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EBV Interferes With the Sensitivity of Burkitt Lymphoma Akata Cells to Etoposide

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ABSTRACT

Burkitt lymphoma (BL) commonly exhibits Epstein–Barr virus (EBV) positivity associated with latent chronic infection. Models of acute EBV infection have been associated with cellular resistance to apoptosis. However, the effect of latent long-term EBV infection on apoptosis induced by drugs is not well defined. To determine this, we have studied the response of the Akata EBV+ cell line (type I latency) to etoposide, before and after downregulating EBV gene expression. We observed that downregulating EBV nuclear antigen-1 (EBNA-1) expression with siRNAs reverted cellular sensitivity to etoposide. In accordance with this finding, Akata EBV+ cells showed increased sensitivity to etoposide, when compared to the Akata EBV− cells. We also observed that Akata EBV+ cells presented increased apoptosis levels and decreased *Bcl-xL* mRNA and protein levels, when compared to the Akata EBV− cells. In addition, Akata EBV+ cells contained less endoplasmic reticulum (ER) than EBV− cells. Finally, downregulation of EBV with EBNA-1 siRNAs caused an increase in the expression of Bcl-xL indicating that EBV is responsible for the differences found between the Akata EBV+ and EBV− cell lines. J. Cell. Biochem. 112: 200–210, 2011.

KEY WORDS: EBV; BURKITT LYMPHOMA; APOPTOSIS; ER; BCL-XL

The Epstein–Barr virus (EBV) is a gammaherpesvirus known to infect more than 95% of the human population [Morgan, 1992; Bornkamm, 2009b]. Infection with EBV usually occurs during childhood without giving rise to symptoms. After primary infection, EBV normally establishes a non-productive latent infection in B cells (latency), although it may periodically undergo lytic cycle producing progeny virus in a small percentage of cells [Roubalova et al., 1985; Leight and Sugden, 2000]. Latent EBV infection has been associated with several human malignancies such as Burkitt lymphoma (BL) (reviewed in [Rezk and Weiss, 2007]).

Several types of viral latency have been described, differing in the viral gene expression programme of the infected cell [Bornkamm, 2009a]. BL tumor cells present type I latency EBV infection, expressing a very restricted subset of viral genes such as the EBV nuclear antigen-1 (EBNA-1), the non-coding EBV-encoded RNAs (EBER-1 and -2), and the *Bam*HI-A rightward transcripts (BARTs). In contrast, EBV immortalized lymphoblasts present type III latency EBV infection expressing all the latent viral gene products which include all the EBNAs (-1, -2, -3A, -3B, -3C, and -LP), the latent membrane proteins (LMP-1, -2A, and -2B), the non-coding EBER-1 and -2 and BART transcripts [Tsurumi et al., 2005; Bornkamm, 2009a, b]. In addition, it is known that almost all BL cells maintained in culture convert viral infection from latency I to latency III [Rowe et al., 1986; Nanbo et al., 2002].

It is possible to observe different phenotypes in cells bearing different types of EBV latency. For example, while BL (latency I) cells are sensitive to induction of apoptosis by several factors, such as cytotoxic drugs, other cell lines such as lymphoblastoid cell lines (latency type III) are highly resistant to apoptosis [Bornkamm, 2009a].

EBV has been traditionally described as protecting cells from apoptosis, allowing survival of the virus in the infected cells [Komano et al., 1998; Nanbo et al., 2002; Kennedy et al., 2003; Sheng et al., 2003; Clybouw et al., 2005; Snow et al., 2006; Tafuku

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et al., 2006]. However, most of these studies were conducted in primary infections, using EBV negative B-cell lymphoma cell lines following infection with EBV, or following the introduction of EBV genes or its complete genome in cells. Furthermore, most studies showing EBV-related resistance to apoptosis were performed using cell lines with EBV in different types of latency, which do not mimic the latency I observed in BL tumors. Therefore, the role of long-term type I latency EBV infection on cellular response to apoptosis, namely on the response to a cytotoxic insult such as etoposide, remains unclear. In order to clarify this question, the response of the type I latency long-term infected cell line (Akata EBV positive, hereon referred to as Akata EBV+) to etoposide was analysed, before and after transfection of siRNAs for the latent viral protein, EBNA-1. We found that downregulation of EBNA-1 in Akata EBV+ cells decreased their sensitivity to etoposide. We also found that Akata EBV+ cells presented decreased *Bcl-xL* mRNA and protein levels, when compared to the Akata EBV negative (hereon referred to as Akata EBV−) cells. *Mcl-1* mRNA was also decreased in the EBV+ cell line. We also noted that Akata EBV+ cells show ultrastructural morphologic differences from the Akata EBV− cells, namely less abundance of ER. In addition, downregulating EBNA-1 expression with siRNAs in Akata EBV+ cells increased expression of Bcl-xL but not of Mcl1.

MATERIALS AND METHODS

CELL CULTURE

Akata EBV+ and Akata EBV− cells were kindly provided by Professor Kenzo Takada, who had previously established the cell lines [Takada and Ono, 1989; Shimizu et al., 1994]. Upon arrival, both cell lines were genotyped at IPATIMUP, which confirmed the same genetic background. Furthermore, EBV DNA was detected in the EBV+ cells but not in the EBV− cells. Both cell lines were routinely cultured in RPMI 1640 with Glutamax supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen) and maintained in a humidified incubator at 37°C with 5% CO_2 in air. Cell number and viability were determined with the Trypan Blue exclusion assay.

TRANSFECTION OF siRNAS AND TREATMENT WITH ETOPOSIDE

For the siRNA experiments, all siRNAs were purchased from Qiagen and resuspended in siRNA suspension buffer, according to the manufacturer's instructions. siRNAs duplex with dTdT 3′overhangs were used for the *EBNA-1* mRNA target sequence 5′-GGAGTTC-CAACCCGAAAT-3′ [Yin and Flemington, 2006]. The All Stars negative control siRNA was used and the treatments with this control siRNA are referred to as negative control siRNAs (CRNAi).

Akata EBV+ cells (5×10^5 cells/well in 24-well plates) were transfected with siRNAs designed for EBNA-1 or with AllStars Negative Control siRNA (Qiagen) using Lipofectamine and Plus Reagent (Invitrogen). The manufacturer's instructions were followed with minor adaptations, using 200 nM siRNAs, 4 μl of both Lipofectamine and Plus Reagent and without FBS during the initial 3 h of transfection. Following this period, 200 μl of medium with enough FBS to bring final FBS concentration to 10% was added to each well, and cells were further incubated. New medium was added to the cells 24 h after transfection. Cells were processed at 24 h, 48 h, or 72 h for protein or RNA extraction.

In the experiments to investigate the response to etoposide following transfection with siRNAs, 20 nM etoposide or its solvent (DMSO) were added to the Akata EBV+ cells 24 h after being transfected with control or EBNA-1 siRNAs. Viability was assessed 48 h after treatment with etoposide, using the Trypan Blue exclusion assay.

To study cellular response to etoposide, 5×10^5Akata cells were treated for 48 h with Etoposide (10–150 nM), with DMSO (solvent) or with medium alone (no drug). Cell growth and viability studies were performed using the Trypan Blue Exclusion assay.

RNA EXTRACTION, REVERSE TRANSCRIPTION, AND REAL-TIME PCR

Total RNA was extracted from both cell lines using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. cDNA was synthesized using 1 μg of total RNA and random hexanucleotide primers (Invitrogen), using 20 U of M-MULV reverse transcriptase (Fermentas), as indicated by the manufacturer. Real-time PCR reactions were performed in a 20 μl mixture containing a diluted aliquot of the cDNA preparation, 1× Power SYBR® Green PCR Master Mix (Applied Biosystems) or the Fast SYBR® Green Master Mix (Applied Biosystems) and the respective set of primers. The primer sequences and concentrations used were the following: *EBNA-1* forward 5′-CGCATCATAGACCGCCAGTA-3′ and reverse 5′-CTGGCCCCTCGTCAGACAT-3′ [Hong et al., 2006], 150 nM of each primer; *Bcl-xL* forward 5′-TTCCTGACGGGCATGATC-3′ and reverse 5′-AGGATGTGGTGGAGCAGA-3′ [Aerbajinai et al., 2003], 300 nM of each primer; *Mcl-1* forward 5′-TAAGGACAAAAC-GGGACTGG-3′ and reverse 5′-ACCAGCTCCTACTCCAGCAA-3′ [Schulze-Bergkamen et al., 2006], 300 nM of each primer; *Hprt1* forward 5′-GCAGACTTTGCTTTCCTTGGTCAG-3′ and reverse 5′-GTCTGGCTTATATCCAACACTTCGTG-3′ [Almeida et al., 2008], 150 nM of each primer; *18S* forward 5′-CGCCGCTAGAGGT-GAAATTC-3′ and reverse 5′-CATTCTTGGCAAAATGCTTTCG-3′, 300 nM of each primer. The amplifications for the reactions using Power SYBR® Green PCR Master Mix were carried out as follows: initial enzyme activation at 95°C for 5 min, 40 cycles at 95°C for 15 s and 60°C for 1 min. For the reactions using Fast SYBR® Green Master Mix the manufacturer's instructions were used. Serial dilutions of the Akata cells cDNA were used for the standard curve calculation. For each primer, a dissociation curve was generated and a single peak obtained; additionally, the PCR efficiency was estimated as being always higher than 90%. Relative *Bcl-xL* and *EBNA-1* mRNA levels were calculated after normalization with the levels obtained for the *18S* and *Hprt1* mRNAs, respectively.

miRNA EXPRESSION ANALYSIS

For miRNA expression analysis, RNA was extracted using TRI Reagent® following the manufacturer's instructions, except for the use of chloroform instead of 1-bromo-3-chloropropane.

Quantitative PCR (qPCR) was carried out with the miScript System (Qiagen). All procedures were performed according to the instructions provided by the manufacturer. Briefly, cDNA was obtained from 1 μg RNA using the reverse transcription (RT) reaction which contained 1 μl miScript Reverse Transcriptase Mix, 4 μl 5× miScript

RT Buffer and 10 μl RNase-free water. Reaction was performed at 37°C for 60 min and then at 95°C for 5 min. qPCR reaction contained 12.5 μl of 2× QuantiTect SYBR Green PCR Master Mix, 2.5 μl miScript universal primer (10×), 2.5 μl specific primer (10×), 1 μl cDNA and 6.5 μl RNase-free water and conditions were the following: 95°C for 15 min and 40 cycles at 94°C for 15 s, 55°C for 30 s and 70°C for 30 s. The primers used, purchased from Qiagen, were specific for this system of miRNA expression analysis: let-*7c* (Hs_let-7c_1) and U6 (Hs_RNAU6B_2, used as control). For each primer, serial dilutions of the cDNA from the Akata cells were used for the standard curve determination. Additionally, a dissociation curve was generated and a single peak obtained. Reactions from three independent experiments were run in duplicate and negative control reactions without RT or template were included.

PROTEIN ISOLATION, QUANTIFICATION, AND WESTERN BLOT

Total protein lysates were obtained after lysing the cells in RIPA complete buffer [50 mM Tris–HCl pH7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40 complemented with 0.1% SDS, 1× EDTA-free protease inhibitor cocktail (Roche) and 1× Phosphatase Inhibitor Cocktail 1 (Sigma)] for 30 min at 4°C. All determinations of protein content were based on the method of Bradford and were carried out by using the Bio-Rad Protein Assay (Bio-Rad), according to the manufacturer's instructions.

To analyse protein expression by Western blot, the same amount of protein (20 μg) per well was loaded into a 12% Bis-Tris gel [Sambrook et al., 1982] and subjected to SDS–PAGE electrophoresis. The separated proteins were transferred electrophoretically to a Amersham nitrocellulose membrane (GE Healthcare) and incubated with the following primary antibodies: rabbit Bcl-xL antibody (1:200, Santa Cruz Biotecnology), rabbit Calnexin antibody (1:500; Stressgen), C/EBP homologous protein (CHOP) antibody (1:200, Santa Cruz Biotechnology), rabbit PARP antibody (1:4,000, Santa Cruz Biotechnology), mouse EA-D antibody (1:1,000, Millipore), mouse Zta monoclonal antibody (1:100, Argene), mouse EBNA-1 monoclonal antibody (1:100, Millipore) or goat Actin antibody (1:2,000, Santa Cruz Biotechnology). The membrane was then incubated with one of the following secondary antibodies: goat anti-mouse IgG-HRP (1:2,000, Santa Cruz Biotechnology), goat anti-rabbit IgG-HRP (1:2,000, Santa Cruz Biotechnology) or donkey anti-goat IgG-HRP (1:2,000, Santa Cruz Biotechnology). Afterwards, the signal was detected with the ECL Western Blotting Detection Reagents (GE Healthcare), the Amersham Hyperfilm ECL (GE Healthcare) and the Kodak GBX developer and fixer twin pack (Sigma) as previously described [Lima et al., 2004].

TUNEL ASSAY

To study apoptosis, the TUNEL assay was carried out using the "in situ cell death detection kit – fluorescein" (Roche) as previously described [Lima et al., 2006]. Apoptosis was assessed at 0 h (cells undergoing exponential growth) and 48 h after centrifuging the cells and replacing the medium. Briefly, cells were fixed in 4% paraformaldehyde and cytospins were then prepared. Cells were permeabilized in ice-cold 0.1% Triton X-100 in 0.1% sodium citrate and incubated with TUNEL reaction mixture (enzyme dilution 1:20). Slides were mounted in Vectashield Mounting Media with DAPI (Vector Laboratories) and cells were observed in a DM IRE 2 microscope (Leica). A semi-quantitative evaluation was performed by counting a minimum of 500 cells per slide. Results are from at least three independent experiments.

TREATMENT OF THE CELLS WITH z-VAD AND MG132

Akata cells (5×10^5/well) were plated in 24-well plates and incubated for 48 h. In the experiments to assess the role of caspases in Bcl-xL protein expression, 50 μM z-VAD-fmk (Bachem AG) or the respective volume of methanol (solvent) were added at 0 h or 24 h after plating. In the experiments to study the role of proteasomal inhibition in Bcl-xL protein expression, 10 μM MG132 (Calbiochem) or the respective volume of DMSO (solvent) were added at 1 h, 2 h, or 4 h prior to cell lysis. In both cases, 48 h after the beginning of the experiment, cells were processed for protein analysis by Western Blot, as described above.

MITOCHONDRIA STAINING AND IMMUNOFLUORESCENCE FOR BCL-xL

To visualize mitochondria, mitochondria were stained with 250 nM Mitotracker Red CMX-Ros (Molecular Probes) for 30 min at 37°C. After being washed with PBS, cells were fixed in 4% paraformaldehyde in PBS and slides were prepared by cytospin.

To visualize Bcl-xL protein expression, cells were fixed in 4% paraformaldehyde and, after cytospin, incubated for 10 min in 50 mM ammonium chloride in PBS. Cells were then permeabilized by treatment with ice-cold 0.2% Triton X-100 in PBS for 5 min. Non-specific antibody binding was blocked by PBS, pH 7.4, containing 5% BSA for 30 min. Slides were incubated with a rabbit antibody for Bcl-xL (sc7615, Santa Cruz Biotechnology) diluted 1:50 in blocking buffer, for 1 h 30 min at room temperature. Cells were then washed with PBS and labeled with Alexa-Fluor 488 goat anti-rabbit IgG antibody (1:500; Invitrogen) for 1 h at room temperature.

All slides were mounted in Vectashield Mounting Media with DAPI (Vector Laboratories). Cells were observed and digital images including overlays were captured with fluorescence microscope ZEISS Axio Imager.Z1 coupled with ApoTome Imaging System.

ELECTRON MICROSCOPY

Akata EBV+ and EBV− cell lines were fixed in 2.1% glutaraldehyde and 2% formaldehyde in phosphate buffer (pH 7.4) at room temperature. After at least 30 min the cells were washed with 0.1 M cacodylate buffer (pH 7.4) and then fixed at room temperature for 2 h with 1% OsO_4 in sodium cacodylate buffer (pH 7.4) at 4°C. Samples were then washed with water and further fixed in 0.5% uranyl acetate for 16 h at 25°C. After dehydration in ethanol the samples were embedded in Epon and ultrathin sections were cut with a Leica Reichert SuperNova ultramicrotome.

Routinely, the sections were doubly contrasted with uranyl acetate-lead citrate. Observations and micrographs were done with a Zeiss EM 902A at 80 kV.

STATISTICAL ANALYSIS

Results are expressed as mean ± SE. Statistical differences were determined using paired Student's *t*-test. A $P \leq 0.05$ was considered statistically significant.

RESULTS

DOWNREGULATION OF EBNA-1 DECREASES SENSITIVITY OF Akata EBV+ CELLS TO ETOPOSIDE

In order to assess the effect of latent EBV infection on cellular response to a cytotoxic insult, the viral EBNA-1, a latent viral protein responsible for the maintenance and replication of the viral genome in the EBV+ cells [Humme et al., 2003], was silenced in the Akata EBV+ cells [Takada and Ono, 1989; Shimizu et al., 1994]. For such, Akata EBV+ cells were transiently transfected (following several procedures for optimization of transfection protocol, Supplementary Fig. 1) with siRNAs designed towards the *EBNA-1* mRNA or CRNAi. To confirm the silencing effect, EBNA-1 mRNA and protein expression levels were analysed by qPCR and Western Blot, respectively. The silencing of EBNA-1 was confirmed 48 h after transfection, at the mRNA level by qPCR analysis (Fig. 1A). Further confirmation was attempted by Western Blot, but the commercially available antibodies did not provide good signal (Fig. 1B). Therefore, further confirmation of EBNA-1 silencing was possible by looking at some viral lytic proteins, which are expressed following etoposide treatment. We did this since we had evidence that etoposide may induce lytic reactivation in Akata cells (submitted for publication),

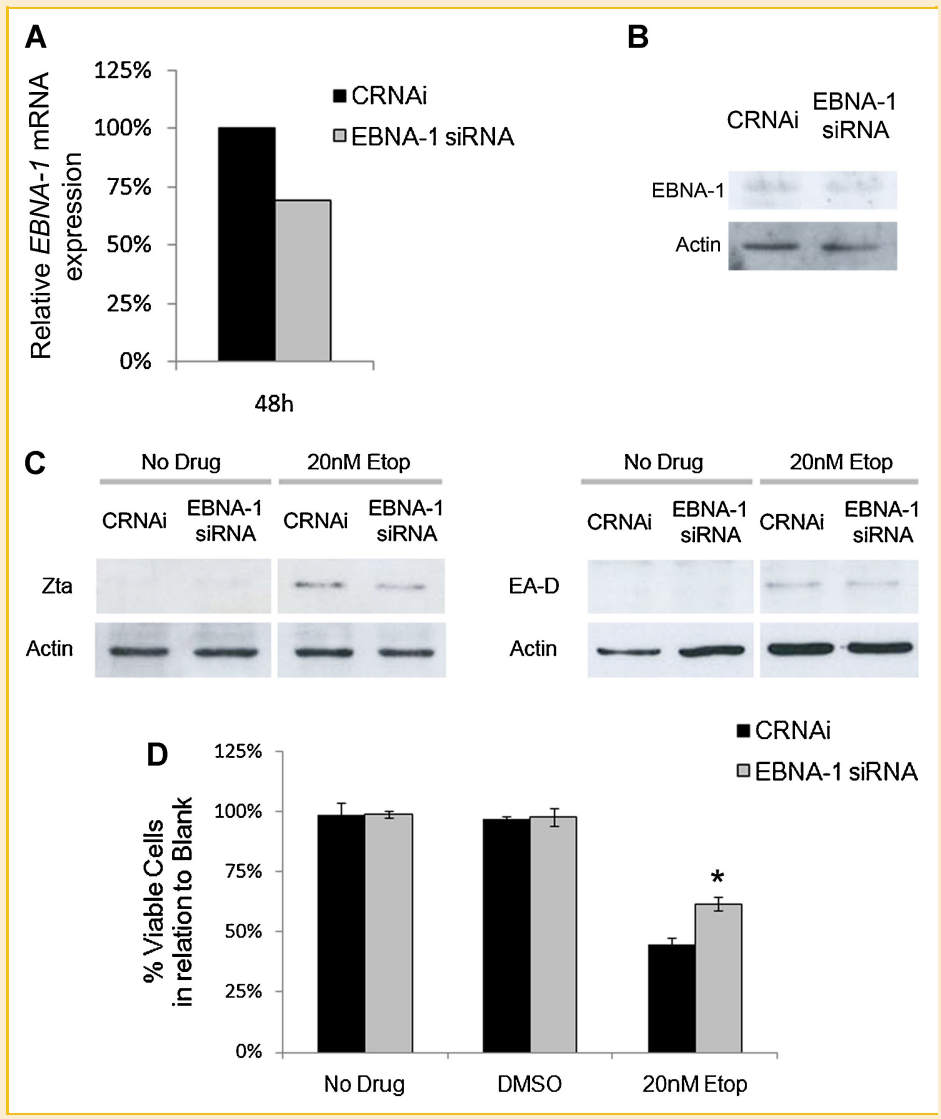

Fig. 1. Downregulation of EBNA-1 decreases Akata EBV+ cells sensitivity to etoposide. Akata EBV+ cells were transfected with EBNA-1 siRNA. A: Real time qPCR analysis of *EBNA-1* mRNA levels in Akata EBV+ cells 48 h following transfection with CRNAi or with siRNAs for EBNA-1. Results, expressed after normalization of the *EBNA-1* values with the values obtained for *Hprt1*, were analysed as % of the control cells (transfected with CRNAi) and represent the mean of two independent experiments performed in triplicate. B: Western Blot analysis of the EBNA-1 and Actin (as loading control) protein expression 72 h following transfection with CRNAi or EBNA-1 siRNAs. C: Western Blot analysis of the induction of expression of viral lytic protein, Zta and EA-D, 72 h following transfection with CRNAi or EBNA-1 siRNAs and 48 h after treatment with 20 nM etoposide. Results are representative of 3 independent experiments. D: Effect of the CRNAi or of the EBNA-1 siRNAs on the response of Akata EBV+ cells to etoposide. Viable cell number was counted with Trypan Blue, 72 h after transfection with CRNAi or EBNA-1 siRNAs and 48 h after treatment with 20 nM Etoposide, DMSO (control) or medium only (i.e., no drug, Blank). Results are represented as a % of the Blank cells considering this as 100%. Results are the mean ± SE of three independent experiments. $^{*}P \leq 0.05$ between the cells transfected with EBNA-1 siRNA and the cells transfected with CRNAi, following treatment with etoposide.

even though other studies which use different concentrations from the one we used (and different cell lines) have not shown such reactivation [Wang et al., 2009]. Western blot analysis of protein extracts 72 h following transfection (48 h after treatment with the drug) showed reactivation of EBV lytic cycle in cells transfected with CRNAi, but much less reactivation in cells transfected with EBNA-1 siRNAs (Fig. 1C). This is observed by the detection of an immediate early viral EBV protein (Zta) and also of an early lytic protein (EA-D) following treatment with the IC50 concentration of etoposide (20 nM).

Once the decrease in EBNA-1 (and other EBV proteins) was confirmed, it was possible to evaluate its effect on cellular response to apoptosis. Etoposide was the drug chosen to promote the apoptotic stimuli, given the fact that this cytotoxic drug is frequently used in the treatment of several cancers, including BL [Perkins and Friedberg, 2008]. The viable cell number 72 h after transfection with the siRNAs (i.e., 48 h after treatment with etoposide) was assessed using Trypan Blue exclusion assay (Fig. 1D). As expected, transfection with EBNA-1 siRNAs or CRNAi had no effect on cellular viability. Also as expected, etoposide treatment (with IC50) of cells previously transfected with CRNAi caused a reduction in the viable cell number to approximately 45%. However, when the cells had been previously transfected with siRNA for EBNA-1, the reduction in the number of viable cells was much lower (to 61%). This indicates that silencing EBNA-1 decreases cellular sensitivity to etoposide.

In order to confirm these findings we decided to compare the effect of etoposide in the Akata EBV+ cell line to the effect in the Akata EBV− cell line, which had been previously obtained by others by serial dilution of the parental Akata EBV+ [Takada and Ono, 1989; Shimizu et al., 1994]. The effect of etoposide on the viable cell number was analysed 48 h after treatment with increasing concentrations of this drug (10, 20, 50, 100 or 150 nM), by counting the number of viable cells after staining with Trypan Blue. As expected, a reduction in the number of viable cells was observed for both cell lines after treatment with increasing concentrations of etoposide. Moreover, the Akata EBV+ cells showed increased sensitivity to etoposide when compared to the Akata EBV− cells (Fig. 2). In fact, the IC50 concentration of etoposide was 20 nM for the Akata EBV+ cell line, while for the Akata EBV− cell line the IC50 concentration of etoposide was 75 nM. This confirmed the previous result obtained with siRNAs in the Akata EBV+ cell line. Therefore, the presence of latent EBV seems to sensitize these EBV+ cells to the effects of etoposide. Furthermore, by using another drug that induces viral reactivation, we confirmed that this difference in the response of the EBV+ and EBV− cells to etoposide was not solely due to the viral reactivation effects, since the other drug induced a much stronger reactivation but not such a strong inhibition in cellular growth (data not shown, in preparation for publication).

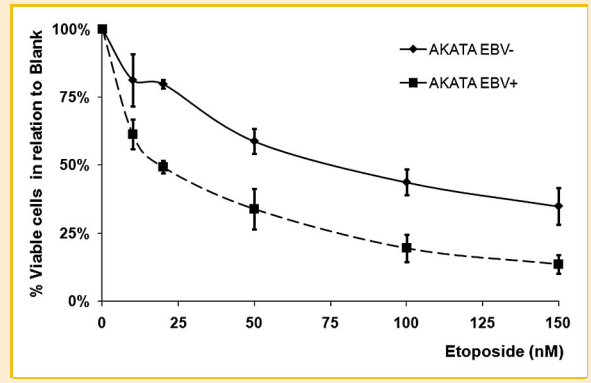

Fig. 2. Akata EBV+ cells are more sensitive to etoposide than Akata EBV− cells. Akata EBV− (solid line) and Akata EBV+ (dashed line) cell lines were treated with increasing concentrations of etoposide (0, 10, 20, 50, 100 or 150 nM). Viable cell number was counted with Trypan Blue, 48 h after treatment with the drug. Results were analysed as a percentage of the Blank cell number (cells treated with medium only), considering this as 100% and are the mean ± SE of at least three independent experiments.

Akata EBV+ CELLULAR BASAL LEVELS OF CLEAVED PARP ARE INCREASED AND OF Bcl-xL ARE DECREASED

Given that etoposide promotes cell death via the apoptotic mitochondrial pathway [Decaudin et al., 1997], we decided to investigate possible differences concerning the basal apoptotic levels of the two cell lines. With this purpose, apoptosis was verified with the TUNEL assay in cells undergoing exponential growth (0 h) as well as 48 h following centrifugation of the cells and incubation with new medium (Fig. 3A). Results showed that, at the 48 h timepoint, Akata EBV+ cells had higher basal levels of apoptosis than the Akata EBV− cells. Consistent with this result, increased expression of the cleaved form of PARP was detected in the Akata EBV+ cells compared to the EBV− cells, both in cells in exponential growth (0 h) and 48 h following centrifugation of those cells and incubation with new medium (Fig. 3B and Supplementary Fig. 2A).

Considering the differences observed in the basal levels of cleaved PARP in the two cell lines, the expression of some apoptotic proteins, namely Bcl-xL, Bcl-2 and Mcl-1, were determined. We observed a statistically significant decrease ($P < 0.05$) in Bcl-xL expression in the Akata EBV+ cells when compared to the Akata EBV− cells (Fig. 3C and Supplementary Fig. 2B). This difference was observed by Western Blot both at 0 h (cells in exponential growth) and at 48 h (following centrifugation and incubation with new medium) and was further confirmed at 0 h by immunofluorescence (Fig. 3D). Regarding the results obtained for Bcl-2 and Mcl-1 proteins expression levels, the differences observed between the two cell lines were not consistently reduced nor were they conclusive (data not shown).

Given that decreased protein expression may be due to increased proteasomal degradation, we investigated whether treatment with proteasome inhibitor MG132 would increase the levels of Bcl-xL, in the EBV+ cell line but not in the EBV− cell line. Results show that for both cell lines and for all the periods of treatment tested, treatment with MG132 did not result in an increase in Bcl-xL protein expression (Fig. 3E). This result indicates that proteasomal degradation was not responsible for the difference in Bcl-xL protein levels observed between the two cell lines.

Another possible cause for lower Bcl-xL levels in the Akata EBV+ cells could be caspase activation [Clem et al., 1998; Fujita et al.,

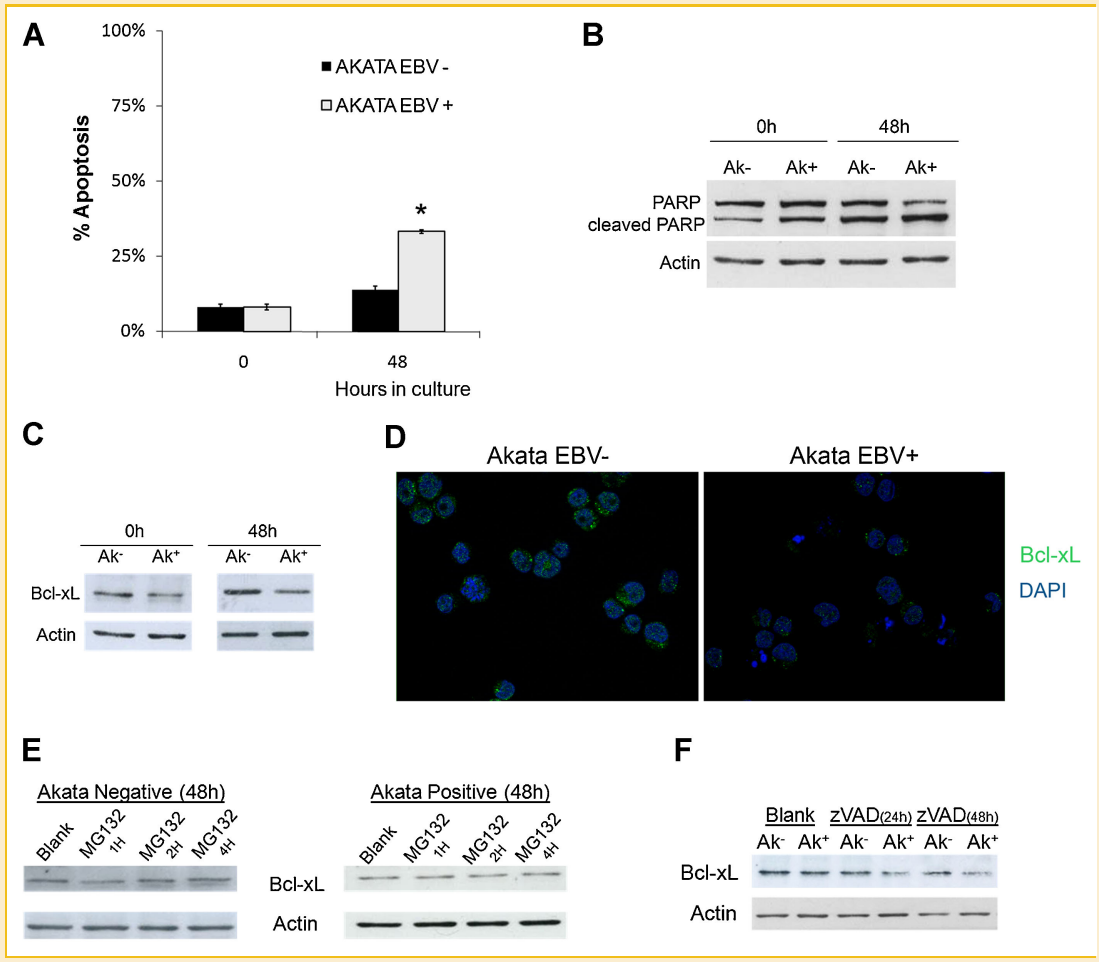

Fig. 3. Akata EBV+ cellular basal levels of cleaved PARP are increased and of Bcl-xL are decreased. A: Increased percentage of apoptotic Akata EBV+ cells 48 h following centrifugation and incubation with new medium, when compared to the Akata EBV− cells, analysed by TUNEL Assay. Results are the mean ± SE of three independent experiments. *represents $P \leq 0.05$ between the Akata EBV− cells and the Akata EBV+ cells. B: Increased cleavage of PARP in the Akata EBV+ cells, compared to Akata EBV− cells. The expression of PARP and its cleaved form in cells in exponential growth (0 h) and in cells 48 h following centrifugation and incubation with new medium (48 h) was analysed by Western Blot. C: Decreased Bcl-xL expression in Akata EBV+ cells analysed by Western blot. D: Immunofluorescence analysis showing the decreased expression of Bcl-xL (green:Alexa fluor488) in Akata EBV+ cells. Cell nuclei were stained with DAPI (blue). E: Western blot analysis of Bcl-xL and Actin (as loading control) expression in Akata EBV− and EBV+ cells 48 h following incubation in new medium and after treatment with 10 μM MG132 for 1 h, 2 h, or 4 h. F: Western blot analysis of Bcl-xL and Actin (as loading control) expression in Akata EBV− and EBV+ cells 48 h following incubation in new medium and after treatment with 50 μM z-VAD for 24 h and 48 h. All blots are representative of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

1998]. Indeed, since Akata EBV+ cells showed increased basal levels of cleaved PARP, we hypothesized that caspase cleavage could be the cause for the lower Bcl-xL protein levels observed in these cells. To investigate this, cells were treated with the pan-caspase inhibitor, zVAD, for 24 h and 48 h. We could clearly observe a decrease in the levels of PARP cleaved form after treating both cell lines with z-VAD (data not shown), thus indicating the successful blockage of caspase cleavage. However, Bcl-xL expression did not increase in the Akata EBV+ cells following treatment with z-VAD. Indeed, even after treatment with z-VAD, Bcl-xL protein expression was always higher in the Akata EBV− cells than in the EBV+ cells (Fig. 3F). Even though there is an apparent decrease in Bcl-xL levels in the Akata EBV+ cells following treatment with z-VAD, this was also observed in the EBV− cells and the relative expression of Bcl-xL in both cell lines is not reverted, as it would be expected if Bcl-xL was degraded by caspases. This result indicates that the lower Bcl-xL levels observed in the Akata EBV+ cells are not a result of the higher level of apoptosis continuously observed in this cell line.

Methylation of CpG islands in promoter regions has been associated with the transcriptional silencing of genes [Bird, 1996]. Furthermore, EBV has been implicated in the methylation status of cellular genes [Flanagan, 2007; Paschos et al., 2009]. In this study, we also sought to verify whether there were alterations in the methylation pattern of the *Bcl-xL* promoter in Akata EBV+ cells that could justify its downregulation. In order to do this, genomic DNA was extracted from both cell lines. After bisulfite treatment, the region of CpG island was amplified and PCR products were cloned and further sequenced to verify differences in their methylation status. Results did not show alterations in the methylation pattern of the DNA region studied (data not shown), thereby indicating that

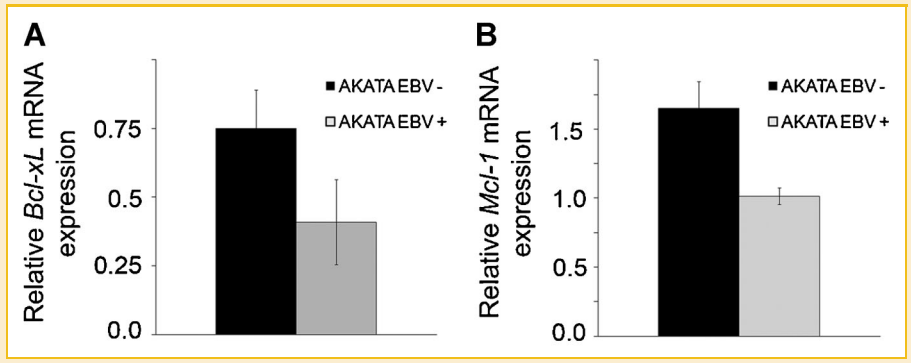

Fig. 4. Decreased Bcl-xL expression in Akata EBV+ cells is already observed at the mRNA level. Real-time qPCR analysis of *Bcl-xL* (A) and *Mcl-1* (B) mRNA levels in Akata EBV− and EBV+ cells. Results, expressed after normalization of *Bcl-xL* or *Mcl-1* values with the values obtained for *18S*, represent the mean ± SE of three independent experiments performed in triplicate.

this is probably not the mechanism responsible for the down-regulation of Bcl-xL found in the Akata EBV+ cells.

Bcl-xL AND Mcl-1 mRNA LEVELS ARE REDUCED IN THE AKATA EBV+ CELLS

Bcl-xL, *Bcl-2*, and *Mcl-1* mRNA levels were analysed by real-time PCR (qPCR) using mRNA extracted from both cell lines (Akata EBV+ and EBV−) in exponential growth. The relative levels of *Bcl-xL* mRNA were lower in the Akata EBV+ cell line than in the EBV− cell line (Fig. 4A). This result not only confirmed the differences previously observed for the two cell lines at the protein level, but also confirmed that those differences can also be observed at the transcriptional level. Regarding *Mcl-1*, the mRNA levels were also lower for the Akata EBV+ cells (Fig. 4B), while for *Bcl-2* mRNA no differences were found between the cell lines (data not shown).

Lrt-7c miRNA IS DOWNREGULATED IN THE AKATA EBV+ CELLS

The levels of *let-7c* miRNA were analysed by qPCR using appropriate primers. Surprisingly, the Akata EBV+ cells had lower *let-7c* miRNA expression than the Akata EBV− cells (fold change >7, data not shown).

AKATA EBV+ CELLS PRESENT STRUCTURAL ALTERATIONS, WHEN COMPARED TO THE AKATA EBV− CELLS, ASSOCIATED WITH THE MITOCHONDRIA AND THE ENDOPLASMIC RETICULUM

Bcl-xL is normally localized in the endoplasmic reticulum (ER) as well as in nuclear membranes and in mitochondria [White et al., 2005; Fiebig et al., 2006]. In order to study if the differences previously observed in total Bcl-xL levels between the two cell lines were also observed at the mitochondria, mitochondrial extracts were prepared following subcellular fractionation. Surprisingly, there was no apparent notable difference between the levels of Bcl-xL in the mitochondrial extracts of both cell lines (Supplementary Fig. 3). Intrigued with this observation, we decided to observe the mitochondria of both cell lines using the mitochondrial dye, Mitotracker Red (Fig. 5A). A more diffuse mitochondrial labeling was observed in the Akata EBV− cells, when compared to a more aggregated labeling observed in the Akata EBV+ cells. These differences could be in the number of mitochondria, in the aggregation of mitochondria or even in mitochondrial integrity but this was not further clarified in this study. However, we analysed the expression levels of some mitochondrial proteins (SDHA, ND1, NDUF3) present in total protein lysates of both cell lines, but found no evidence for the possibility of EBV+ cells harbouring less mitochondria (Supplementary Fig. 4).

To further understand the differences between the two cell lines we observed structural differences by electron microscopy. Surprisingly, the major difference observed between the two cell lines was related to the ER. In fact, the Akata EBV+ cells had consistently less ER than the Akata EBV− cells (Fig. 5B). In order to confirm the observation that the levels of ER were indeed decreased in the Akata EBV+ cell line when compared to the Akata EBV−, the expression of proteins associated with the ER, such as Calnexin and CHOP (involved in the unfolded protein response - UPR), were analysed by Western Blot. Figure 5C shows an evident reduction in the levels of both these proteins in the Akata EBV+ cells when compared to the EBV− cells, thereby confirming the previous observation that ER is less abundant in the Akata EBV+ cell line.

DOWNREGULATION OF EBNA-1 RESULTS IN AN INCREASE IN THE BCL-xL EXPRESSION

The results shown so far demonstrate that Akata EBV+ cells have increased sensitivity to etoposide, decreased Bcl-xL expression and decreased amount of ER, when compared to the Akata EBV− cells. To conclude whether these differences between the two cell lines were due to the presence (or absence) of the virus, the effect of silencing EBNA-1 on the expression of Bcl-xL protein in Akata EBV+ cells was evaluated. Figure 6A shows an increase in the levels of the *Bcl-xL* mRNA 48 h following transfection with EBNA-1 siRNAs. This was further confirmed by analysing the expression of Bcl-xL protein at 48 h and 72 h following transfection by Western Blot (Fig. 6B) and also at 72 h by immunofluorescence (Fig. 6C). Therefore, silencing EBNA-1 caused an increase in the expression of Bcl-xL.

DISCUSSION

There is a strong association of EBV with BL. In fact, BL was the first human cancer for which a viral association has been shown,

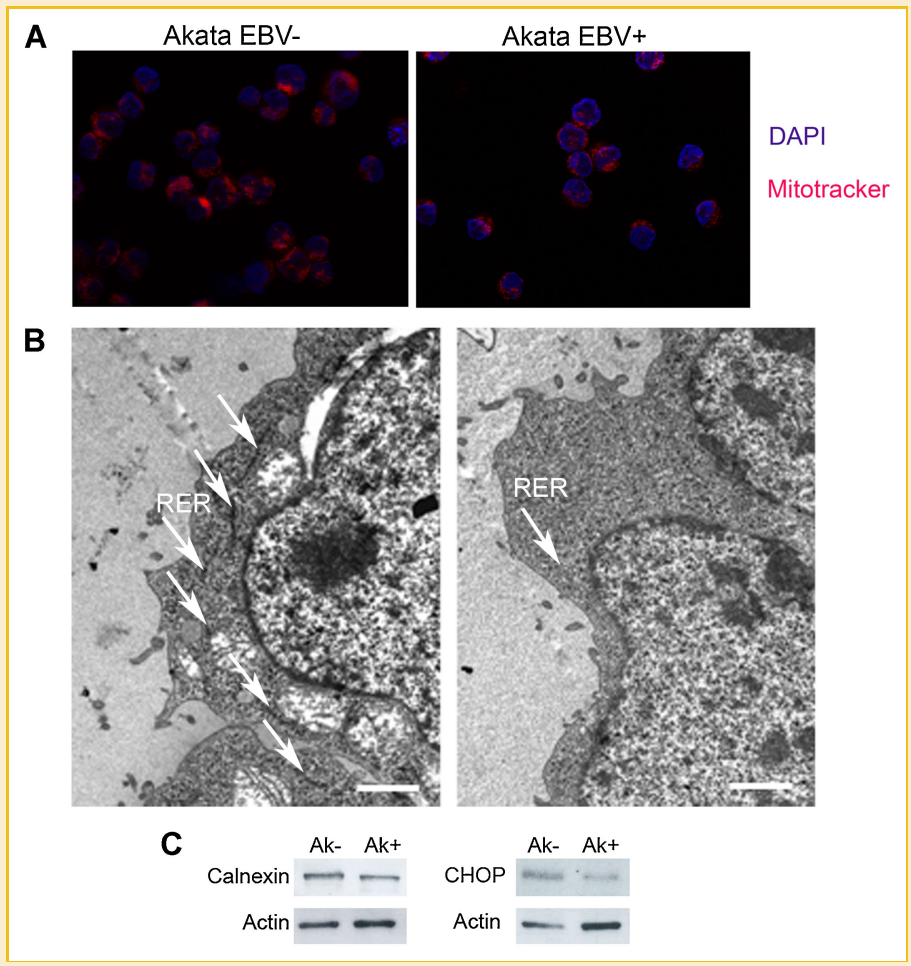

Fig. 5. The Akata EBV+ cells present structural alterations when compared to the Akata EBV− cells. A: Fluorescence microscopy of Akata EBV− and EBV+ cells showing Mitotracker Red stained mitochondria (Red) and DAPI stained nuclei (Blue). B: electron microscopy of Akata EBV− and EBV+ cells, showing a stronger presence of ER in the Akata EBV− cells than in the the Akata EBV+ cells. The arrows indicate the ER. Bar = 1,000 nm. C: Western Blot analysis showing the expression of the ER associated proteins, Calnexin, and CHOP, in total cell extracts from Akata EBV− and EBV+ cells. Blots are representative of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

reviewed in [Pattle and Farrell, 2006; Kelly and Rickinson, 2007]. However, the features of the virus and its ability to acquire different types of latency have presented a challenge to the development of strategies to overcome the effects of EBV infection. The Akata cell lines (EBV+ and EBV−) were chosen for the present study since Akata EBV+ cells retain latency I infection after long-term culture *in vitro*, thereby resembling BL tumors [Rowe et al., 1986; Rowe et al., 1987; Nanbo and Takada, 2002]. Also, for the purpose of comparison between EBV+ cells and its EBV− variants, these cells are probably the best experimental system available [Bornkamm, 2009b]. EBV gene expression in Akata EBV+ cells mimic the infection found in BL tumors, in which most of the EBV genes are not expressed and only one viral protein, EBNA-1, is required for virus maintenance (latency type I). Indeed, EBNA-1 protein is required for episomal maintenance and it is expressed in all EBV+ tumors [Leight and Sugden, 2000; Hung et al., 2001; Bornkamm, 2009b].

The present results show that EBNA-1 (or another latter expressed viral protein) affects cellular response to etoposide. Indeed, although the downregulation of EBNA-1 expression with siRNAs in Akata EBV+ cells caused no effect on cellular viability, it clearly decreased the sensitivity of Akata EBV+ cells to etoposide. Likewise, when comparing the response of the Akata EBV+ with the EBV− cell line, we also observed that the presence of EBV increases sensitivity to etoposide. These results were, at a first glance, unexpected, because EBV+ cells have been described as being resistant to apoptosis and this being related to the presence of EBV [Komano et al., 1998]. However, much of the work showing that EBV renders cells more resistant to various apoptotic stimuli resulted from work in cells following primary infection or following the expression of some viral gene products [Fagard et al., 2002; Clybouw et al., 2005; Snow et al., 2006]. Several of those studies have been conducted in Akata EBV+ cells obtained after re-infection of Akata EBV− cells (which had lost the virus). Therefore, those studies do not reflect how EBV affects cells and their response to apoptosis after a long-term period of infection. In fact, other studies are in agreement with our results, such as the studies conducted with TSA, in which Akata EBV+ cells were more prone to apoptosis than EBV− cells [Kook et al., 2005;

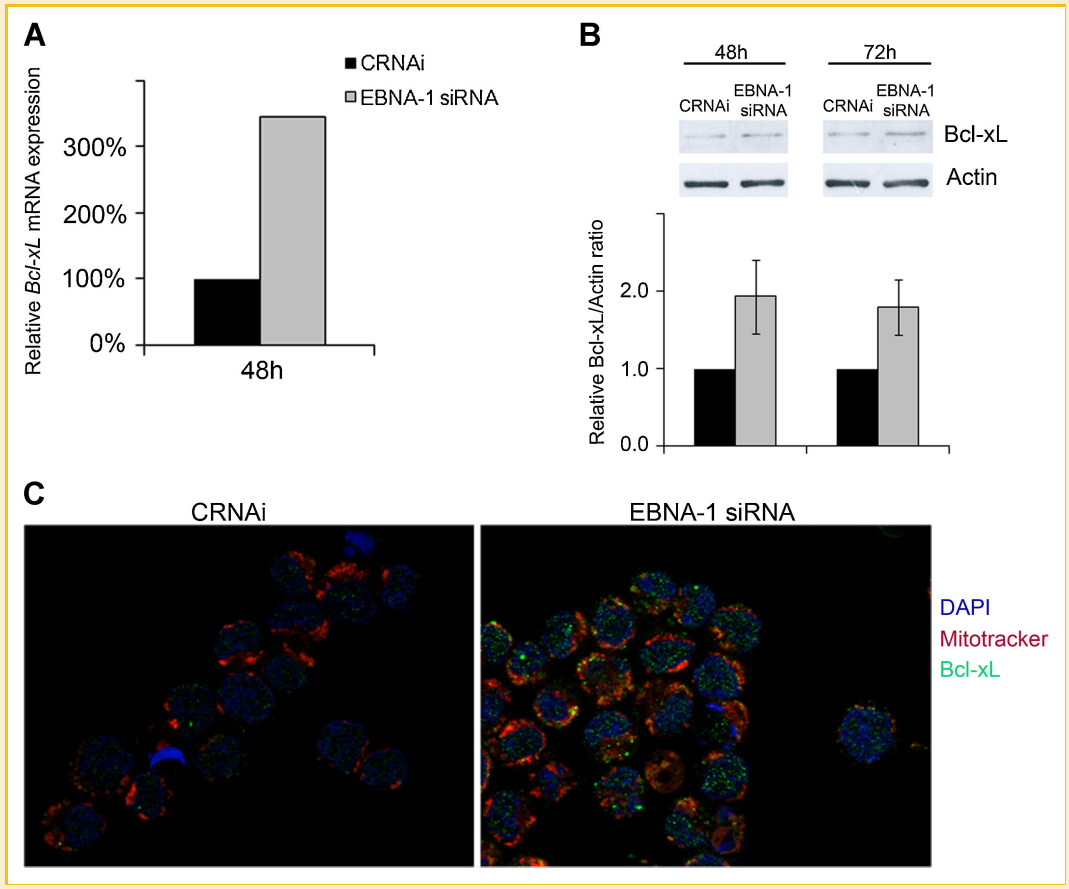

Fig. 6. Downregulation of EBNA-1 increases Bcl-xL expression. Akata EBV+ cells were transfected with EBNA-1 siRNA. A: Real time quantitative PCR analysis of *Bcl-xL* mRNA levels in Akata EBV+ cells following transfection with EBNA-1 siRNAs. Results, expressed after normalization of the *Bcl-xL* values with the values obtained for *18S*, were analysed as % of the control cells (transfected with CRNAi) and represent the mean of two independent experiments performed in triplicate. B: Western Blot analysis of the effect of the EBNA-1 siRNAs on the expression of Bcl-xL in Akata EBV+ cells. Results are representative of three independent experiments. The bands were analyzed by densitometry and are represented in the bar graph. Each bar represents the mean ± SE of the ratios Bcl-xL/Actin, obtained from the intensity of the bands from three independent experiments. C: Immunofluorescence analysis of the effect of the EBNA-1 siRNAs on the expression of Bcl-xL in Akata EBV+ cells, showing Bcl-xL (Green), Mitotracker Red stained mitochondria (Red), and DAPI stained nuclei (Blue). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Son et al., 2006]. Furthermore, in a flow cytometry analysis of a panel of BL cell lines following treatment with etoposide, Akata EBV+ cells were classified as being "sensitive" to this drug [Karpova et al., 2006]. In addition, a previous study showed that EBNA-1 increased the sensitivity of ovarian cancer cells to chemotherapeutic anticancer drugs targeting topoisomerase II, such as etoposide [Chuang et al., 2003].

The fact that we found EBV+ cells to be more sensitive than EBV− cells to etoposide, a drug that induces cell death by apoptosis, together with the knowledge that several viral proteins (including some EBV proteins) are known to modulate the apoptotic response of cells to infection (reviewed in [Galluzzi et al., 2008]), prompted us to study possible differences in the levels of apoptosis of both cell lines. Our results showed that Akata EBV+ cells presented increased basal levels of cleaved PARP (at 0 h), when compared to Akata EBV− cells. This was consistently observed even though there were no differences in the TUNEL assay results between the two cell lines at 0 h, possibly because PARP cleavage is an early marker of apoptosis [Kaufmann et al., 1993], which precedes DNA degradation and TUNEL labeling [Zhao et al., 2003]. In fact, at the 48 h time-point, both assays (PARP cleavage and TUNEL) provided positive results, indicating that other stimulus such as centrifugation of cells or depletion of growth factors may be triggering apoptosis in the EBV+ cells but not in the EBV− cells, even though both cell lines were subjected to the exact same conditions.

Additionally, the expression of the anti-apoptotic Bcl-xL (mRNA and protein) was decreased in the Akata EBV+ cells (at both 0 h and 48 h). This may justify the differences in apoptosis levels observed between the cell lines. In fact, it is accepted that a small difference in the amount of anti-apoptotic proteins, particularly in Bcl-xL, may result in large differences in cell survival [Fiebig et al., 2006]. We confirmed that the observed lower levels of Bcl-xL were not a result of the increased apoptosis/caspase cleavage observed in the EBV+ cells, neither were due to an increase in proteasomal degradation. Furthermore, bisulfite treatment followed by DNA sequencing of the CpG island region did not reveal alterations on the methylation pattern of the studied promoter region of Bcl-xL. Therefore, these do not seem to be the mechanisms involved in Bcl-xL downregulation.

In addition, we attempted to see if Bcl-xL was downregulated due to the expression of miRNAs that target the Bcl-xL mRNA (miR-491, let-7c and let-7g miRNAs) [Nakano et al., 2010; Shimizu et al., 2010]. It was not possible to proceed with the analysis for miR-491 and let-7g, due to possible interference of some viral RNAs with the primers used. The results obtained for let-7c showed a downregulation of this miRNA in the Akata EBV+ cells compared to the EBV− cells. Therefore, this miRNA is not responsible for the Bcl-xL downregulation in this cell line. Interestingly, a study of O'Hara et al. [2009] has shown that let-7 family of miRNAs were significantly downregulated in primary effusion lymphoma and in Kaposi sarcoma, both associated with the HHV8 infection. These authors state that the downregulation of multiple tumor suppressor miRNAs is an alternative mechanism of cellular transformation. Therefore, this may also be occurring in the cell line studied. We intend to further investigate this in future work.

Given the fact that Bcl-xL expression was found to be decreased in Akata EBV+ cells when compared to EBV− cells, we decided to investigate if EBV interferes with the expression of the anti-apoptotic protein Bcl-xL. Results from RNAi experiments to downregulated EBNA-1 expression clearly show that Bcl-xL expression increases following EBNA-1 downregulation. Whether the mechanism through which EBV decreases cellular Bcl-xL levels is through EBNA-1 or another latter expressed viral protein (since viral reactivation may occur), still remains to be addressed. Nevertheless, this study shows a clear evidence for an association between EBV latent infection and downregulation of Bcl-xL. In addition, the fact that EBV is associated with lower Bcl-xL levels (a cellular anti-apoptotic protein) may account for the observed differences in sensitivity to etoposide. Whether this is a mechanism through which the virus is able to render cells (after long-term infection) more sensitive to death induced by drugs which induce the lytic cycle, and thereby more prone to disseminate virus progeny, also remains to be addressed.

Bcl-xL is known to exist in the mitochondria, in nuclear membranes and in the ER [Tagami et al., 2000; White et al., 2005]. Our results in the Akata EBV+ versus EBV− cells show evidence of obvious morphological alterations in mitochondria and in ER. A previous study has shown scant ER in an EBV+ cell line [Hillman et al., 1977]. We found evidence of lower amount of ER in EBV+ cells when compared to EBV− cells. This could justify the lower levels of Bcl-xL found in the EBV+ cells. However, the reason why the EBV+ cells presented altered mitochondria and less ER is not understood. Future work will attempt to further elucidate this. Interestingly, there is published evidence that Bcl-xL interferes with mitochondrial fusion and fission in neurons [Berman et al., 2009].

In conclusion, our study suggests that latent (type I) EBV infection decreases Bcl-xL expression and increases cellular sensitivity to etoposide. We are currently investigating if these results obtained in the Akata cells are seen in other cells with this type of latency.

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