitored by whole body imaging using the IVIS Imaging system. After tumor establishment, the mice received IP injections of the vehicle (DMSO, left panel) or Nutlin-3 (right panel) every second day for 14 days. A representative experiment of three is shown. Quantification of *in vivo* bioluminescence signal (**b**) or measurement of the weight of the animals (**c**) treated with seven doses of Nutlin-3 or vehicle during a 14-day treatment period. The graphs show collective data from three independent experiments. Asterisk indicates tumor cell implantation. Horizontal lines represent median values. $^{**}P < 0.01$; $^{***}P < 0.001$, Student's *t*-test.

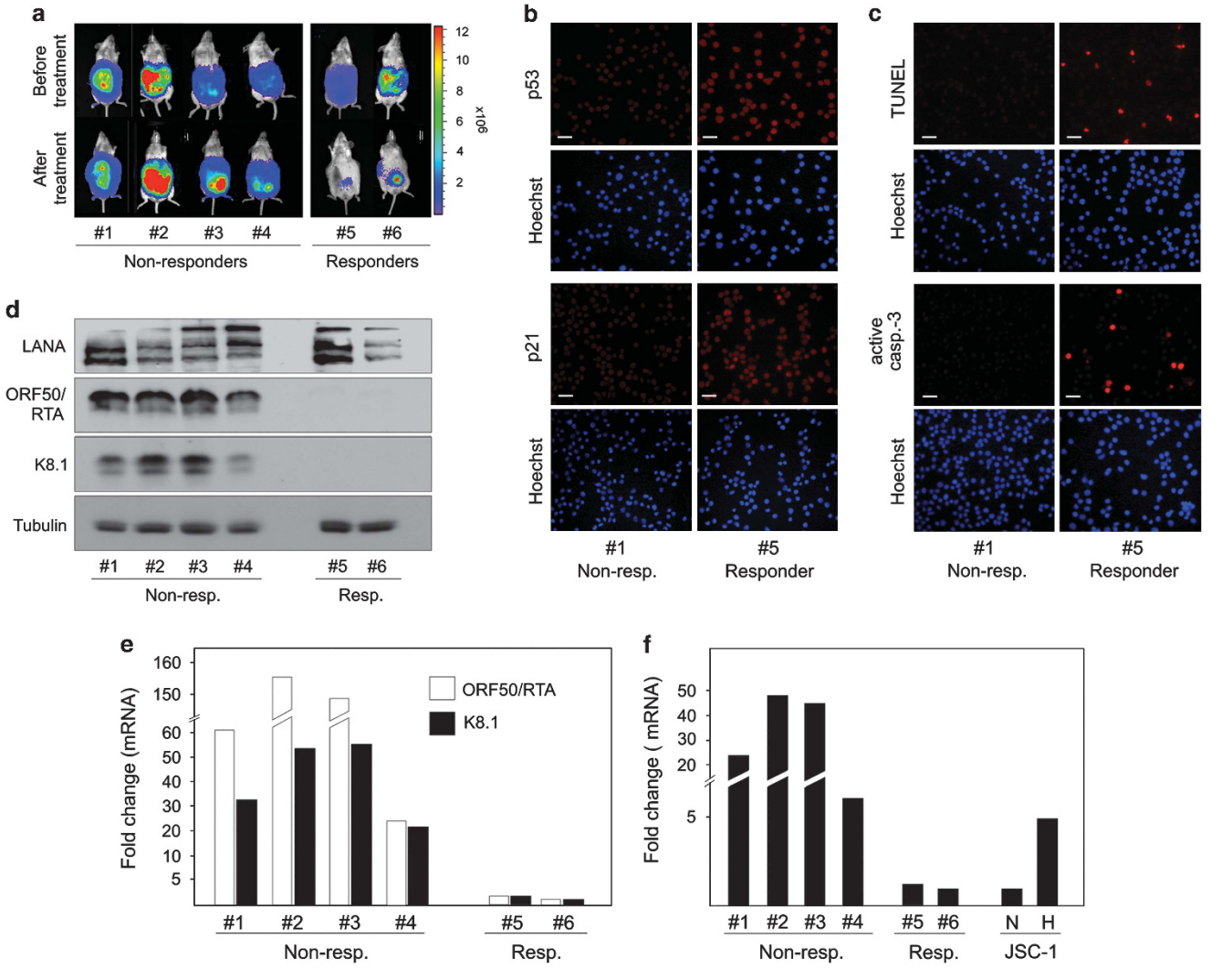

Figure 2. Viral lytic replication compromised anti-tumor potential of Nutlin-3. (**a**) Mice with advanced PEL tumors showing significant bioluminescent signal and evident ascites formation were treated with Nutlin-3. Non-responder (left panel) and responder (right panel) mice are shown before (upper panels) and after the treatment with seven doses of Nutlin-3 (bottom panels). Tumor cells were collected from the ascites of mice shown in (**a**), and analyzed for the expression of p53 and p21CIP1 (**b**) as well as for TUNEL and active caspase-3 (**c**). Nuclei were visualized by Hoechst staining. (**d**) Tumor cells were collected from the ascites of the mice shown in (**a**), and analyzed by immunoblotting for the expression of LANA, ORF50/RTA and K8.1. Tubulin served as a loading control. (**e**) Total RNA of tumor cells isolated from mice shown in (**a**) was assayed for the expression of ORF50/RTA, K8.1 and LANA transcripts by qRT–PCR. Transcripts for human β-actin were used as an internal control. The bar graph represents levels of ORF50/RTA and K8.1 transcripts, which were normalized to the LANA transcripts in the same tumor. (**f**) The same total RNA as in (**e**) was assayed for the CA9 transcripts by qRT–PCR. Transcripts for human GAPDH were used as an internal control. The amount of assayed transcripts from different tumors was normalized to that of the responder #6, which was set to one (**e**, **f**). Scale bars in panels (**b**, **c**), 20 μm.

gene.[18] Data shown in Figure 2d indicate a dramatic increase in both early and late lytic gene products in the non-responders but not in the responder mice. This was further confirmed at mRNA level by quantitative real-time PCR (qRT–PCR) analysis, which showed 22- to 156-fold and 20- to 58-fold increase for ORF50/RTA and for K8.1, respectively, in the non-responder mice (non-resp.) over the responders (resp.; Figure 2e).

The luciferase expression in the PEL tumor xenografts used to monitor the tumor growth and response to Nutlin-3 was under the regulation of NF-κB promoter. PEL cells are known to have a constitutively activated NF-κB pathway.[19] The effect of NF-κB activation on latency and lytic reactivation is reported to be quite complex and dependent on the cellular context.[20] Therefore, we sought to exclude the possibility that NF-κB signaling pathway would be affected upon induction of viral replication in tumors of the non-responder mice. To this end, BC-3/NF-κB-luc and BC-3 cells were induced to lytic replication by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) treatment for 48 h. As a control, we treated cells with the vehicle (dimethyl sulfoxide, DMSO). The treated cells were then subjected to subcellular fractionation and immunoblotting to examine the nuclear and cytoplasmic distribution of the NF-κB subunit p65 (RelA). Increased nuclear localization of p65 was used as an indication of NF-κB activation,[21] over the constitutive level. As a positive control, PEL cells were treated for 3 h with 100 μm etoposide, a topoisomerase II inhibitor, that has been shown to activate NF-κB pathway in the chronic myeloid leukemia cells.[22] The amount of p65 detected in the nuclear fraction did not change upon induction of lytic replication (TPA treatment) of the BC-3/NF-κB-luc or BC-3 cells in contrast to the etoposide-stimulated cells where the nuclear levels of p65 increased by 8- and 12-fold, respectively (Supplementary Figures S2a and S2b). These data indicate that induction of viral lytic replication does not lead to further NF-κB activation.

What could trigger the spontaneous reactivation in the tumors of the non-responder mice? As viral replication can be induced by hypoxia,[23,24] and hypoxia is a common feature in tumors, we assessed the hypoxic status of the ascites from responders and non-responders using qRT–PCR for the expression of the carbonic anhydrase 9 (CA9), an endogenous marker for hypoxia.[25] JSC-1 cells cultured under hypoxia (H) or normoxia (N) for 48 h were used as positive and negative controls, respectively (Figure 2f). The mRNA levels of CA9 were 7- to 50-fold higher in ascites of the non-responder mice compared with those of the responder mice (Figure 2f), indicating that the induction of viral lytic replication in these mice could be due to increased hypoxia in the tumor microenvironment.

KSHV lytic replication impairs stabilization of p53 and Nutlin-3-induced apoptosis in PEL cells

To further study the role of viral lytic replication in the attenuation of the p53 apoptotic response, we first induced lytic replication in BC-3 and JSC-1 cells with TPA. The efficiency of induction of lytic gene expression was confirmed by western blot analyses for vIL-6, a delayed early lytic protein (Figure 3a), and indirect immunofluorescence using antibodies against another delayed early lytic marker, ORF59 (data not shown). Intriguingly, the TPA-pretreated BC-3 and JSC-1 cells showed an approximately fourfold reduction in the levels of stabilized p53 protein as well as impaired accumulation of the p53 targets, p21 and Bax, after the Nutlin-3 treatment when compared with the non-TPA-treated cells (Figure 3a, lanes 2 vs 4 and 6 vs 8). Importantly, the observed decrease in p53 levels was not due to an adverse TPA effect, since KSHV-negative lymphoblastoid cells (IHE) showed no decrease in the p53 levels in response to TPA (Supplementary Figure S3). In addition to abrogation of the p53 stabilization in response to Nutlin-3, the induction of lytic replication affected the basal level of p53 protein in the JSC-1 cells, where prominent expression of vIL-6, an indication of lytic replication, coincided with the lower basal levels of p53 (Figure 3a, lane 7).

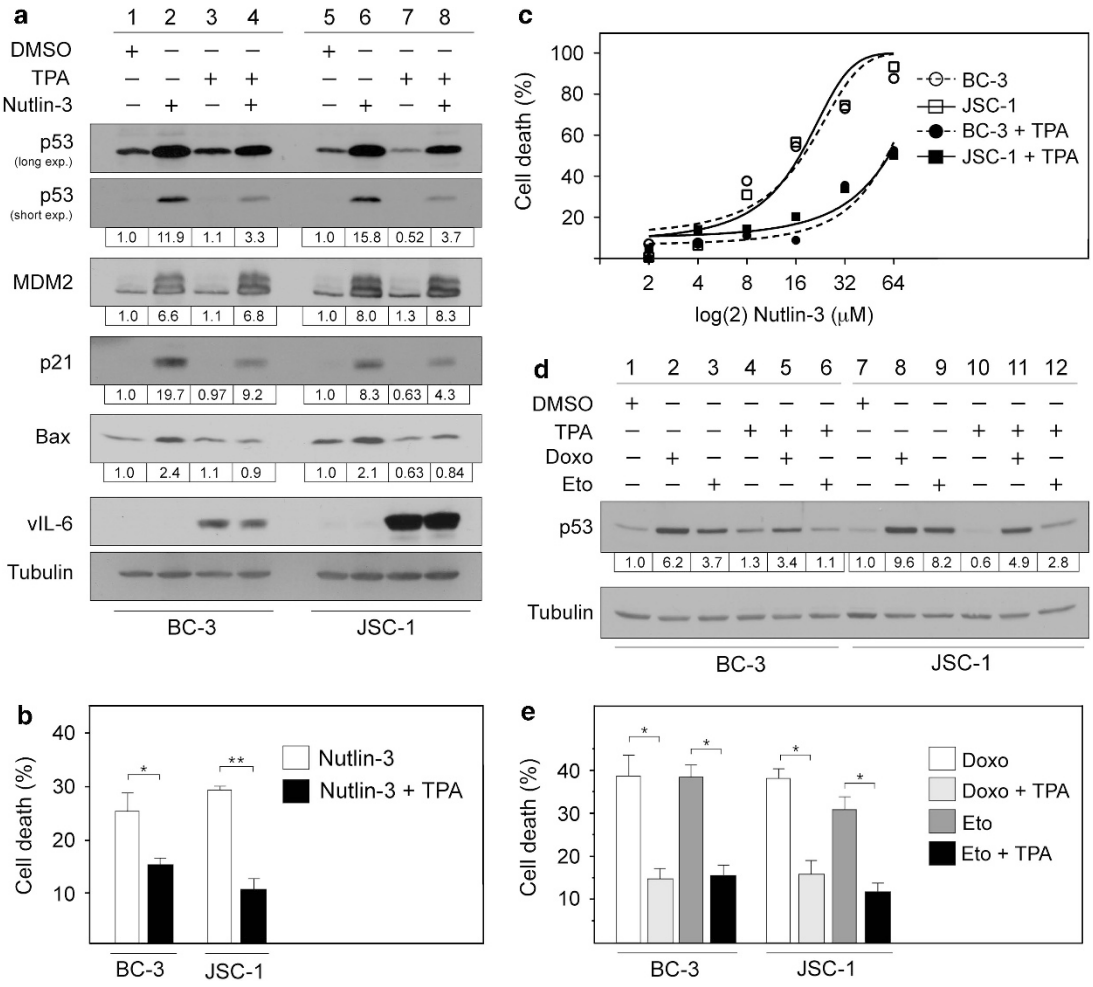

Figure 3. Nutlin-3-induced p53 stabilization and cytotoxicity were attenuated in TPA-treated PEL cells. (**a**) BC-3 and JSC-1 cells were pretreated with vehicle (DMSO) or TPA (20 ng/ml) to induce viral replication for 18 h followed by incubation in the presence or absence of Nutlin-3 (7 μM) for additional 6 h. Whole-cell extracts were separated by SDS–PAGE followed by immunoblotting with antibodies against p53 (short and long exposures), MDM2, p21, Bax, vIL-6 and tubulin. BC-3 and JSC-1 cells were pre-incubated with DMSO or TPA as in (**a**). Cells were then treated with 7 μM (**b**) or with a series (**c**) of Nutlin-3 concentrations ranging from 0 to 64 μM. Cell death was assessed by trypan blue exclusion at 48 h of the treatment. Values represent the percentage of dead cells induced by Nutlin-3 treatment. The percentage of dead cells in the DMSO control was subtracted as a background. (**d**) BC-3 and JSC-1 cells were pretreated with DMSO or TPA as in (**a**) and then incubated with 1 μM doxorubicine (Doxo) or 10 μM etoposide (Eto) for 24 h. Whole-cell extracts were separated by SDS–PAGE followed by immunoblotting with anti-p53 antibodies. Tubulin served as a loading control. (**e**) Cells described in (**d**) were assayed for cell death by trypan blue exclusion. The percentage of dead cells in the DMSO control was subtracted as a background. Results are presented as the mean of two independent experiments ± s.d. $^{*}P<0.05$; $^{**}P<0.01$, Student's *t*-test.

To recapitulate the attenuation of the p53-dependent apoptosis observed in the non-responder mice in cell culture, we pretreated BC-3 and JSC-1 cells with TPA for 18 h to induce lytic replication, followed by treatment with Nutlin-3 in the presence of TPA for 48 h. Figure 3b shows that induction of lytic replication led to a significant decrease (41 and 64%) in the apoptotic response to the Nutlin-3 treatment in BC-3 and JSC-1 cells, respectively. Moreover, induction of viral replication dramatically reduced the IC_{50} of Nutlin-3 at 48 h in BC-3 (17.8–58.2 μM) and JSC-1 (16.9–59.9 μM) cells (Figure 3c).

Induction of lytic replication compromises p53-dependent apoptosis by genotoxic agents

Next, we assessed if viral lytic replication in PEL cells would attenuate p53-dependent apoptosis induced by genotoxic anticancer agents routinely used for the treatment of PEL.[26,27] To this end, TPA-induced BC-3 and JSC-1 cells were treated with doxorubicin and etoposide, at doses previously shown to induce p53-mediated apoptotic response in PEL cells.[28,29] Western blot analysis of the doxorubicin- and etoposide-treated BC-3 and JSC-1 cells revealed about 45 and 70% reduced levels of the stabilized p53, respectively, in the TPA-induced lytic cells over the DMSO-treated latent PEL cells (Figure 3d, lanes 2 and 3 vs 5 and 6 and lanes 8 and 9 vs 11 and 12). Furthermore, as determined by the trypan blue exclusion, induction of lytic replication in both BC-3 and JSC-1 cells led to a significantly impaired apoptotic response to etoposide and doxorubicin (Figure 3e). Taken together, these results suggest that induction of viral lytic replication compromises p53-dependent apoptosis regardless of the mechanism that contributes to activation of p53.

Lytic replication hampers apoptotic response to p53-restoration regardless of the mechanism of viral reactivation

To investigate further the emerging link between the impaired apoptotic response to Nutlin-3 treatment and viral lytic replication, we induced viral replication by tetracycline treatment in a BCBL-1-derived cell line (BCBL-1 TREx-RTA) with inducible ORF50/RTA expression.[30] ORF50/RTA induction resulted in viral replication in 53% of the cells as determined by staining with an early lytic marker ORF59 (data not shown). These cells were treated with 15 μM Nutlin-3 and analyzed by immunofluorescence and western blotting. We used higher Nutlin-3 concentration since in the BCBL-1 cell line growth inhibition is compromised at low doses of Nutlin-3 due to the heterozygous mutant *TP53* allele.[31] Similar to the TPA-induced PEL cells, stabilization of the p53 levels by Nutlin-3 was compromised in the reactivated BCBL-1 TREx-RTA cells (Supplementary Figures S4a and S4b). Lytic replication also led to a reduced p53 levels (Supplementary Figure S4b) and markedly impaired Nutlin-3-induced cell death (Supplementary Figure S4c).

To explore whether lytic replication induced by hypoxia (a more physiological manner than TPA induction) could attenuate apoptosis by p53 reactivation, BC-3 and JSC-1 cells were cultured under hypoxic conditions (1% O_2) for 96 h, and analyzed for viral reactivation by immunofluorescence for ORF59. When the number of ORF59-positive cells was quantified, a 7- to 8-fold increase in the ORF59-positive cells (Supplementary Figure S5a), and induction of vIL-6 (Supplementary Figure S5b, lane 3) were observed in the hypoxia-treated cells compared with the cells cultured under normoxic control environment (21% O_2), reflecting an efficient induction of lytic replication. To study the cytotoxicity of Nutlin-3 in hypoxia-reactivated BC-3 and JSC-1 cells, we grew them under hypoxic conditions for 18 h followed by Nutlin-3 co-treatment for 48 h. In accordance with the data obtained with TPA, about sixfold decrease in the Nutlin-3-induced cytotoxicity was observed in the hypoxia-reactivated BC-3 and JSC-1 cells, whereas treatment of the KSHV-negative lymphoblastoid cell line (CZE) with Nutlin-3 under hypoxia had no effect on cell death (Figure 4a). Similar to lytic induction by TPA or the inducible RTA, reduced p53 levels were detected when viral lytic replication was induced by hypoxia (Supplementary Figure S5b, lane 4), and the basal level of p53 was lower in hypoxia than in cells grown under normoxic conditions (Supplementary Figure S5b, lane 3).

Inhibition of viral replication restores Nutlin-3 cytotoxic activity in reactivated PEL cells

Next, we studied whether inhibition of viral replication by two widely used antiviral drugs could restore the apoptotic potential of Nutlin-3 in hypoxia-reactivated PEL cells. First, we confirmed the efficacy of ganciclovir and cidofovir (GCV and CDV)[32] to inhibit KSHV replication. As shown in Figure 4b, treatment of JSC-1 cells with GCV or CDV reduced the expression of late lytic genes K8.1 and ORF25 by ~40 and 60%, respectively. Then, we treated JSC-1 cells cultured under hypoxia with GCV, CDV or with the vehicle control for 2 days followed by co-treatment with Nutlin-3 and GCV or CDV for 3 more days. Intriguingly, we found that inhibition of the hypoxia-induced viral replication by both GCV and CDV in JSC-1 cells resulted in restoration of the p53 accumulation (Supplementary Figure S6) and Nutlin-3-induced cell death (Figure 4c). Davis *et al*.[33] reported that GCV becomes toxic to PEL cells when the cells are exposed to hypoxia. We, however, did not detect any increase in cell death in PEL cells treated with GCV or CDV under hypoxia (data not shown). This apparent discrepancy may be due to the high concentration of GCV (100–500 μM) used by Davis *et al*.[33] as compared with 5 μM used in our study. These results provide further support that KSHV viral replication compromises Nutlin-3-induced cytotoxicity in PEL cells.

Although GCV and CDV restored Nutlin-3 apoptotic potential; they both act at a late stage of the lytic cascade by inhibiting the viral DNA polymerase.[34,35] Spontaneous viral reactivation leads to expression of early lytic gene products that include chemokines and pro-inflammatory cytokines that could potentially contribute to tumor cell microenvironment (reviewed in Nicholas[36]). We have recently demonstrated that both Pim 1 and Pim 3 kinases are critical regulators of the KSHV lytic replication, and that silencing of these kinases by RNAi leads to inhibition of the lytic replication cascade at an early stage.[37,38] To examine whether inhibition of viral lytic replication by Pim inhibition could restore the apoptotic response to Nutlin-3, we treated TPA-induced PEL cells with a selective Pim kinase inhibitor DHPCC-9.[39,40] The efficacy of DHPCC-9 to inhibit induction of viral lytic replication was first addressed by immunofluorescence for the early lytic marker ORF59. When the number of ORF59-positive cells was quantified, a maximum of 50% inhibition of reactivation was obtained with 5 μM DHPCC-9, whereas higher doses of the inhibitor led to unspecific toxicity in the cells (Supplementary Figure S7). Pretreatment of BC-3 cells with 5 μM of the DHPCC-9 for 4 h followed by co-treatment with TPA (20 ng/ml) for an additional 24 h resulted in a 68% decrease in the expression ORF50/RTA protein in the lytically reactivated cells over the cells treated with vehicle (DMSO) alone (Figure 4d, lanes 7 and 9). As expected, co-treatment of TPA and CDV had only a moderate effect on the expression of ORF50/RTA, since CDV inhibits viral replication at a later stage of the cascade (Figure 4d, lanes 7 and 11). Following TPA treatment, the DHPCC-9, CDV or DMSO pretreated BC-3 cells were co-incubated with Nutlin-3. Western blot analysis revealed restoration of the p53 protein levels in the TPA-induced, Nutlin-3-treated PEL cells when reactivation was inhibited by either CDV or the Pim kinase inhibitor DHPCC-9 (Figure 4d, lane 8 vs lanes 10 and 12). Furthermore, suppression of the lytic gene expression by the Pim kinase inhibitor not only restored the Nutlin-3-induced apoptosis but also increased it from 30 to 36%, which was 25% more efficient than that obtained with CDV (Figure 4e). These

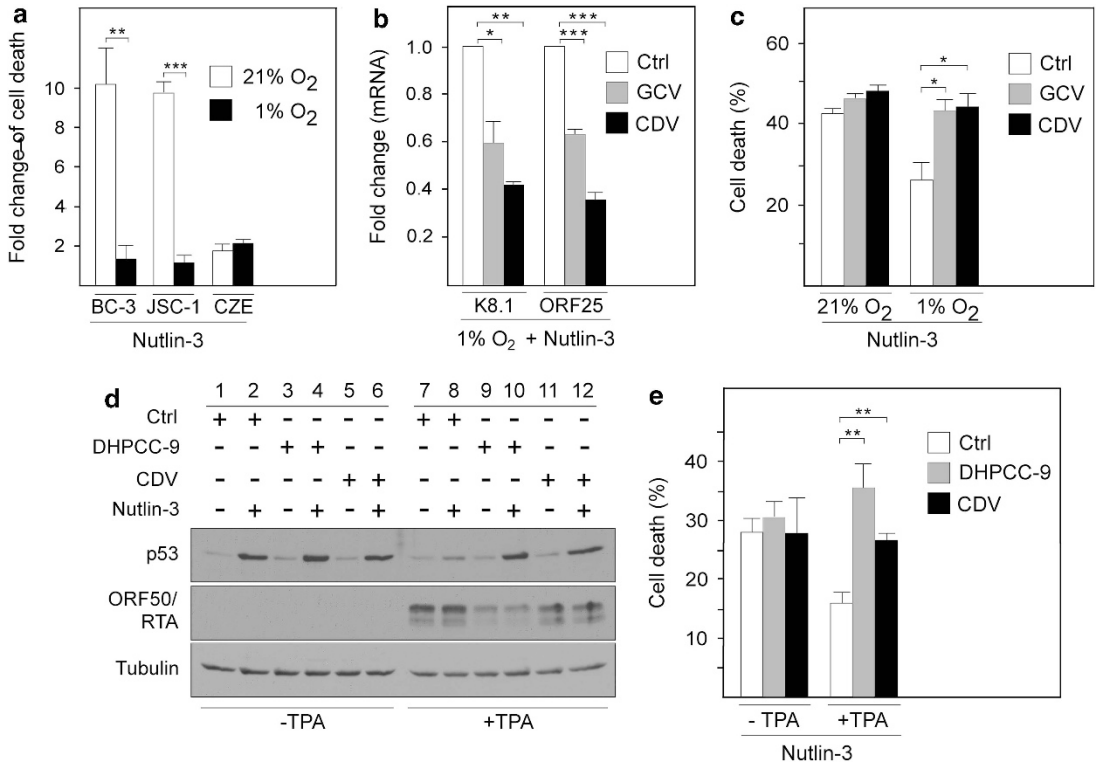

Figure 4. Inhibition of viral lytic replication restored apoptosis by p53 reactivation. (**a**) KSHV-infected PEL cells (BC-3 and JSC-1) and an EBV-transformed lymphoblastoid cell line (CZE) were pre-incubated at normoxia (21% O_2) or hypoxia (1% O_2) for 18 h followed by treatment with 7 μM Nutlin-3. Cell death was assessed by trypan blue exclusion at 48 h of the treatment. The graphs represent data from two separate experiments ± s.d. (**b**) JSC-1 cells were exposed to hypoxia for 48 h in the absence or presence of GCV (5 μM) or CDV (10 μM). Cells were co-treated with 7 μM Nutlin-3 and GCV or CDV for 72 h, and total RNA was assayed for the expression of late lytic transcripts K8.1 and ORF25 by qRT–PCR. Transcripts of human β-actin served as an endogenous control. The level of the lytic transcripts in GCV- or CDV-treated cells (GCV; CDV) was normalized to that in the control cells (Ctrl), which was set to one. The graphs represent data from two independent experiments ± s.d. (**c**) Quantification of cell death in JSC-1 cells treated as in (**b**), was assessed by trypan blue exclusion. The percentage of dead cells in the DMSO control was subtracted as a background. (**d**) BC-3 cells were pre-incubated with 5 μM Pim kinase inhibitor (DHPCC-9), 10 μM CDV or vehicle (DMSO) for 4 h followed by co-treatment with TPA (20 ng/ml) for 18 h. Next, cells were treated with 7 μM Nutlin-3 for additional 6 h. Cells were harvested and the whole-cell extracts were separated by SDS–PAGE followed by immunoblotting with antibodies against p53 and ORF50/RTA. Tubulin served as a loading control. (**e**) BC-3 cells were pretreated with DMSO, CDV or DHPCC-9 in the presence or absence of TPA as in (**d**). Cells were then treated with Nutlin-3 (7 μM), and cell death was determined by trypan blue exclusion at 48 h of the treatment. Values represent the percentage of dead cells induced by Nutlin-3 treatment. The percentage of dead cells in the DMSO control was subtracted as a background. Each value represents the mean of three independent experiments ± s.d. $*P<0.05$; $**P<0.01$; $***P<0.001$, Student's *t*-test.

results support the importance of inhibition of viral reactivation at the early stage in restoration of the Nutlin-3 efficacy.

Decrease in the p53 protein level in lytic PEL cells is proteasome-mediated

The p53 tumor suppressor is a short-lived protein whose stability is primarily regulated by the MDM2-mediated proteasomal degradation.[8] To examine the mechanism of the attenuated stabilization of p53 in response to Nutlin-3 upon viral lytic replication, we treated the cells with a 26S proteasome inhibitor, MG132. As proteasome inhibitors such as bortezomib have been suggested to induce lytic replication,[41] we first ruled out that MG132 would trigger lytic replication at the dose used in our study (Supplementary Figure S8a). Next, JSC-1 cells were incubated either under normoxia or hypoxia with a vehicle control (MG132−) or with 1 μM MG132 (MG132+). As shown in Supplementary Figure S8b, MG132 treatment in hypoxia (lane 7) resulted in a 8.3-fold increase in the total p53 level compared with the vehicle (lane 3), suggesting that the decrease in p53 protein levels in the lytically reactivated PEL cells was proteasome-mediated. Of note, treatment with Nutlin-3 in the presence of MG132 did not result in additional increase in the total p53 level (lane 8), probably due to already substantial inhibition of p53 degradation.

DISCUSSION

The present study shows a critical novel finding concerning the future therapeutic use of p53 reactivation in KSHV lymphomas by demonstrating that spontaneous induction of viral lytic replication in the tumors drastically attenuated the Nutlin-3-induced p53-dependent apoptotic response. Attenuation of the cell death response in the lytic PEL cells was not restricted to p53 reactivation by the MDM2 inhibitor Nutlin-3, but was also observed upon induction of apoptosis by genotoxic agents. Importantly, the undesired resistance was overcome and the sensitivity of cells to Nutlin-3-induced apoptosis was restored by inhibition of viral replication at early phase by Pim kinase inhibitor[39,40] or by suppression of late stages of the lytic cascade with well-known anti-herpes agents, GCV or CDV.[32]

Although majority of the PEL cells are latently infected with KSHV, and only a limited set of viral genes is expressed,[42] a spontaneous transition from latent phase to lytic replication can occur, leading to expression of lytic gene products including chemokines and pro-inflammatory agents/factors (reviewed in Ganem[43]).

Hypoxia has been reported to reactivate KSHV in PEL cells, and to potentiate reactivation of KSHV by TPA.[23] Indeed, we demonstrate here a dramatic increase in the mRNA levels of CA9, a hypoxia marker, in the ascites of the mice refractory to the Nutlin-3 treatment. Therefore, it seems that the microenvironment in the PEL IP tumors becomes hypoxic with increased tumor mass resulting in spontaneous induction of viral lytic replication. Interestingly, inhibition of recurrence of pleural effusions, either alone or with adjunctive low-dose chemotherapy or HAART, has been described to date for both GCV and CDV.[44–46] The findings of this study may be particularly important if p53 restoration is to be considered as a future therapeutic modality for the MCD. MCD represents another devastating lymphoproliferative syndrome, where all MCD lesions in the HIV-seropositive patients are KSHV-positive, whereas only about half of the HIV-negative ones are infected with KSHV (reviewed in Ablashi *et al.*[1]). KSHV-associated MCD has a significantly higher proportion of lytic cells than PELs, and is characterized by periodic KSHV lytic replication with high viremia in the peripheral blood.[47]

Taken together, our findings demonstrate the importance of understanding the particular features of the disease in question; that is, in the case of PEL, the potential of predominantly latent virus to reactivate and reveal an unexpected resistance to the treatment. The data presented here implicate viral lytic replication as a factor, which can hamper the otherwise efficient apoptotic response of p53 restoration *in vivo*. The results further demonstrate that the attenuated apoptotic response is due to proteasomal degradation of p53 upon induction of lytic replication most likely endorsed through paracrine mechanisms (our unpublished data). It would thus be important to monitor the viral replication phase in the tumors of PEL patients to avoid undesired resistance to treatment and to design better treatment modalities where inhibition of paracrine loops induced by viral reactivation would be combined with specific therapies such as the MDM2 inhibitors.

MATERIALS AND METHODS

Cells and treatments

BC-3/NF-κB-luc[16] and BC-3 PEL cell lines were kindly provided by Dr Cesarman (Cornell Medical College, New York, NY, USA). CZE and IHE (p53 wt) Ebstein-Barr virus (EBV)-transformed lymphoblastoid cells were grown as detailed earlier.[15] JSC-1 PEL lymphoma cell line was from ATCC (Manassas, VA, USA). Tetracycline-inducible BCBL-TREx-RTA cells were kindly provided by Dr Jung (University of Southern California, Los Angeles, CA, USA). The lymphoma cell lines were cultured as previously described.[15,30] KSHV reactivation was induced as described earlier for the BC-3 and JSC-1 cells.[48] BCBL-1 TREx-RTA cells were induced with tetracycline as described.[30] Hypoxia studies were performed by incubating cells in an incubator containing 1% O_2 and 5% CO_2 (INVIVO 400 Workstation; Ruskinn Technology Ltd, Bridgend, UK). Inhibition of lytic gene expression was carried out with 5 μM GCV (Sigma, St Louis, MO, USA) or 10 μM CDV (Gilead Sciences Inc., Foster City, CA, USA). Selective inhibition of the Pim family kinases was carried out by treatment of BC-3 cells with 5 μM of small-molecule compound 1,10-dihydropyrrolo[2,3-a]carbazole-3-carbaldehyde (DHPCC-9).[39,40]

Treatment of cultured cells with Nutlin-3 or genotoxic drugs

PEL cell lines suspended in the growth medium at 3×10^5 cells/ml were incubated with 7 μM Nutlin-3 (Cayman Chemical, Ann Arbor, MI, USA), 1 μM doxorubicin (Sigma), 10 μM etoposide (Sigma) or the solvent (vehicle; 0.1%. DMSO; Sigma) for the indicated time points. Cell death was determined by trypan blue exclusion assay (Sigma).

In vivo studies

IP PEL tumors were established by implanting the BC-3/NF-κB-luc cells to female SCID and NOD/SCID mice (Taconic Europe) as previously described.[16] To monitor the tumor growth, D-luciferin (SYNCHEM OHG, Felsberg, Germany) in phosphate-buffered-saline (PBS) (100 mg/kg) was injected intraperitoneally and mice were imaged using the IVIS Imaging system (Xenogen, Alameda, CA, USA). Data were analyzed with The Living Image software (version 3.1, Xenogen, Alameda, CA, USA). For quantitative analysis, the intensity of the signal was measured as total photon/s/cm^2/steradian (p/s/cm^2/sr). In all, 20–40 mg/kg Nutlin-3 (Cayman Chemical) or the vehicle control (DMSO) was administered intraperitoneally every second day. Animal studies were conducted according to the guidelines of the Provincial Government of Southern Finland, and approved by the Experimental Animal Committee.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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