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# Epstein-Barr virus-encoded LMP1 promotes cisplatin-induced caspase activation through JNK and NF-κB signaling pathways

Xiangning Zhang [a,1], Duangmanee Sanmun [b,c], LiFu Hu [a], Bengt Fadeel [b], Ingemar Ernberg [a,*]

[a] Department of Microbiology, Tumor and Cell Biology, Box 280, Karolinska Institutet, 171 77 Stockholm, Sweden
[b] Division of Biochemical Toxicology, Institute of Environmental Medicine, Karolinska Institutet, Stockholm, Sweden
[c] Department of Clinical Microscopy, Faculty of Medical Technology, Mahidol University, Bangkok, Thailand

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**Abstract**

Our previous studies have shown that Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) potentiates chemotherapeutic agent-induced apoptosis in human cell lines of epithelial origin: cervical carcinoma-derived HeLa cells and nasopharyngeal carcinoma-derived TW03 cells. LMP1 acted upstream of caspase-dependent mitochondrial perturbation, and the effect was mapped to the C-terminal signaling domain of LMP1, designated CTAR2. CTAR2 is known to engage the c-Jun N-terminal kinase (JNK) and NF-κB pathways, and we show here that SP600125, a selective JNK inhibitor, suppresses LMP1 potentiation of cisplatin-induced mitochondrial damage and caspase activation in HeLa cells. Moreover, the potentiation of cisplatin-triggered caspase activation was blocked by Bay11-7082, a potent inhibitor of NF-κB. Similar results were obtained when a dominant negative form of IκB, a specific repressor of NF-κB, was co-expressed with LMP1. The current data support the notion that LMP1 modifies stress-induced apoptosis in epithelial cells through molecular interactions downstream of its C-terminal signaling domain.


*Keywords:* Apoptosis; Epstein-Barr virus; Caspase; c-Jun-N-terminal kinase; Latent membrane protein 1; Nuclear factor-κB

Epstein-Barr virus (EBV) encodes a number of proteins with transforming ability. Among these proteins, latent membrane protein 1 (LMP1) is essential for EBV-mediated transformation of resting primary human B cells into indefinitely proliferating lymphoblastoid cell lines [1]. EBV is associated with human lymphoid tumors, such as Burkitt's lymphoma (BL) arising in immunocompetent individuals, and lymphoblastoid cell-like lymphomas in immunocompromised patients. Importantly, early studies showed that LMP1 confers apoptosis resistance in B cells through transcriptional regulation of the anti-apoptotic protein Bcl-2 [2]. Recent studies have yielded a more complex picture insofar as LMP1 was shown to upregulate expression of multiple genes with opposing activities in B cell proliferation and apoptosis [3].

EBV infection is also tightly associated with the undifferentiated form of nasopharyngeal carcinoma (NPC), a human tumor of epithelial origin. Thirty to sixty percent of the EBV-associated NPCs are also positive for LMP1 [4,5]. LMP1 inhibits terminal differentiation of human epithelial cells [6]. LMP1 also affects apoptosis in epithelial cells, but the outcome appears to vary in a context-dependent manner [7–10]. Moreover, in several NPC cell lines, LMP1 was shown to repress DNA repair and enhance sensitivity to DNA-damaging agents, as assayed by micronucleus formation [11]. The latter findings support an additional role for LMP1 in the pathogenesis of NPC, insofar as disruption of DNA repair by LMP1 may contribute to genomic instability of epithelial cells.

* Corresponding author. Fax: +46 8 31 94 70.
*E-mail address:* ingemar.ernberg@ki.se (I. Ernberg).
[1] Present address: Department of Pathophysiology, Guangdong Medical College, Zhanjiang, Guangdong, People's Republic of China.

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The cellular effects of LMP1 are mostly attributed to the two functional domains in its cytoplasmic region: a membrane proximal domain, termed the C-terminal activation region 1 (CTAR1), and a membrane distal domain, CTAR2 [12]. Activation of NF-κB is the hallmark of the biological activities of LMP1 [13], and CTAR1 has been shown to contribute to 30% of the NF-κB activation and to the initiation of cell proliferation exerted by LMP1 in some cell types [14]. CTAR2, in turn, contributes to 70% of the NF-κB activation as well as to the activation of c-Jun-N-terminal kinase (JNK) [15].

Our previous studies have shown that LMP1 regulates apoptosis in the epithelial cell line HeLa in a stimulus-dependent manner [9]. Hence, expression of LMP1 diminished tumor necrosis factor (TNF)-induced apoptosis, but enhanced apoptosis triggered by other forms of cellular stress, such as etoposide. We also observed that the regulation of cellular stress-induced apoptosis by LMP1 occurred upstream of caspase-dependent mitochondrial perturbation, and this effect was mapped to CTAR2 [10]. The latter observations suggested that molecular interactions involving the membrane distal functional domain of LMP1 may be responsible for the regulation of stress-induced apoptosis. However, the underlying signaling pathways or factors involved have remained elusive. In the present study, we investigated the involvement of NF-κB and JNK in the regulation of stress-induced apoptosis in human cell lines of epithelial origin.

## Materials and methods

*Cell lines and plasmids.* HeLa cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). Raji, an EBV-positive BL-derived cell line [16], was from the Department of Microbiology, Tumor, and Cell Biology at Karolinska Institutet. HeLa cells were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μl/ml streptomycin, 2 mg/ml geneticin, 500 μg/ml hygromycin B, and 1 μg/ml tetracycline (Stratagene, La Jolla, CA, USA). Raji cells were cultivated in RPM1-1640 medium (Gibco) supplemented with 10% FCS and antibiotics. The κB.ConA.LUC reporter plasmid contains three NF-κB enhancer element upstream of a conalbumin promoter driving a luciferase gene [17], and the β-galactosidase-encoding plasmid pCMV βGal was obtained from Dr. Martin Rowe (University of Wales, Cardiff, UK). Dual JNK reporter plasmids pFA+pFR from Strategene were kindly provided by Dr. Aris Eliopoulos (University of Birmingham, Birmingham, UK). Transient transfection of a plasmid expressing a dominant negative (DN) inhibitor of NF-κB (IκB) [termed Gly-Ala repeat (GAr) IκB] (the generous gift of Dr. Anatoly Sharipo, Karolinska Institutet) [18] was performed using Lipofectamine 2000 (Invitrogen, Paisley, Scotland) according to instructions from the manufacturer.

*Transfection procedures.* Transfections with tetracycline transactivator (tTA) expressor plasmid and responder plasmid with inserts of LMP1 cDNA were done as previously described [9]. Cells with tetracycline-regulated LMP1 expression were selected by subcloning. For reporter assays, monolayer cells were plated in 6-well tissue culture plates, and incubated overnight at 37 °C. Cells were then co-incubated with 4 μg/ml lipofectin (Invitrogen) mixed with 300 ng κB. ConA.LUC reporter plasmid or 50 ng pFA+pFR and 300 ng pCMV βGal plasmids. The introduced genes were expressed for 24 h during which time the HeLa cells were cultured in the presence or absence of tetracycline. Cells were then harvested in cell culture lysis reagent (CCLR) (Promega, Madison, WI, USA). Transfection efficiency was normalized by assessment of β-galactosidase activity, as described previously [12].

*Western blotting.* To monitor the tetracycline-regulated expression of LMP1 in HeLa cells, the same cell lysate mentioned above was mixed with an equal volume of sampling buffer (65 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol with protease inhibitors) and heated by boiling at 95 °C, whereupon total proteins were separated by 7.5% polyacrylamide gel electrophoresis (PAGE). After electroblotting, the membranes were probed using the anti-LMP1 antibody S12 [10] or the anti-β-actin antibody AC-15 (SIGMA, St. Louis, MO, USA). Horseradish peroxidase (HRP)-linked anti-mouse goat immunoglobulins (Bio-Rad, Hercules, CA, USA) were used as secondary antibodies and membranes were developed with ECL reagents, as recommended by the manufacturer (Amersham, Uppsala, Sweden).

*Apoptosis induction.* Apoptosis of HeLa cells was triggered by the addition of 5 μg/ml of cisplatin (Sigma). For pharmacologic inhibition of JNK and NF-κB, cells were pre-incubated with 10 μM SP600125 (Calbiochem, Nottingham, UK) or Bay11-7082 (Calbiochem), respectively, while control cells were cultivated in the presence of solvent alone (DMSO). Effectiveness of apoptosis induction/inhibition was routinely monitored by the assessment of detached and floating cells in the culture medium (data not shown). The apoptosis assays described below were performed on floating cells pooled with the remaining, adherent cells detached by trypsinization.

*Mitochondrial membrane potential.* The drop of mitochondrial membrane potential (MMP) upon apoptosis induction was determined as previously reported [10], using the cationic fluorescent dye tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes, Leiden, The Netherlands). Data were acquired using a FACScan (Becton–Dickinson, San Jose, CA, USA), operating with the CellQuest software (Becton–Dickinson), and results are depicted as the percentage of cells with depolarized mitochondria.

*Caspase activation.* Caspase-2 and caspase-3 activities were measured as reported previously [10]. Briefly, cell lysates and peptide substrates (VDVAD-AMC or DEVD-AMC) (Calbiochem) were combined in a standard reaction buffer [19] and added to a 96-well plate. Cleavage of the fluorogenic substrates was monitored by AMC liberation in a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden). Fluorescence units were converted to pmol of AMC using a standard curve generated from free AMC. Data were analyzed by linear regression and are displayed as pmol AMC release per min.

## Results

To evaluate the role of JNK activation for LMP1 modulation of apoptosis, HeLa cells were transiently transfected with JNK reporter plasmids (Fig. 1A). LMP1 induction of JNK was observed, and the addition of SP600125 [20] decreased JNK activation, as expected (Fig. 1B). Cells were then pre-treated with SP600125 before administration of cisplatin to trigger apoptosis. As seen in Fig. 1C, the drop of MMP was increased in LMP1-expressing cells, in line with our previous studies [10]. Importantly, SP600125 abrogated the dissipation of MMP in LMP1-expressing cells, but not in the LMP1-negative cells. Furthermore, induction of caspase-2 (Fig. 1D) and caspase-3 (Fig. 1E) activity upon exposure of the LMP1-expressing cells to cisplatin was blocked by the JNK inhibitor. Together, these data suggest that stress (cisplatin)-induced apoptosis in LMP1-expressing HeLa cells is JNK-dependent.

We also asked whether NF-κB-dependent pathways were involved in stress-induced apoptosis in our model

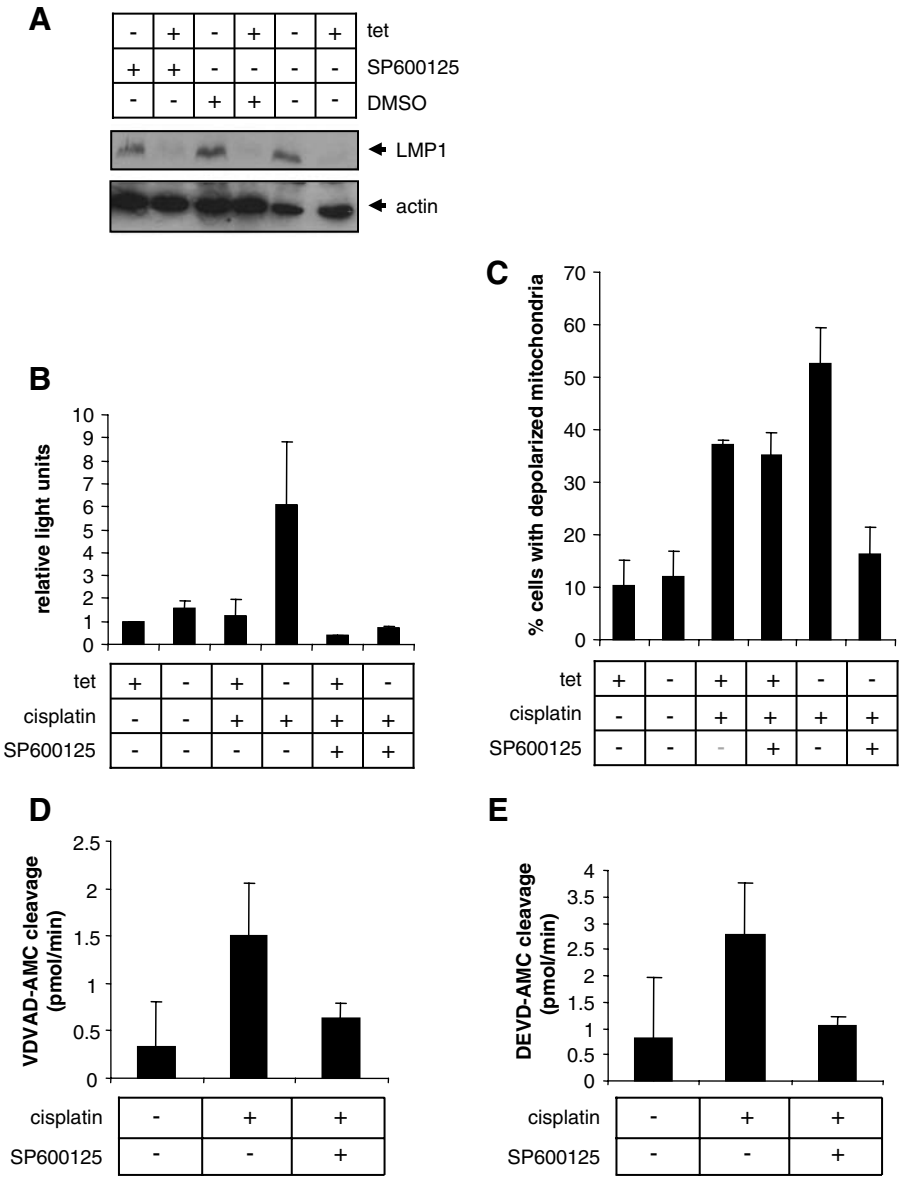


Fig. 1. LMP1 potentiation of stress-induced apoptosis is JNK-dependent. (A) HeLa cells co-transfected with dual pFA+pFR plasmids were cultivated in the presence or absence of tetracycline (tet), and immunoblotting was then performed with specific anti-LMP1 antibodies. The membranes were re-probed using actin-specific antibodies to verify equal loading of protein. Note that neither the JNK inhibitor, SP600125, nor solvent alone (DMSO) interfered with LMP1 induction. (B) HeLa cells transfected with dual JNK reporter plasmids were incubated with or without tet, and pre-treated for 15 min with SP600125 (10 μM) prior to administration of cisplatin (5 μg/ml) for 24 h. Luciferase activity was then determined. Transfection efficiency was normalized by assessment of βGal activity. (C) HeLa cells incubated in the presence or absence of tet were pre-treated with SP600125 (10 μM) or solvent alone before administration of cisplatin (5 μg/ml). MMP was then determined by assessment of TMRE fluorescence. (D) LMP1-expressing cells pre-treated with the JNK inhibitor, SP600125 (10 μM) or DMSO were challenged with cisplatin (5 μg/ml) for 24 h, and caspase-2 activity was assessed in a fluorogenic assay. (E) Cells treated as in D were harvested and tested for activation of caspase-3-like enzyme activity. Data shown in (B)–(E) are mean values ± SD ($n = 3$).

system. To this end, HeLa cells were pre-treated with Bay 11-7082, an inhibitor of IκB phosphorylation and degradation [21], and then challenged with cisplatin. Activation of caspase-2 was seen in the LMP1-negative cell cultures, and this was further enhanced in cells expressing LMP1 (Fig. 2A). Bay11-7082 completely suppressed the activation of both caspase-2 (Fig. 2A) and caspase-3 (Fig. 2B) in the LMP1-expressing cells. However, only a partial inhibition of the cisplatin-induced drop in MMP was seen (Fig. 2C). The latter findings suggest that NF-κB-mediated enhancement of apoptosis by LMP1 may transpire via mitochondria-dependent and -independent routes.

To provide further support for a role of NF-κB in this model, HeLa cells were transfected with a DN IκB mutant. Introduction of the DN IκB plasmid or the corresponding empty vector pRC/CMV did not alter the expression of LMP1 (Fig. 3A). However, activation of NF-κB by LMP1 was potently inhibited by expression of the IκB DN mutant (Fig. 3B). LMP1-expressing HeLa cells transfected with the DN IκB construct or control vector were

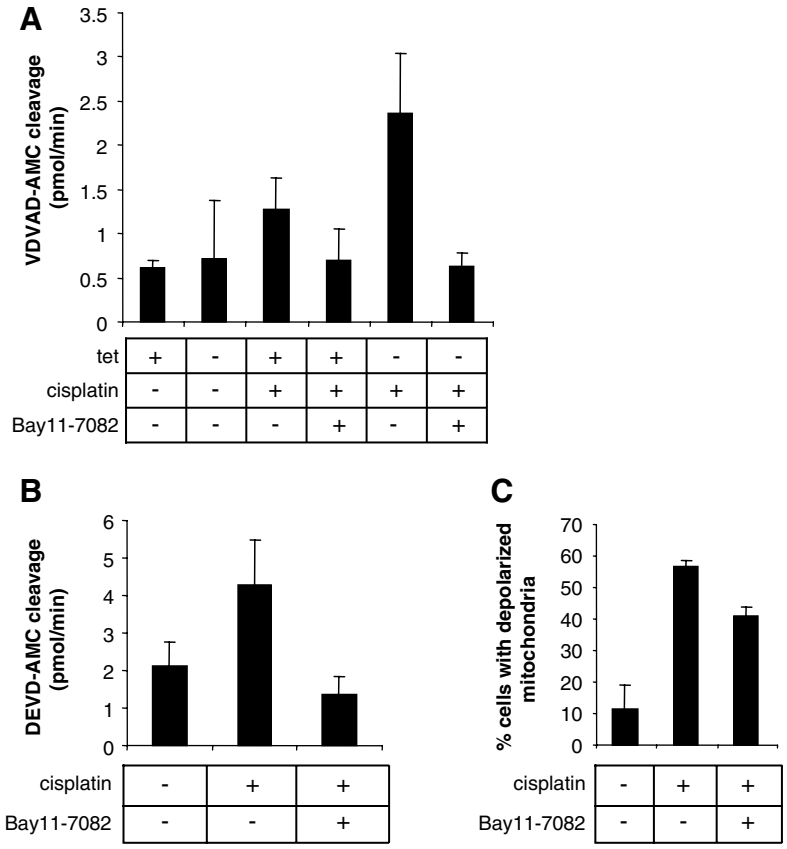


Fig. 2. LMP1-mediated enhancement of stress-induced caspase activation is blocked by the NF-κB inhibitor, Bay11-7082. (A) HeLa cells were cultured in the presence or absence of tetracycline (tet) and then pre-treated with Bay11-7082 (10 μM) for 30 min before administration of cisplatin (5 μg/ml) for 24 h. Caspase-2 activation was then quantified. (B) LMP1-expressing cells treated with Bay11-7082 (10 μM) or DMSO were challenged with cisplatin (5 μg/ml) for 24 h, and caspase-3-like enzyme activity was evaluated. (C) Cells treated as in B were harvested and the dissipation of MMP was assessed. Note the partial protection afforded by the NF-κB inhibitor, Bay11-7082. Data displayed in (A)–(C) are mean values ± SD ($n = 3$–4).

then challenged with cisplatin. As seen in Fig. 3C, cisplatin-triggered caspase-3 activity was completely blocked by the IκB DN construct. The dissipation of MMP, on the other hand, was only partially inhibited when the IκB DN construct was expressed (Fig. 3D). These results thus lend further support to the notion that the regulation of stress-induced apoptosis by LMP1 is, to some extent, NF-κB dependent.

## Discussion

We have previously demonstrated that LMP1 enhances chemotherapeutic drug-induced apoptosis in cell lines of epithelial origin [10]. The experiments reported herein suggest that both NF-κB and JNK signaling pathways contribute to the LMP1-dependent modulation of apoptosis and activation of caspases.

Caspase-2 has been shown to act as an upstream regulator of mitochondrial permeabilization induced by chemotherapeutic agents, such as etoposide and cisplatin [22]. Moreover, JNK, one of the stress-activated protein kinases, was previously found to contribute to caspase-2-dependent mitochondrial perturbation in the context of DNA damage-induced apoptosis [23]. Conversely, caspase-2 may act upstream of JNK, in doxorubicin-treated cells, leading to the alterations of mitochondria and execution of apoptosis [24]. In our study, a selective JNK inhibitor, SP600125, strongly reduced JNK activation in both LMP1-positive and -negative cells. This compound was therefore used to explore the role of JNK in apoptosis modulation. SP600125 suppressed cisplatin-triggered activation of caspase-2 and caspase-3, and also decreased the dissipation of MMP, most notably in LMP1-expressing cells. In fact, the protective effect was considerably less pronounced in LMP1-negative cells (cultivated in the presence of tetracycline), suggesting a specific JNK-dependent component in the LMP1-mediated enhancement of apoptosis.

NF-κB has been shown to initiate transcription of a number of genes with anti-apoptotic function, such as FLICE-inhibitory protein (FLIP) [25] and inhibitor of apoptosis proteins (IAPs), which serve as endogenous caspase inhibitors [26]. However, NF-κB activation may also promote apoptosis under certain conditions [27,28]. Hence, although LMP1 may inhibit apoptosis through the transcription of anti-apoptotic genes in some cell types [29], LMP1 could also enhance apoptosis, at least in part, via

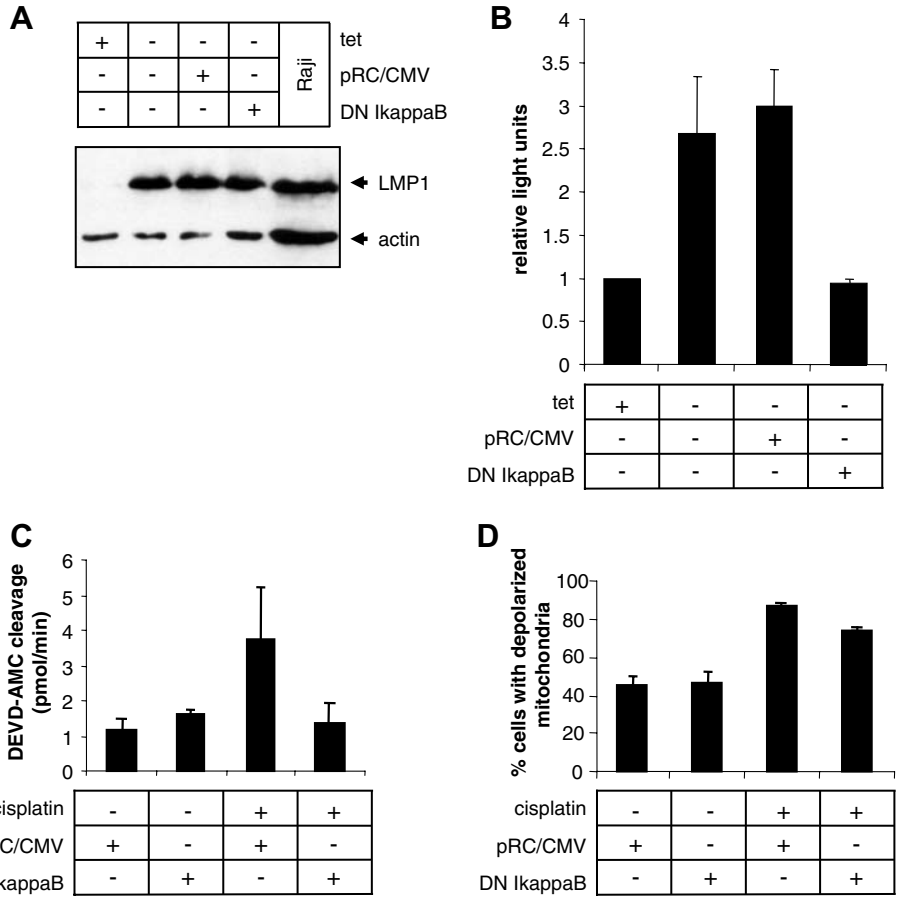


Fig. 3. Cisplatin-triggered activation of caspases is regulated by LMP1 in an NF-κB-dependent manner. (A) Hela cells were co-transfected with the pCMV βGal plasmid and the DN IκB plasmid or the corresponding control vector, pRC/CMV, and cultured in the presence or absence of tetracycline (tet) for 24 h. Tet-regulated LMP1 expression was then evaluated by immunoblotting using specific anti-LMP1 antibodies; anti-actin antibodies were used to control for equal loading of protein. BL-derived Raji cells were included as a positive control for LMP1 expression. (B) NF-κB-driven luciferase activity was determined in lysates of HeLa cell, co-transfected with the κB. ConA.LUC reporter and βGal plasmids, cultured as indicated, and calibrated on the basis of the βGal activity. Data shown are mean values ± SD ($n = 3$). (C) LMP1-expressing HeLa cells transfected with the control vector, pRC/CMV or the DN IκB plasmid were cultured in the absence or presence of cisplatin (5 μg/ml) for 24 h. Caspase-3-like enzyme activity was then quantified using a fluorogenic assay. (D) MMP was determined in LMP1-expressing HeLa cells incubated as described in C. Data shown in C and D are mean values ± SD ($n = 3$).

NF-κB activation. We found that cisplatin-induced apoptosis was reduced by DN IκB, when co-expressed with LMP1. The addition of Bay117082 also reduced caspase-2 and caspase-3 activation in LMP1-expressing cells challenged with cisplatin. This effect was not evident, however, in LMP1 non-expressing HeLa cells. These data suggest that the apoptotic regulation by NF-κB depends upon the expression of LMP1. In line with these findings, others have previously shown that NF-κB is required for cell death induction by LMP1 in 293T and Rat-1 cells [30]. However, the identity of the specific target genes that promote NF-κB-dependent apoptosis in LMP1-expressing cells remains to be determined.

In conclusion, our study provides evidence that EBV-encoded LMP1 can potentiate cisplatin-triggered apoptosis, and suggests that both JNK and NF-κB signaling pathways are involved. These findings could have implications for the clinical management of EBV-associated malignancies, such as nasopharyngeal carcinoma (NPC). Indeed, one may predict, on the basis of the current data that LMP1-positive tumors should be more responsive to treatment with drugs that activate the apoptosis pathways delineated above. Further investigations using, for instance, NPC-derived cell lines as a model system, are required to address this hypothesis.

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