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Disruption of ATR Signaling by Epstein–Barr Virus Latent Membrane Protein 1 Sensitizes Nasopharyngeal Carcinoma Cells to Cisplatin

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Keywords: DNA damage response signaling | Epstein–Barr virus | latent membrane protein 1 | nasopharyngeal carcinoma

ABSTRACT

Nasopharyngeal carcinoma (NPC) occurs with high incidence in Southeast Asia where almost all tumors are associated with Epstein–Barr virus (EBV) infection. Cisplatin is used in combination chemotherapy. In this study, we determined that the EBV oncoprotein, latent membrane protein 1 (LMP1), perturbs DNA damage response (DDR) signaling, activation of cell cycle checkpoints, and sensitivity to cisplatin in NPC cells (HK1). Hypersensitivity was validated by LMP1 knockdown and CRISPR/Cas9 targeting in HK1-EBV cells with latent EBV infection. The conserved PxQxT motif (in CTAR1) and Y384 residue (in CTAR2) were required for the hypersensitivity. Inhibition of ATR (VE821 or AZD6738), but not ATM (KU55933 or AZD0156), phenocopied the G1 arrest and hypersensitivity. Attenuation of DDR signaling and hypersensitivity by LMP1 or ATR inhibition was also observed in the C17 NPC cell line with restored stable LMP1 expression. LMP1 expression in NPC tumors is highly variable. Publicly available RNA-sequencing data from microdissected NPC tumors showed that LMP1 expression in the primary tumors was the lowest in cisplatin-treated patients that experienced recurrence. These findings could have clinical significance in stratifying NPC patients such that tumors with limited or variable LMP1 expression might benefit from ATR inhibitor therapy.

1 | Introduction

Nasopharyngeal carcinoma (NPC) is a head and neck cancer with a global incidence of over 170 000 cases/year that is endemic to Southeast Asia and Southern China [1]. Although EBV-infected nasopharyngeal epithelial cells are rarely detected, almost all endemic NPCs are associated with latent and clonal infection with Epstein–Barr virus (EBV) [2]. EBV immortalizes primary B-cells, but the infection or expression of viral genes is not sufficient to confer immortalization in epithelial cells [3, 4]. Multiple risk factors are thought to be involved including EBV infection, virus and host genetics, and the consumption of dietary carcinogens such as nitrosamines [5]. The genetic landscape of NPC is complex but may converge on immune escape and conditions that favor EBV persistent infection [6]. EBV reactivation can contribute to genomic instability [7], however, latent infection and the expression of Type II latency transcripts can also interfere with DNA repair [7–12]. EBV latent infection in nasopharyngeal cells fine tunes the histone bivalent switch (reduced activating H3K4me3 and increased suppressive

H3K27me3) such that a number of DNA damage repair genes are repressed [8, 10, 13–16]. Furthermore, expression of EBV latent membrane protein 1 (LMP1) is associated with the inhibition of DNA repair and hypersensitivity to cisplatin [10, 13–16]. Interestingly, NPC tumors express variable levels of LMP1 which can differ within and between tumors [17, 18]. LMP1 could therefore sensitize an NPC tumor to DNA-damaging agents but tumors with low or heterogeneous levels of LMP1 may escape cisplatin cytotoxicity, which could increase the risk of cancer recurrence.

LMP1 is a constitutively active CD40 signaling mimic that promotes oncogenic mechanisms through interaction with tumor necrosis factor receptor-associated factors (TRAFs) [18]. The N-terminal transmembrane domains are required for homo-oligomerization and localization to lipid rafts [19]. The C-terminus encodes three signaling domains known as C-terminal activation regions (CTARs) which are conserved across all LMP1 strain variants [20, 21]. High physiological levels of LMP1 have been associated with G1 cytostatic arrest, which has been observed by induced expression in B-cells and epithelial cells [22, 23]. In serially passaged cells, stable tolerable expression of LMP1 has been shown to promote cell cycle progression through inactivation of Rb mechanisms [22, 24, 25]. Thus, the impairment of the DNA damage response (DDR), or the activation of cell cycle checkpoints, could influence cellular sensitivity and the recovery from DNA-damaging agents. While LMP1 is known to inhibit the repair of UV- and bleomycin-induced DNA damage which primarily results in the cumulation of DNA bulky adducts and strand breaks, it is not clear how LMP1 sensitizes cells to the DNA cross-linker, cisplatin [10, 15, 16].

In this study, the effect of LMP1 on cisplatin-induced activation of cell cycle checkpoints and DDR signaling was investigated. In this study with HK1 (with and without EBV infection) and C17 cells (with EBV infection), we show that LMP1 increases sensitivity to cisplatin with attenuated ATR signaling. While the bulk NPC tumor is frequently sensitive to cisplatin, the risk of relapse in up to 36% of patients indicates that refractory cells remain [26]. Thus, the inhibition of ATR could be considered for patients with NPC tumors exhibiting negligible or heterogeneous levels of LMP1.

2 | Materials and Methods

2.1 | Generation of Cell Lines

The origin and culture conditions for the HK1, HK1-EBV, and C17 cell lines are described in Supporting Information: Materials and Methods. To avoid variability from random selection of a few cell clones, all lentiviral inducible and retroviral stable cell lines were generated from pooled cells that survived selection and were maintained in selection. HK1 LMP1 inducible cell lines were created by lentivirus transduction. Lentiviruses expressing LMP1 or LMP1 mutants were packaged in 293T cells cotransfected with the packaging plasmids pMD2.G and psPAX2 (Addgene #12259, #12260) and the pLVX-TRE3G (neo^R, Clontech) transactivator plasmid or the pLVX-TRE-tightBI ($puro^R$) responsive plasmid containing the gene of interest. HK1 cell lines were selected and maintained in 800 μg/mL G418 and 1 μg/mL puromycin. Doxycycline-inducible LMP1 expression was tested using increasing concentrations of doxycycline (0, 10, 50, or 100 ng/mL) for 48 h, followed by immunoblot analysis of whole cell extracts with anti-HA antibodies. Doxycycline was refreshed every 48 h for all assays with longer time points. The HK1-EBV CRISPR/Cas9 sgLMP1 cell line was created by lentiviral transduction and puromycin selection as previously described [27]. HK1-EBV LMP1 shRNA (shLMP1) and LMP1 (China1) cell lines, and the C17 pBabe and LMP1 (Ch1 strain) stable cell lines were created by retroviral transduction as previously described [28, 29], and maintained in 1 μg/mL puromycin. Parental and stable cell lines were authenticated by STR profiling using the Promega PowerPlex16HS Assay at the University of Arizona Genetics Core. All cell lines were performed with mycoplasma-free cells as determined by PCR.

3 | Results

3.1 | LMP1 Disrupts Cell Cycle Progression by Promoting G1 Arrest

LMP1 (B95-8 strain)-inducible cell lines were established in the "HK1" NPC cell line. HK1 is derived from an NPC tumor and is devoid of HeLa contaminant that is often found in other NPC cell lines [30]. Parental HK1 cells were stably transduced with a doxycycline-inducible LMP1 fused to a N-terminal HA-tag which reached maximal induction by 50–100 ng/mL of doxycycline (Supporting Information: Figure S1A). We reasoned that cells expressing cytostatic levels of LMP1 would be outcompeted in serial passage. Thus, checkpoint-arrested cells would only be revealed by inducible expression of LMP1.

We first established the cell cycle effects of LMP1 in the absence of induced DNA damage. HK1 cells expressing inducible LMP1 were treated with the CDK1 ATP-competitive inhibitor (CDK1i) RO3306. Treatment with RO3306 synchronizes cells in G2–M and any cells arrested in G1 or S phase would become apparent [31]. In vehicle control (DMSO)-treated cells, only a fraction of cells was in G2–M (21%–24%, Supporting Information: Figure S1B). Treatment with RO3306 resulted in a large proportion (57%) of HK1 vector control cells synchronized in G2–M, with only 4% of cells remaining in G1 (Supporting Information: Figure S1B). However, 11% of the LMP1-induced cells remained in G1, compared to the 3% of cells in G1 without doxycycline (Supporting Information: Figure S1B). LMP1 upregulated the expression of the G1–S checkpoint protein p21, without activation of the DDR signaling proteins (pATR, pChk1, pChk2, p-p53[S15]) or induction of the CDK inhibitor proteins, p27 and p18 (Supporting Information: Figure S1A). However, p21 and PUMA (a Bcl-2 antagonist that can be activated in a p53-dependent or -independent manner [32]) transcripts were upregulated by LMP1 (Supporting Information: Figure S1C). Interestingly, expression of p53 and its transcriptional target MDM2 were not affected (Supporting Information: Figure S1A,C). These results indicate that in the absence of induced DNA damage, LMP1 promotes G1 arrest in a p53-independent manner. To avoid potential cytotoxicity from chronic LMP1 expression, subsequent experiments in HK1 were conducted by induced expression of LMP1 or by knockdown in EBV-infected cells.

3.2 | LMP1 Disrupts Cisplatin-Induced Cell Cycle Arrest

Disruption of cell cycle checkpoints could increase sensitivity to genotoxins. Cisplatin primarily induces intrastrand DNA cross-links which can occur at all phases of the cell cycle [33, 34]. To explore if LMP1 affects checkpoint activation and cell cycle arrest in response to cisplatin, HK1 LMP1-inducible cells were analyzed for cell cycle profiles before and after treatment for 72 h, the time required to fully arrest HK1 cells in S and G2–M. Several cisplatin doses were tested (inhibitory concentrations: IC50, IC70, and IC90) but only the high cytotoxic dose (7 μM, IC90) resulted in complete arrest in S and G2–M, with only 3%–5% of cells remaining in G1 (Figure 1A, left panel). In contrast, 44% of the LMP1-induced cells remained in G1, compared to 4% without doxycycline (Figure 1A, right panel). This increase in the G1 population correlates in a dose-dependent manner with LMP1 induction (Figure 1B).

Cells exposed to cisplatin could arrest in the first cell division or later generations after accumulating DNA damage, which may be manipulated by LMP1. To distinguish between these possibilities, cell division was monitored with the CellTrace dye (CFSE) in which the mean fluorescence intensity (MFI) is reduced to half with every cell division. HK1 LMP1 cells not exposed to cisplatin underwent five divisions during a period of 72 h, regardless of doxycycline induction (Figure 1C). However, LMP1 cells treated with 7 μM (IC90) of cisplatin only underwent three divisions, but this did not differ in the presence or absence of doxycycline (Figure 1C). This indicates that the aberrant LMP1-induced G1 arrested population undergo an equivalent number of cell divisions. Given that the CFSE distribution is equivalent and unimodal in the LMP1 condition, we conclude that the aberrant DNA damage-induced G1 population did not experience checkpoint escape.

3.3 | Cisplatin-Treated LMP1-Expressing Cells Display Low Levels of γH2AX

Cell cycle arrest in G1 can be cytoprotective if DNA repair mechanisms are intact [35]. To examine if LMP1-induced G1 arrest protects cells from cisplatin-induced DNA damage, LMP1-inducible HK1 cells were treated with cisplatin in the presence or absence of doxycycline, and costained for the DNA damage marker γH2AX and propidium iodide. The MFI values for γH2AX were compared for cells in G1 and G2–M. In both HK1 vector control and LMP1 cells not exposed to cisplatin, the level of γH2AX correlates with the DNA content such that the MFI for cells in G2–M (with DNA content "2n") is double that

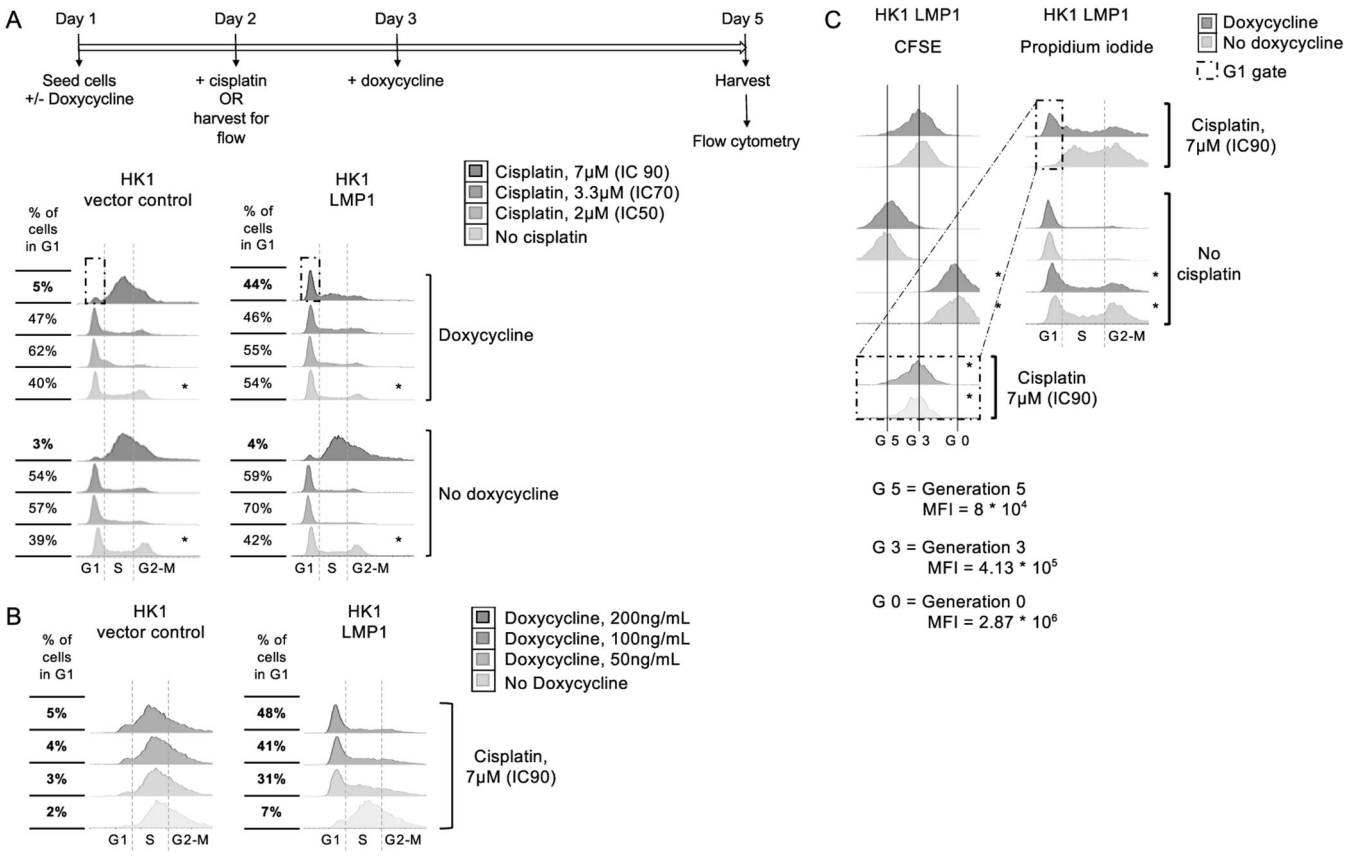


FIGURE 1 | LMP1 disrupts cisplatin-induced cell cycle arrest. (A) HK1 cells treated with doxycycline (50 ng/mL, 24 h) to induce LMP1 expression were exposed to increasing doses of cisplatin for 72 h and harvested for cell cycle analysis. (B) HK1-inducible cell lines were treated with increasing doses of doxycycline and analyzed for cell cycle profiles after exposure to cytotoxic levels of cisplatin (IC90, 7 μM) for 72 h. (C) HK1 cells expressing LMP1 (50 ng/mL doxycycline) with or without cisplatin treatment were analyzed for cell division and cell cycle profiles with the CFSE cell tracker dye and propidium iodide costaining. The mean fluorescence intensity (MFI) value for each cell division is indicated for Generation 0 (G0), G3, and G5. *Cells harvested at the start of the assay, before exposure to cisplatin.

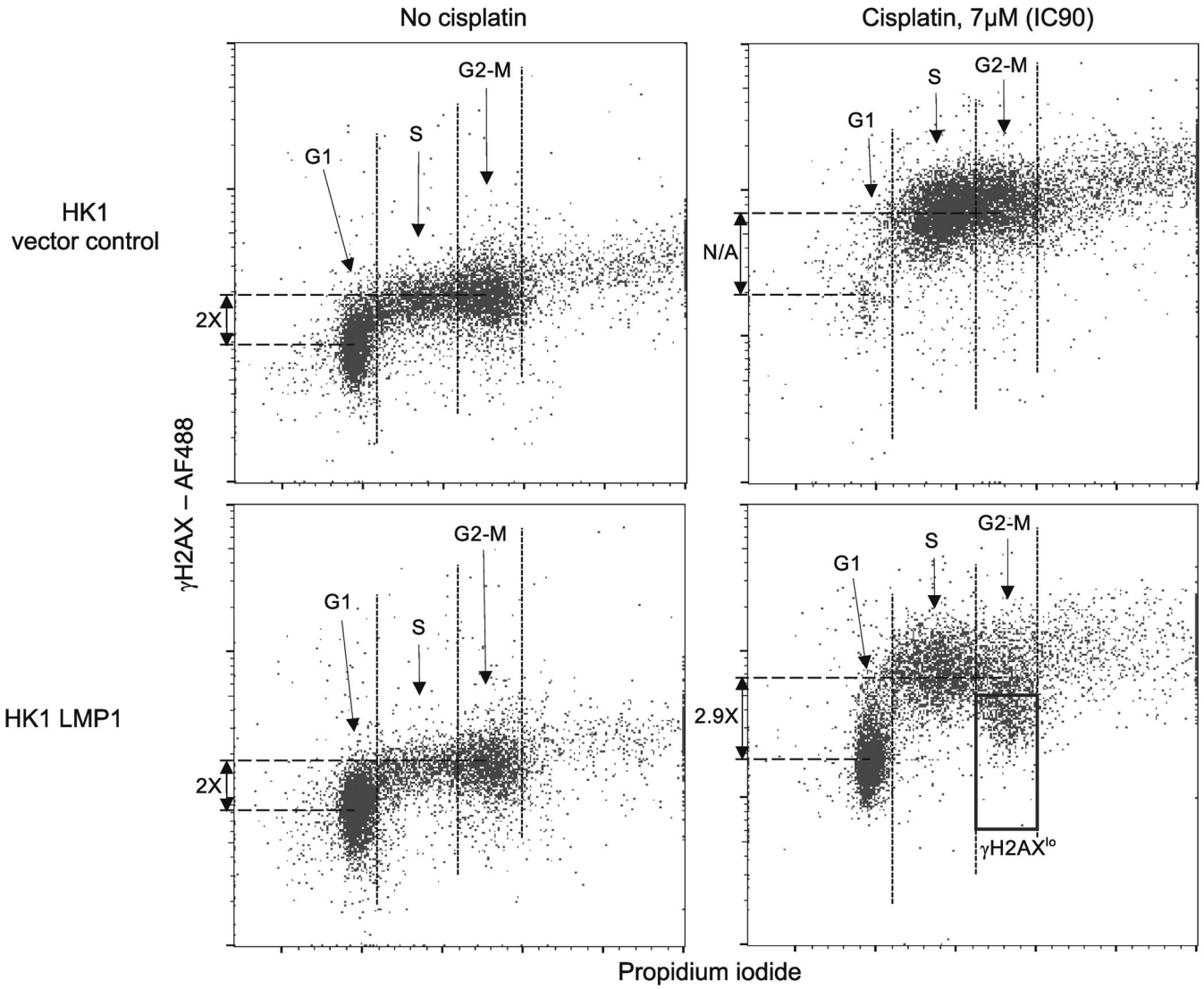


FIGURE 2 | Cells arrested in G1 show lower levels of the DNA damage marker γH2AX. HK1 vector control and LMP1-expressing cells (50 ng/mL doxycycline) were analyzed for γH2AX levels in different phases of the cell cycle, after exposure to 7 μM (IC90) of cisplatin for 72 h. The discontinued lines mark the mean fluorescence intensity for cells in G1 or G2–M. *x* and *y* axes are set to the same scale for all plots. N/A (top right panel), HK1 vector control cells treated with cisplatin do not yield a G1 population and therefore a change in MFI could not be determined.

of cells in G1 (with DNA content "n") (Figure 2, left panels). As expected, the overall γH2AX signal increased in all cells upon treatment with cisplatin (Figure 2, right panels). However, LMP1-expressing cells showed an additional G2–M subpopulation that expressed low levels of γH2AX (γH2AX^{lo}). In cisplatin-treated LMP1-expressing cells where a distinct G1 population could be identified, the level of γH2AX no longer correlated precisely with DNA content such that the difference in γH2AX signal in G1 versus G2–M cells was 2.9-fold (Figure 2, lower right panel). These data indicate that LMP1-expressing cells have low γH2AX.

3.4 | LMP1 Attenuates DNA Damage Signaling in Response to Cisplatin

Reduced γH2AX could result from less DNA damage or a failure to recognize DNA damage. To distinguish between these possibilities, cisplatin-treated cells were evaluated for DDR signaling and for the removal of DNA-cisplatin adducts immediately after treatment and during the recovery period (Figure 3). In vector control cells, DDR signaling proteins (ATR, ATM, Chk1, and Chk2), and ATM/ATR substrates ([pS/pT]QT motif) were activated with cisplatin treatment and remained activated to some degree up to the 5 days recovery. Similarly, the DNA damage-responsive p53 (S15) was phosphorylated upon cisplatin treatment, but the signal resolved in the recovery period. Interestingly, the p-p53(S15) and total p53 levels were consistently lower across all recovery times in LMP1-expressing cells. The DNA damage markers γH2AX and pRPA32 spiked upon exposure to cisplatin, then decreased in 1–3 days of recovery. Strikingly, all the DDR signaling proteins evaluated were downregulated in LMP1-expressing cells (Figure 3A). The efficient removal and repair of cisplatin-DNA adducts could explain the resolution of DDR signaling. However, the removal of platinum moieties from genomic DNA was less efficient in LMP1-expressing cells at the end of the recovery period (Figure 3B, $p = 0.0003$). Taken together, these results indicate

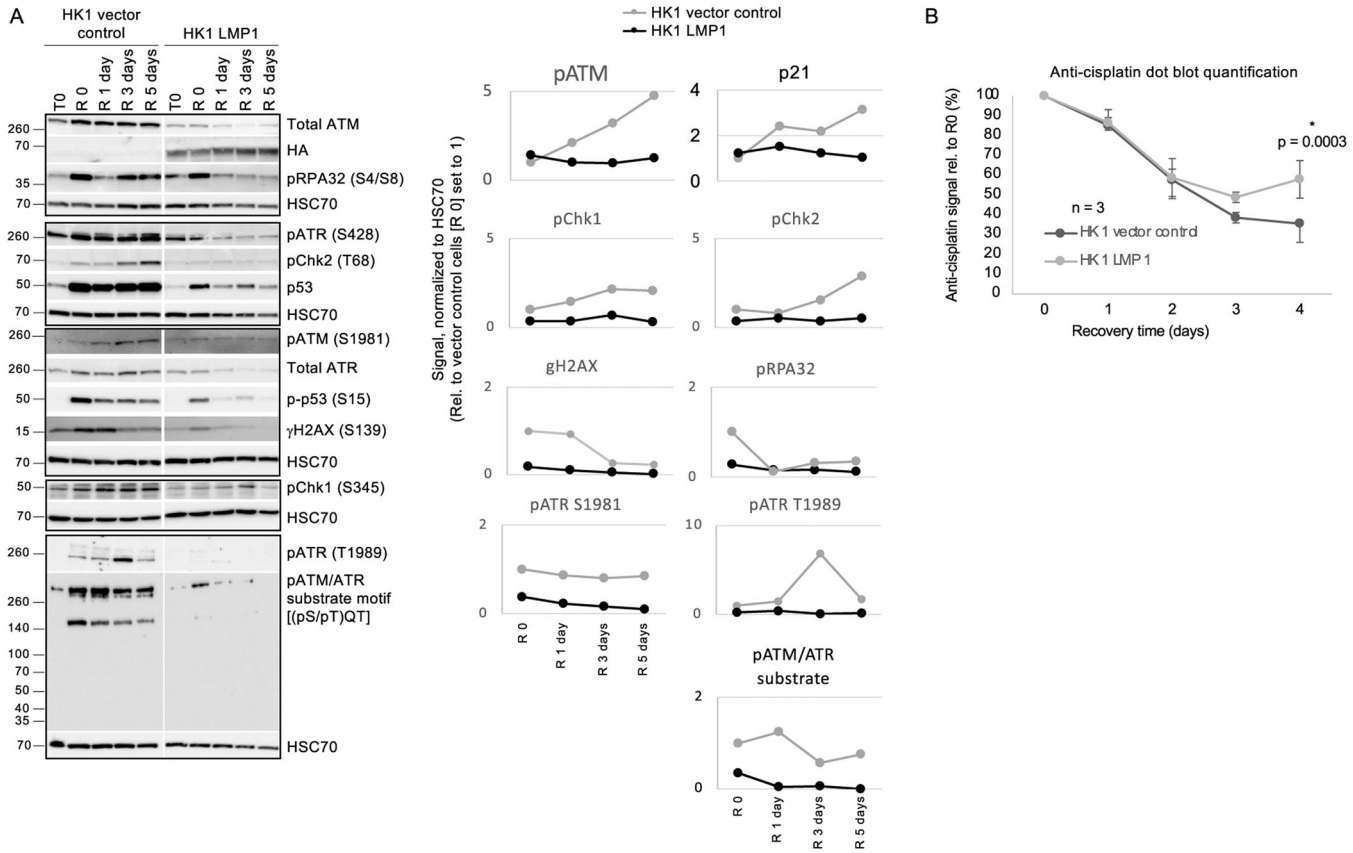


FIGURE 3 | LMP1 attenuates DNA damage response signaling and the removal of cisplatin-DNA adducts. HK1 vector control and LMP1-expressing cells (100 ng/mL doxycycline) were analyzed for (A) DNA damage response signaling and G1/S checkpoint proteins by immunoblot. (B) Residual cisplatin-DNA adducts were analyzed by a genomic DNA dot blot and probed with anti-cisplatin DNA adduct antibody. Shown is the background-subtracted signal normalized to the starting signal at recovery time zero (R0).

that decreased levels of γH2AX in LMP1-expressing cells are likely to have resulted from attenuated DDR signaling and not from the more efficient removal of cisplatin-DNA adducts.

3.5 | CTAR1 and CTAR2 Signaling Residues Are Required for LMP1-Induced G1 Arrest

To provide mechanistic insight, a panel of LMP1 C-terminal deletion mutants lacking one or more CTAR domains, and point mutations in the CTAR1 or CTAR2 domains, with confirmed inducible expression, was assembled (Figure 4A) [36]. An additional N-terminal mutant (LMP1 $FWLY_{38–41}$) located in transmembrane domain 1 that is defective for oligomerization and trafficking to lipid rafts was included to test if residues beyond the known CTAR1 and CTAR2 signaling motifs could be involved [19]. Maximal LMP1 expression was achieved with the LMP1 full-length (FL) construct but only the LMP1 A5, $Y_{384}G$ dual mutant showed comparable expression of the FL protein by HA-tag (Figure 4A, arrows). Titration of the doxycycline dose did not achieve comparable levels in the other mutants. Thus, the LMP1 A5, $Y_{384}G$ mutant was further characterized for G1 arrest and p53 activation upon cisplatin treatment. The LMP1 A5, $Y_{384}G$ mutant was attenuated for G1 arrest ($p \leq 0.05$, Figure 4B). The ablation of p53 can lead to prolonged cell cycle arrest but eventual cytotoxicity in cisplatin-treated cells [37]. The G1 arrest profiles of the LMP1 mutants correlated inversely with p53 activation such that the LMP1 A5, $Y_{384}G$ mutant had restored p53 activation comparable to the vector control (Figure 4C). These results indicate that both the CTAR1 PxQxT motif and the Y_{384} residue in CTAR2 are involved in aberrant cisplatin-induced G1 arrest, and the subversion of p53 mechanisms.

3.6 | LMP1-Induced Sensitivity Is Augmented by Consecutive Rounds of Cisplatin Treatment

LMP1 confers sensitivity to various DNA-damaging agents [15, 38]. To study the effect of LMP1 on cisplatin-induced acute cytotoxicity, HK1 cells were treated with increasing doses of cisplatin and cell viability was measured immediately after treatment. The dose–response curves with the corresponding IC50 values are shown in Figure 5A. In vector control cells, the dose–response curves were not significantly different, and the best-fit curves by linear regression were shared for all the doxycycline doses. In all three experiments with LMP1 cells, the IC50 value was > 2-fold lower and therefore sensitized in the presence of doxycycline, and different by the dose–response curve without doxycycline ($p < 0.0001$, Figure 5A). In vector control cells, inhibition of ATR (VE821) or Chk1 (PF477736) resulted in a consistent decrease in IC50 with the ATR inhibitor having the most sensitizing effect in any one experiment (dose–response curves $p < 0.0001$, Figure 5B). Interestingly, the sensitizing effect was not observed

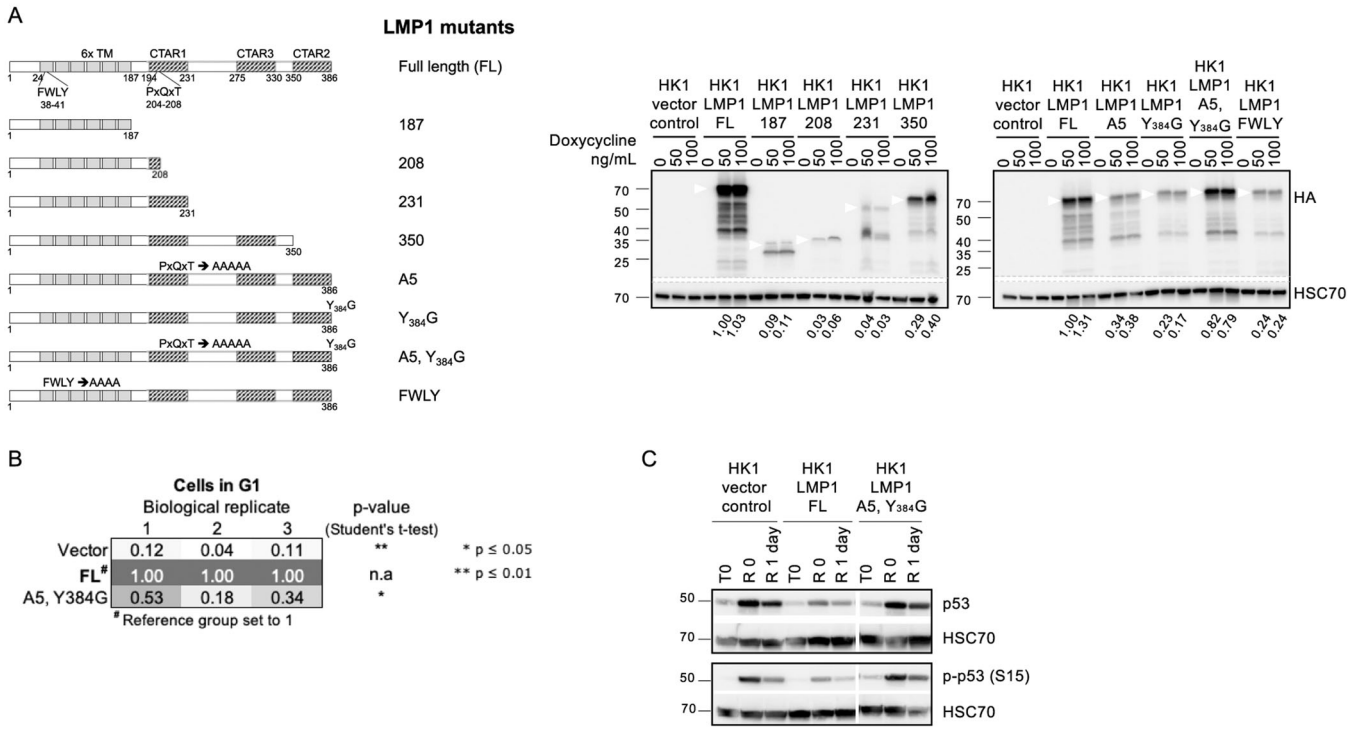


FIGURE 4 | LMP1 functional mapping by cell cycle profile and phosphorylation of p53 (S15) in response to cisplatin. (A) Schematic of LMP1 deletion and point mutants. LMP1-inducible HK1 cells were analyzed for LMP1 expression by immunoblot for the HA-tag. (B) Cell cycle profiles and (C) p53 activation were compared for the full-length LMP1 (FL) and LMP1 A5, $Y_{384}G$ mutant (50 ng/mL doxycycline) post-cisplatin treatment (IC90, 7 μM for 72 h).

with the ATM or Chk2 inhibitors (Figure 5B). These results were validated with clinical ATR and ATM inhibitors (AZD6783 and AZD0156, respectively) which are bioavailable ATP-competitive inhibitors with distinct structures from VE821 and KU55933 (Figure 5C) [39, 40]. The relative fold-change varied by experiment but the direction of change and the rank order among the inhibitor treatments was consistent within any experiment.

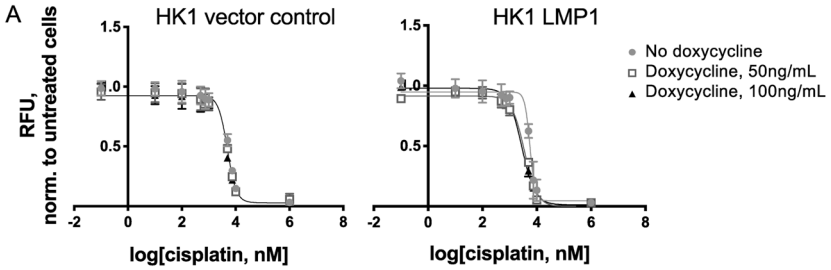
Chemotherapeutic regimens consist of multiple rounds of chemotherapy. To assess if LMP1 augments sensitivity to consecutive rounds of cisplatin, HK1 vector control or LMP1-expressing cells were treated with a cytotoxic dose of cisplatin (IC90), allowed to recover for 1 week, and subjected to cytotoxicity kill curves. The IC50 values of the vector control cells were similar in the absence or presence of doxycycline. However, the IC50 value in LMP1-induced cells was 7.66-fold lower than without doxycycline, and different by the dose–response curve ($p < 0.0001$, Figure 6A). These results demonstrate that LMP1-induced sensitivity is exacerbated by consecutive rounds of cisplatin treatment.

3.7 | The CTAR1 PxQxT Motif and CTAR2 Y_{384} Residue Are Required for LMP1-Induced Hypersensitivity to Cisplatin

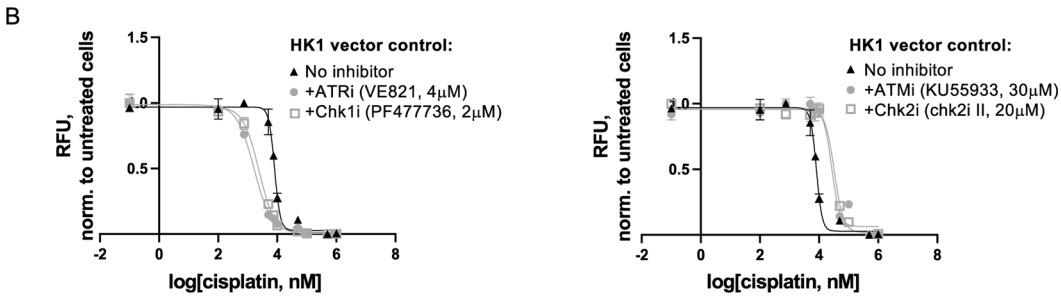
To determine if there is a correlation between G1 arrest and cisplatin sensitivity, cells expressing FL or LMP1 A5, $Y_{384}G$ mutant were compared after two consecutive cisplatin treatments. The dose–response curves and IC50 values are shown in Figure 6B. The IC50 of the LMP1 A5, $Y_{384}G$ mutant was 2.01-fold lower than the vector control (dose–response curve, $p < 0.0003$) and 1.83-fold higher than LMP1 FL (dose–response curve, $p < 0.0001$), correlating with an attenuated G1 arrest and restoration of p-p53(S15) activation (Figure 4B,C). These results summarized in Figure 6C show that G1 arrest is a proxy for LMP1-induced cisplatin sensitivity, and that the conserved PxQxT motif and Y_{384} residue are required for these effects.

3.8 | LMP1 Eliminates the Recovery of Cisplatin-Treated Cells

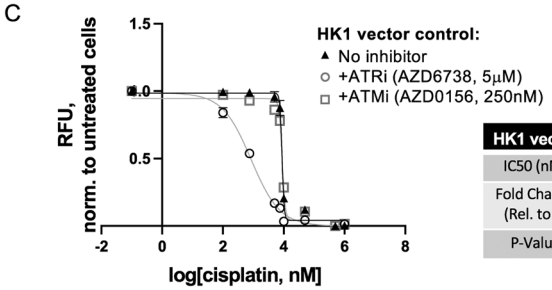
To study the long-term effects of LMP1-induced sensitivity, HK1 vector control and LMP1-expressing cells were treated with a cytotoxic dose of cisplatin (7 μM, IC90) and cell viability was monitored immediately after treatment and at weekly intervals during a recovery period of 6 weeks. Vector control cells recovered steadily compared to LMP1-expressing cells which showed a delayed recovery, with the lowest viability at Day 21 (i.e., 16 days post-recovery) (Supporting Information: Figure S2, top and bottom left panels). By Day 50, both vector control and LMP1-expressing cells recovered and reached comparable viability. Given the variable cell viability in the recovered LMP1-expressing cells, we hypothesized that these cells remaining at Day 21 would be more sensitive to cisplatin than vector control cells in a repeat treatment. Sequential cisplatin treatment as occurs in chemotherapy could result in a more complete elimination in the presence of LMP1. Interestingly, in rare cases (1/20 wells at Day 35, and 2/20 wells at Day 50) vector control cells recovered, which did not occur in LMP1-expressing cells (Supporting Information: Figure S2, top



Cell line	HK1 vector control			HK1 LMP1		
Doxycycline, ng/mL	0*	50	100	0*	50	100
IC50 (nM), n=3	5163	3887	4013	5930	2787	2533
P-value (α = 0.05)	n.s			< 0.0001		
Fold change (Rel. to *)	n.a.	-1.33	-1.29	n.a.	-2.13	-2.34



HK1 vector	No inhibitor*			ATRi (VE821)			Chk1i (PF477736)			ATMi (KU55933)			Chk2i (chk2i II)		
IC50 (nM)	8111	9154	13578	1634	3763	1876	2395	5922	3272	26512	57387	41990	32596	54677	51246
Fold Change (Rel. to *)	n.a.			-4.96	-2.43	-7.24	-3.39	-1.55	-4.15	+3.27	+6.27	+3.09	+4.02	+5.97	+3.77
P-Value	n.a.			<0.0001			<0.0001			<0.0001			<0.0001		



HK1 vector	No inhibitor*			ATRi (AZD6738)			ATMi (AZD0156)		
IC50 (nM)	8058	7433	8824	304.3	297.1	846	14010	14854	8907
Fold Change (Rel. to *)	n.a.			-26.48	-25.02	-10.43	+1.74	+2.00	+1.01
P-Value	n.a.			<0.0001			<0.0001		

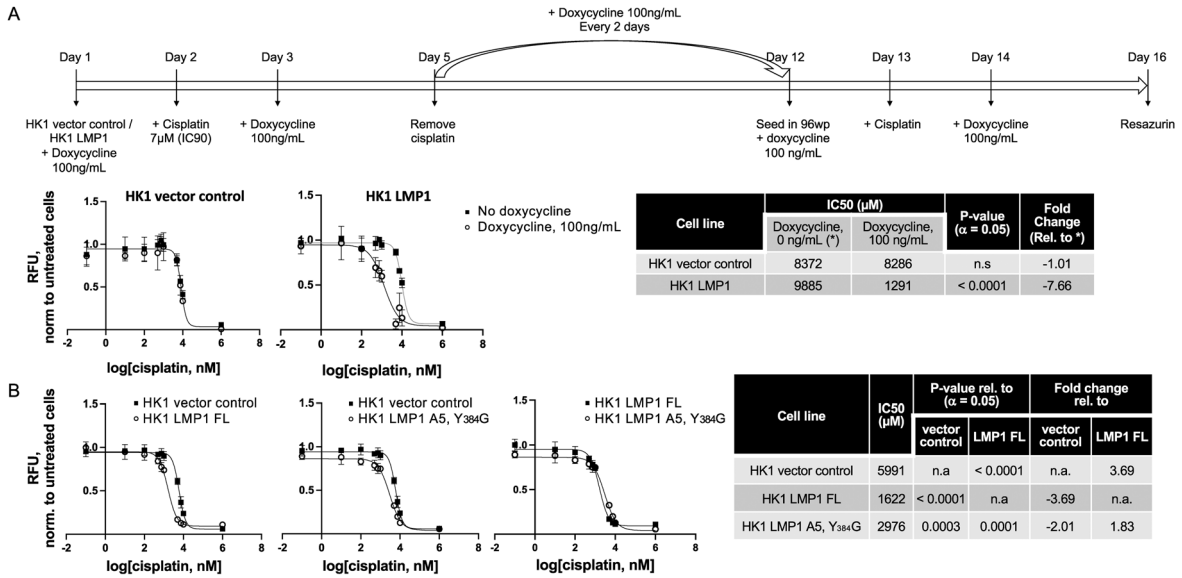
FIGURE 5 | LMP1 sensitizes HK1 cells to cisplatin. (A) HK1 vector control or LMP1-expressing cells were treated with increasing doses of cisplatin for 72 h. Shown are dose–response (best-fit) curves from a representative experiment. The IC50 values and the dose–response curves by two-way ANOVA analysis from three independent experiments are shown in the table. (B and C) Cisplatin sensitivity is phenocopied in HK1 vector control cells treated with the ATR (VE821 and AZD6738) or Chk1 (PF477736) inhibitors, but not with the ATM (KU55933 and AZD0156) or Chk2 (chk2i II) inhibitors. Shown is one representative experiment from three independent experiments quantified in the table.

and bottom right panels). These results demonstrate that LMP1 has the potential to eliminate residual tumor cells that escape initial cisplatin acute cytotoxicity.

3.9 | LMP1 Sensitizes EBV-Infected Cells to Cisplatin Treatment

EBV-infected cells expressing basal levels of LMP1 can be stimulated by IL6 to trigger the STAT3-responsive, epithelial-specific LMP1 promoter (pTR-L1) [41]. ATM is frequently downregulated in NPC tumors, which is a target of EBV miRNAs [9, 11]. To determine if LMP1 is sensitizing in the presence of EBV infection, EBV-infected HK1 cells (HK1-EBV) with or without stable expression of LMP1-China1 (Ch1) strain, or LMP1 inhibition by shRNA knockdown or CRISPR/Cas9 targeting, were subjected to cisplatin cytotoxicity assays after one round of exposure. Notably, HK1-EBV cells are almost exclusively latent and defined by Type II latency as occurs in NPC tumors [42]. These conditions were evaluated in the absence or presence of IL6, with or without an ATM inhibitor (ATMi, KU55933). LMP1-Ch1 is matched to the LMP1 strain in the parent EBV (Akata) used to derive the HK1-EBV cell line [42]. Dose–response curves showed that EBV infection consistently sensitized HK1 cells to cisplatin (HK1 vs. HK1-EBV, $p < 0.0001$; fold-change range = −1.28 to −1.63) regardless of IL6 or ATMi treatment (Figure 7 and Supporting Information: Figure S3A). This sensitivity was further enhanced by the introduction of LMP1-Ch1 (HK1-EBV-pB vs. HK1-EBV-Ch1, $p < 0.0001$; fold-change range = −1.29 to −1.91, Figure 7 and Supporting Information: Figure S3A). The stable expression of LMP1, shRNA knockdown (shLMP1), or stable expression of a CRISPR/Cas9 LMP1 targeting construct (sgLMP1) were verified by immunoblot (Supporting Information: Figure S3B).

Reciprocally, knockdown or CRISPR/Cas9 targeting of endogenous LMP1 conferred resistance to cisplatin (HK1-EBV-shsc vs. HK1-EBV-shLMP1, $p < 0.0001$; HK1-EBV-Cas9 vs. HK1-EBV-sgLMP1, $p < 0.0001$, Figure 7). We noted that IL6 treatment had only a modest induction of endogenous LMP1 (Supporting



Cell line	IC50 (μM)		P-value (α = 0.05)	Fold Change (Rel. to *)
	Doxycycline, 0 ng/mL (*)	Doxycycline, 100 ng/mL		
HK1 vector control	8372	8286	n.s	-1.01
HK1 LMP1	9885	1291	< 0.0001	-7.66

Cell line	IC50 (μM)	P-value rel. to (α = 0.05)		Fold change rel. to	
		vector control	LMP1 FL	vector control	LMP1 FL
HK1 vector control	5991	n.a	< 0.0001	n.a.	3.69
HK1 LMP1 FL	1622	< 0.0001	n.a	-3.69	n.a.
HK1 LMP1 A5, Y384G	2976	0.0003	0.0001	-2.01	1.83

C

	Expression	G1 arrest (mean %)	p-p53 (S15)	IC50
Vector control	-	- (2.33)	+++	5.99
LMP1 FL	+++	+++ (25.27)	+	1.62
LMP1 A5	+	+++ (21.53)	+	1.84
LMP1 Y384G	+	+++ (19.8)	+	2.47
LMP1 A5, Y384G	+++	+ (8.97)	+++	2.98

FIGURE 6 | LMP1 sensitizes HK1 cells after two consecutive rounds of treatment with cisplatin. (A) HK1 inducible cell lines with/without doxycycline induction (100 ng/mL, 24 h) were treated with 7 μM (IC90, 72 h) of cisplatin. Cells recovering after 1 week were treated with increasing doses of cisplatin for 72 h. Dose–response curves were generated by plotting the concentration of inhibitor (cisplatin) on a logarithmic scale vs. response (RFU normalized to untreated cells). The IC50 was defined as the concentration of cisplatin that results in a 50% reduction of the cell viability. (B) Sensitivity of the LMP1 A5, $Y_{384}G$ mutant to cisplatin treatment was compared to the vector control or LMP1 FL. (C) Summary of LMP1 functional mapping phenotypes in response to cisplatin.

Information: Figure S3C), and the sensitivity of HK1-EBV cells was not enhanced by IL6 or ATMi treatment (IC50 positive fold-change: 1.03–1.10, Supporting Information: Figure S4). These results demonstrate that endogenous LMP1 is sufficient to sensitize latently infected EBV cells and that infected cells overexpressing LMP1 are the most sensitized to cisplatin.

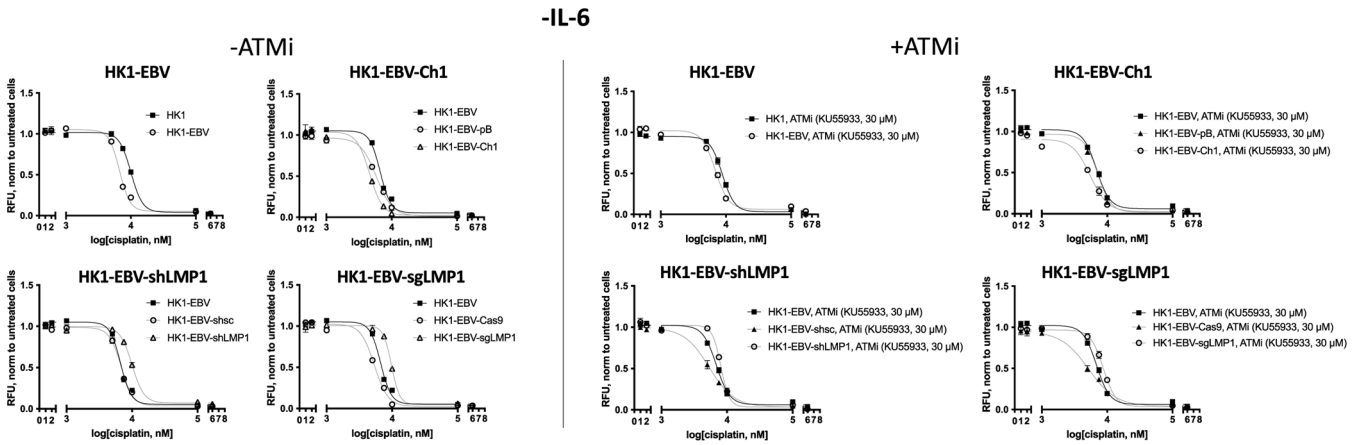
3.10 | Inhibition of ATR Phenocopies LMP1-Induced Cisplatin Hypersensitivity

Treatment with the ATR inhibitor (ATRi, VE821) but not the ATM inhibitor (ATMi, KU55933) sensitized HK1 and HK1-EBV cells (Supporting Information: Figure S5A). Similarly, treatment with the clinical ATR inhibitor (AZD6738), but not the ATM inhibitor (AZD0156), sensitized HK1 and HK1-EBV cells (Supporting Information: Figure S5B). Interestingly, treatment with the ATRi was sufficient to induce G1 arrest in HK1 cells with or without cisplatin treatment (Supporting Information: Figure S6). These data demonstrate that ATR inhibition in EBV-infected cells phenocopy LMP1-induced cisplatin hypersensitivity, and that G1 arrest can be a downstream effect of ATR inhibition.

To explore if the sensitizing effect of LMP1 and ATR inhibition could be observed in other NPC cell lines, LMP1 expression was restored in the C17 cell line by stable expression. The parental C17 cell line has negligible LMP1 and ATM expression [11, 43]. LMP1 expression reproducibly sensitized C17 cells, which was phenocopied by ATR inhibition (VE821 and AZD6738) but not Chk2 inhibition (Chk2i II) (Supporting Information: Figure S7A–C). Inhibitors to ATM (KU55933) and Chk1 (PF477736) were toxic to C17 cells, thus, kill curves could not be determined. However, the clinical ATM inhibitor (AZD0156) was well tolerated and did not sensitize C17 cells (Supporting Information: Figure S7C). In the presence of LMP1, DDR signaling was attenuated such that the activation of p-ATM/ATR substrates, pRPA32, pChk1, and pChk2 were decreased during cisplatin treatment and in the recovery period, compared to the pBabe vector control (Supporting Information: Figure S7D).

3.11 | Evaluation of LMP1 and ATR Expression Levels From Microdissected NPC Tumors

To evaluate clinical significance, RNA-seq data from microdissected NPC tumors were analyzed for LMP1 and ATR expression (Supporting Information: Figure S8) [44]. In NPC patients who received cisplatin, primary tumors with high LMP1 expression were observed only in the nonrecurrent group (Supporting Information: Figure S8A). Furthermore, ATR expression was equivalent in primary tumors compared to tumor adjacent tissue or panendoscopy control tissue from cancer-free donors, regardless of whether the tumor had detectable LMP1 expression (Supporting Information: Figure S8B,C). This is consistent with the hypothesis that NPC tumors with high LMP1 are less likely to recur. From these data, we can infer that the



-ATMi	HK1 vs HK1-EBV						HK1-EBV-pB vs HK1-EBV-Ch1						HK1-EBV-shsc vs HK1-EBV-shLMP1						HK1-EBV-Cas9* vs HK1-EBV-sgLMP1	
	HK1*			HK1-EBV			HK1-EBV-pB*			HK1-EBV-Ch1			HK1-EBV-shsc*			HK1-EBV-shLMP1			Cas9*	sgLMP1
IC50 (nM)	9654	10746	11439	6673	7250	7802	5847	6292	7162	4595	3938	5129	6818	6442	7556	9988	10135	9576	5357	9761
Fold Change (Rel. to *)	n.a.			-1.45	-1.48	-1.47	n.a.			-1.27	-1.60	-1.40	n.a.			1.46	1.57	1.27	n.a.	1.82
P-Value	n.a.			<0.0001			n.a.			<0.0001			n.a.			<0.0001			<0.0001	

+ATMi	HK1 vs HK1-EBV						HK1-EBV-pB vs HK1-EBV-Ch1						HK1-EBV-shsc vs HK1-EBV-shLMP1						HK1-EBV-Cas9* vs HK1-EBV-sgLMP1	
	HK1*			HK1-EBV			HK1-EBV-pB*			HK1-EBV-Ch1			HK1-EBV-shsc*			HK1-EBV-shLMP1			Cas9*	sgLMP1
IC50	8809	9041	11177	6863	6897	7662	7098	7351	7570	5487	5294	3963	5339	7579	7531	8039	11423	9924	5178	8738
Fold Change (Rel. to *)	n.a.			-1.28	-1.31	-1.46	n.a.			-1.29	-1.39	-1.91	n.a.			1.51	1.51	1.32	n.a.	1.69
P-Value	n.a.			<0.0001			n.a.			<0.0001			n.a.			<0.0001			<0.0001	

FIGURE 7 | LMP1 sensitizes HK1-EBV cells to cisplatin. HK1-EBV stable cell lines expressing recombinant LMP1 China 1 (Ch1) strain or knockdown of endogenous LMP1 (by shRNA or CRISPR/Cas9) were treated with increasing doses of cisplatin for 72 h, in the absence of IL6 stimulation of the endogenous LMP1 promoter, with or without ATMi (KU55933, 30 μM). Shown are dose–response (best-fit) curves from a representative experiment. Comparison of the average IC50 values and the dose–response curves by two-way ANOVA analysis, from three independent experiments, are shown in the table.

manipulation of ATR signaling by LMP1 is posttranscriptional or posttranslational. A proposed model for LMP1 disruption of cisplatin-induced DDR signaling is summarized in Figure 8.

4 | Discussion

EBV-associated NPC is often treated with concurrent radiation and chemotherapy. For locoregionally advanced NPC, cisplatin-based chemotherapy with or without sequential chemotherapy is recommended [45]. The standard regimen for recurrent and/or metastatic NPC is platinum-based chemotherapy with gemcitabine [46]. In multiple epithelial cell lines (EBV-negative: CNE2, TW02, TW04; EBV-positive: CG-1), LMP1 has been reported to be sensitizing to cisplatin [16, 38]. Interestingly, cisplatin sensitivity is reversed by EBV BART miRNAs (Cluster 1) which are abundantly expressed in NPC tumors and are known to downregulate and bind the 3′UTR of LMP1 [15]. While it is known that NPC tumors harbor clonal EBV infection [2], the expression of LMP1 is highly variable as illustrated by histology and single-cell RNA-sequencing [6, 18, 47]. We posit that LMP1 intra- and intertumoral heterogeneity could compromise cisplatin efficacy, and that tumor cells expressing negligible levels of LMP1 may emerge as refractory cells. Here, we propose that in cases where the NPC tumor has limited or variable LMP1 expression, complementary treatment with an ATR (or Chk1) inhibitor could result in more complete elimination of the tumor and/or reduce the risk of recurrence.

LMP1 can manipulate DNA repair by repression of DDR signaling, deregulation of cell cycle checkpoints, or potentially activation of DNA damage-induced apoptosis [10, 48, 49]. Most studies have focused on the acute response to cisplatin upon one round of treatment. In this study, we measured both the acute and long-term response to cisplatin from recovered cells after sequential rounds of treatment. A minor but consistent change in the dose–response curves can yield a difference by two-way ANOVA analysis. Thus, we also compared the discrete IC50 values to interpret the magnitude of change which is a relative value that should be compared within an experiment. This led to the discovery that ATR or Chk1 inhibition has a sensitizing effect, phenocopying the LMP1 hypersensitivity effect. Curiously, the recovery assay provided a categorical score by comparison of the number of recovered wells and demonstrated that the absence of LMP1 can lead to the rare emergence of cisplatin-refractory cells (Supporting Information: Figure S2). Collectively, these functional assays offer an in-depth analysis on the sensitizing effect of LMP1 in paired EBV-infected and uninfected NPC cell lines. We note that NPC studies are often limited by the availability of authenticated and paired cell lines with/without EBV infection. While many NPC-derived cell lines are contaminated with HeLa cells, the unique HK1 and HK1-EBV cell lines and the C17 cell line are devoid of HeLa contaminants [50, 51].

Functional mapping studies of LMP1 revealed that cisplatin sensitivity is a conserved property of LMP1. LMP1 variants can

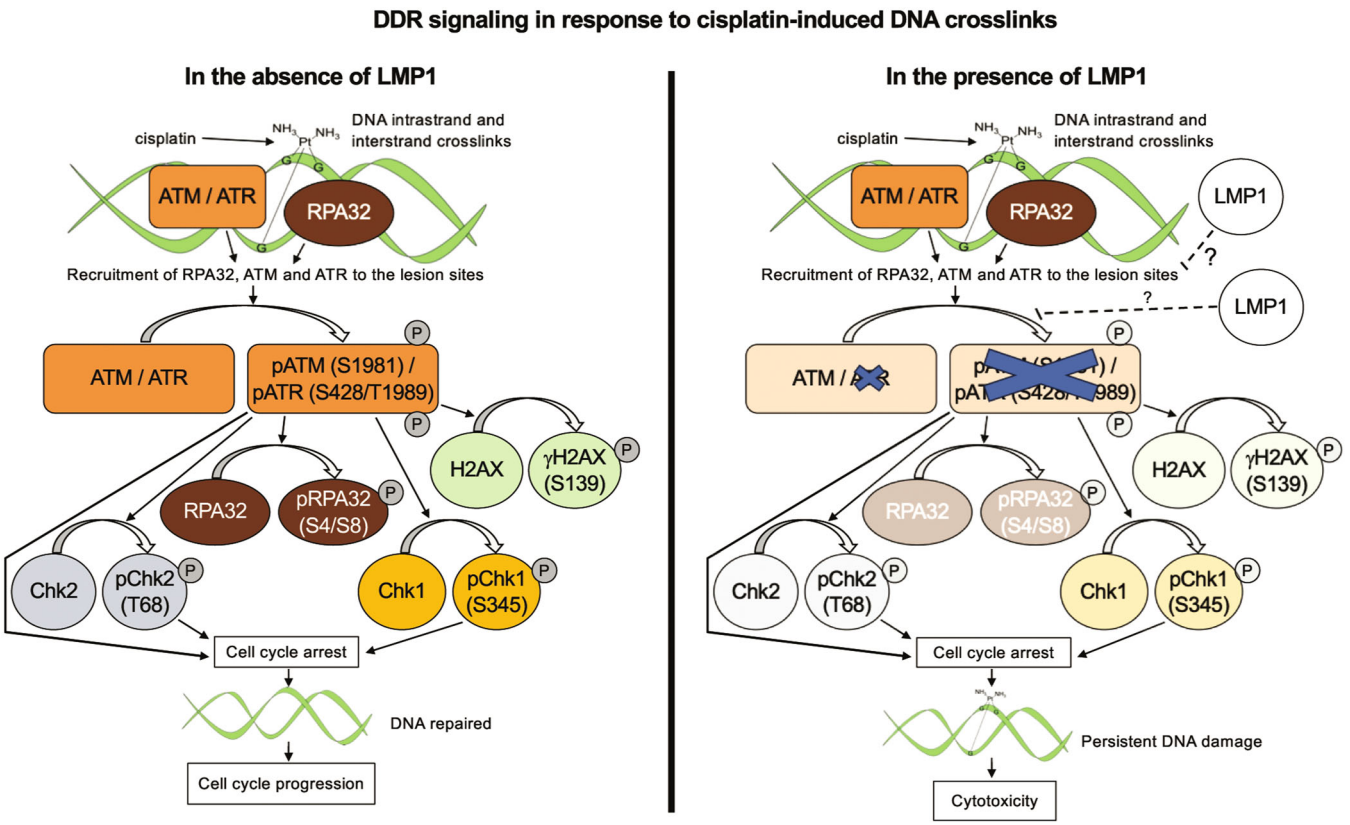


FIGURE 8 | Model of cisplatin-induced DNA damage response (DDR) signaling with or without LMP1. Cisplatin generates interstrand and intrastrand DNA crosslinks. RPA32 binds to the lesion sites and recruits mainly ATR but also ATM. Upon recruitment to the lesion sites, these kinases are activated and trigger DDR signaling that includes phosphorylation of RPA32 and other targets such as Chk1, Chk2, and H2AX. Signaling mediated by ATM and ATR leads to checkpoint activation, cell cycle arrest and recruitment of repair proteins. If DNA damage is repaired, the cell cycle progresses. In NPC tumors where ATM expression is downregulated, LMP1 disrupts the response to DNA damage by attenuating the activation of ATR and Chk1, which results in reduced efficiency of DNA repair, persistent DNA damage, cell cycle arrest, and cytotoxicity.

be classified by amino acid sequence into at least seven phylogenetically distinct strains, of which China 1 is the most frequently detected in tumors from NPC-endemic regions [21]. We tested both the B95-8 and China 1 LMP1 strains and mapped cisplatin sensitivity to the conserved signaling motifs in CTAR1 (PxQxT) and CTAR2 (Y384) (Figure 6). Because these phenotypes could not be separated by mapping to distinct domains in LMP1, we asked if ATR (or Chk1) inhibition could phenocopy LMP1 because recovering LMP1-expressing cells were attenuated for ATR signaling (Figure 3). Given that ATR and Chk1 inhibition phenocopied cisplatin sensitivity (Figure 5B and Supporting Information: Figure S5), these data are consistent with the hypothesis that attenuated ATR signaling by LMP1 imposes G1 checkpoint arrest and enhanced cisplatin sensitivity. Given that LMP1-expressing HK1 cells retain residual cisplatin-DNA adducts (Figure 3), our results are in agreement with prior studies that LMP1 impairs DNA repair triggered by multiple types of DNA-damaging agents including radiation, UV-adducts, and DNA cross-linking [10, 14, 16]. Nucleotide excision repair (NER) is the major repair mechanism for cisplatin adducts [52]. Although NER has not been extensively studied in NPC, EBV infection in immortalized nasopharyngeal epithelial cells promotes histone bivalent switch modifications in the DNA repair genes of base excision repair, homologous recombination, nonhomologous end-joining, and mismatch repair pathways, although genes in NER were not named [8]. Delineation of defects in DNA repair mechanisms will require the assembly of cisplatin-resistant xenografts and/or the generation of isogenic cell lines for further study. The lack of in vivo evidence supporting that LMP1-positive NPC tumors have attenuated ATR signaling is a limitation of the current study. However, in the absence of appreciable ATM expression in NPC tumors [9, 11], we propose that a drug-induced or LMP1-induced inhibition of ATR in animal studies is warranted. It would be interesting to query if recurrent NPC tumors are enriched for cells lacking LMP1 expression, as suggested from the Smart-3SEQ analysis of microdissected NPC tumors who's original LMP1 expression is exclusively low in cisplatin-treated patients that experienced recurrence.

Author Contributions

Conceptualization, Gabriella Zarkovic, Phillip Ziegler, and Kathy Ho Yen Shair; methodology, Gabriella Zarkovic, Phillip Ziegler, Masahiro Shuda, and Kathy Ho Yen Shair; formal analysis, Gabriella Zarkovic, Phillip Ziegler, Jennifer Hye-Rim Lee, Brooke Dresden, Amit Kumar, and Kathy Ho Yen Shair; investigation, Gabriella Zarkovic, Phillip Ziegler, Jennifer Hye-Rim Lee, Brooke Dresden, Amit Kumar, and Masahiro Shuda; resources, Gabriella Zarkovic, Phillip Ziegler, and Amit Kumar; data curation, Gabriella Zarkovic, Phillip Ziegler, Jennifer Hye-Rim Lee, Brooke Dresden, and Kathy Ho Yen Shair; writing – original draft preparation, Gabriella Zarkovic and Kathy Ho Yen Shair; writing – review and

editing, Gabriella Zarkovic, Masahiro Shuda, and Kathy Ho Yen Shair; supervision, Kathy Ho Yen Shair; project administration, Kathy Ho Yen Shair; funding acquisition, Kathy Ho Yen Shair. All authors have read and agreed to the published version of the manuscript.

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Ethics Statement

The authors have nothing to report.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Publicly available transcriptome data from Tay et al. [44] were provided from the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject) under accession number PRJNA413176. All other data are available upon reasonable request.

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Supporting Information

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