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Treatment with a BH3 mimetic overcomes the resistance of latency III EBV (+) cells to p53-mediated apoptosis

A Pujals^{1,2}, B Renouf^{1,2,3}, A Robert¹, S Chelouah¹, É Hollville^{1,4} and J Wiels^{*,1}

P53 inactivation is often observed in Burkitt's lymphoma (BL) cells due to mutations in the p53 gene or overexpression of its negative regulator, murine double minute-2 (MDM2). This event is now considered an essential part of the oncogenic process. Epstein–Barr virus (EBV) is strongly associated with BL and is a cofactor in its development. We previously showed that nutlin-3, an antagonist of MDM2, activates the p53 pathway in BL cell lines harboring wild-type p53. However, nutlin-3 strongly induced apoptosis in EBV (–) or latency I EBV (+) cells, whereas latency III EBV (+) cells were much more resistant. We show here that this resistance to apoptosis is also observed in latency III EBV (+) lymphoblastoid cell lines. We also show that, in latency III EBV (+) cells, B-cell lymphoma 2 (Bcl-2) is selectively overproduced and interacts with Bcl-2-associated X protein (Bax), preventing its activation. The treatment of these cells with the Bcl-2-homology domain 3 mimetic ABT-737 disrupts Bax/Bcl-2 interaction and allows Bax activation by nutlin-3. Furthermore, treatment with these two compounds strongly induces apoptosis. Thus, a combination of Mdm2 and Bcl-2 inhibitors might be a useful anti-cancer strategy for diseases linked to EBV infection.

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Subject Category: Cancer

The p53 tumor suppressor is a transcription factor that is activated in response to DNA or cell damage, such as oncogene activation, radiation, mitotic stress, ribosomal stress or chemical challenge.¹ When activated, p53 has a key role in protecting against tumor development, by inducing cell cycle arrest or apoptosis.² This physiological function of p53 is frequently altered in malignant cells, through mutation, cytoplasmic sequestration, interaction with viral oncoproteins or an increase in interactions with its main cellular regulator murine double minute-2 (MDM2).³ MDM2 is an E3 ubiquitin ligase that binds p53 and induces its ubiquitin-dependent degradation.⁴ MDM2 also inhibits the transcriptional activity of p53 through direct protein–protein interactions.⁵ MDM2 overproduction, leading to p53 inactivation, has been observed in various types of cancer.^{6,7} Given the key role of p53 in determining cell fate, several strategies for disrupting the p53–MDM2 interaction have been explored.^{8,9} Potent, stable and selective small-molecule antagonists of MDM2 have recently been synthesized. These molecules include nultins, *cis*-imidazoline compounds that act by binding to the p53 pocket of MDM2, thereby overcoming the inhibitory effects of this molecule and inducing the release and activation of p53.¹⁰ Nutlin treatment has been shown to enhance apoptosis

in various tumor cells *in vitro*,^{11,12} to inhibit tumor growth *in vivo*^{10,13} and to act in synergy with genotoxic drugs or irradiation in cancer therapy (reviewed in Vassilev¹⁴).

The anti-cancer activity of p53 results principally from the ability of this molecule to induce apoptosis through the intrinsic mitochondrial pathway.¹⁵ The crucial event in the initiation of this pathway is mitochondrial outer membrane permeabilization (MOMP), leading to the release of signaling molecules from the intermembrane space into the cytosol.¹⁶ This process is controlled by members of the B-cell lymphoma 2 (Bcl-2) protein family.¹⁷ This family has pro-apoptotic members, classified into ‘effectors’ (Bcl-2-associated X protein (Bax) and Bak), which participate directly in membrane permeabilization, and ‘Bcl-2-homology domain 3 (BH3)-only members’ (Puma, Noxa, Bad, Bim and Bid), which act as ‘direct activators’ or ‘sensitizers’ of the apoptotic pathway.¹⁸ However, this family also has anti-apoptotic members, such as Bcl-2, B-cell lymphoma-extra large (Bcl-xL) and myeloid cell factor-1 (Mcl-1), which antagonize MOMP. The levels of the pro-apoptotic Bcl-2 family proteins Bax, Noxa, Bid and Puma are directly regulated by p53.¹⁹

The p53 pathway is frequently disrupted in Burkitt's lymphoma (BL) cells, and this disruption is now considered

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Abbreviations: Bax, Bcl-2-associated X protein; Bcl-xL, B-cell lymphoma-extra large; Bcl-2, B-cell lymphoma 2; BH3, Bcl-2-homology domain 3; caspase, cysteinyl aspartate-specific protease; DiOC₆(3), 3'-dihexyloxycarbocyanine iodide; BL, Burkitt's lymphoma; EBV, Epstein–Barr virus; PI, propidium iodide; LCL, lymphoblastoid cell lines; LMP, latent membrane protein; Mcl-1, myeloid cell factor-1; MDM2, murine double minute-2; MFI, mean fluorescence intensity; MOMP, mitochondrial outer membrane permeabilization; NF-κB, nuclear factor-κB; PARP, poly (ADP-ribose) polymerase

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as an essential part of the tumorigenic process.²⁰ In a previous study, we therefore investigated the consequences of activating wt p53 and determined whether Epstein-Barr virus (EBV) infection of the cells had an effect on these consequences.²¹ The EBV genome is present in the vast majority of BL cases. The virus remains latent in infected tumor cells, producing only a very small number of viral proteins.²² Most EBV-infected BL cells harbor the latency I phenotype (Epstein-Barr nuclear antigen 1 (EBNA1) is the only viral protein produced). However, cases with the latency III profile (all latent EBV proteins are produced: EBNA1, 2, 3a, 3b, 3c, EBNA leader protein, latent membrane protein 1 (LMP1) and 2 (LMP2)) have also been identified.²³ Latent EBV does not specifically target p53, but it nonetheless interferes with cell cycle checkpoints regulated by p53 in the G1/S and G2/M phases²⁴ and modulates p53-induced apoptosis.²⁵ We have shown that, regardless of EBV status, the treatment of BL cells with nutlin-3 induces p53 activation, as shown by the induction of p21^{WAF1} MDM2 and Bax. However, although nutlin-3-treated EBV (–) BL cells display massive apoptosis, latency III EBV (+) cells are much more resistant.²¹

In this study, we investigated the mechanism responsible for the resistance to p53-mediated apoptosis induced by EBV in lymphoid cells. We analyzed two types of EBV (+) latency III cells: BL cells and lymphoblastoid cell lines (LCLs), a recognized model for the study of post-transplantation lymphoproliferative disorders. Both types of cell were found to be more resistant to treatment with nutlin-3 than EBV (–) cells. In EBV (–) cells, Bax was activated by nutlin-3 treatment, promoting apoptosis. By contrast, in EBV (+) latency III cells, Bax accumulated in mitochondria but was poorly activated by nutlin-3. Our findings indicate that Bcl-2 is selectively overproduced in these cells and forms stable complexes with Bax. Treatment with nutlin-3 has no significant impact on these complexes. Finally, we also demonstrated that ABT-737, a potent and selective small molecule that binds and antagonizes Bcl-xL, Bcl-2 and Bcl-w, sensitizes EBV (+) latency III cells to nutlin-3 treatment.

Results

Latency III EBV (+) cells are more resistant than EBV (–) cells to p53-dependent apoptosis. We have previously shown that, in BL cell lines, the induction of apoptosis by nutlin-3 depends on the EBV status of the cells: EBV (–) and EBV (+) latency I cells are highly sensitive to this antagonist of MDM2, whereas EBV (+) latency III cells are much more resistant.²¹ We investigated whether other EBV (+) latency III cells were also resistant to p53-dependent apoptosis by evaluating the response to nutlin-3 treatment in LCLs. All cell lines were treated with 10 μ M nutlin-3 for 24 h and apoptosis was assessed by flow cytometry after labeling the cells with annexin-V-FITC and propidium iodide (PI). Nutlin-3 induced slightly higher levels of apoptosis in LCL (52 \pm 10%, 49 \pm 4%, 48 \pm 7% apoptotic cells for RPMI8866, Priess and Remb1 cells, respectively) than in latency III BL cell lines (40 \pm 4%, 18 \pm 5%, 36 \pm 2%, for BL2/B95, Seraphina and LY47 cells, respectively) but these levels of apoptosis remained lower

than those in EBV (–) BL cell lines (76 \pm 4%, 95 \pm 4% for BL2 and BL28 cells, respectively; Figure 1a).

We characterized the apoptotic cell death induced by nutlin-3 in more detail by measuring the loss of mitochondrial membrane potential ($\Delta\psi_m$) by flow cytometry with the carbocyanine dye 3'-dihexyloxacarbocyanine iodide (DiOC₆(3)). Loss of $\Delta\psi_m$ occurs early in apoptosis and was therefore measured after 16 h of treatment with nutlin-3. In EBV (–) BL cells treated with nutlin-3, mitochondrial depolarization was observed in 53 \pm 6 % of BL2 cells and 89 \pm 5% of BL28 cells (Figure 1b). By contrast, in EBV (+) latency III cells (BL and LCL), the loss of $\Delta\psi_m$ was much milder, with 25 \pm 2%, 9 \pm 3%, 5 \pm 4%, 19 \pm 4%, 22 \pm 1%, 23 \pm 2 % mitochondrial depolarization in BL2/B95, Seraphina, LY47, RPMI8866, Priess and Remb1 cells, respectively.

We then carried out western blotting to assess the cleavage of poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme directly targeted by caspase-3 (cysteiny aspartate-specific protease-3). After 7 h of treatment with nutlin-3, the 85 kDa PARP cleavage product was observed in EBV (–) BL2 and BL28 cell extracts, whereas no cleavage was observed in EBV (+) BL and LCL extracts, except for Remb1 cells, in which low levels of cleavage were detected (Figure 1c). After 24 h of treatment, PARP was almost totally (BL2) or totally (BL28) cleaved in EBV (–) cells. By contrast, in EBV (+) cells, PARP cleavage occurred either at very low rates (BL2/B95, RPMI8866, Priess and Remb1 cells) or not at all (Seraphina and LY47 cells). Thus, both BL and LCL latency III EBV (+) cells are more resistant than EBV (–) cells to p53-mediated apoptosis.

The activation of p53 increases Bax levels similarly in EBV (–) and EBV (+) cell lines. In susceptible EBV (–) BL cells, nutlin-3-induced apoptosis involves permeabilization of the mitochondrial outer membrane. This mechanism is generally controlled and mediated by Bcl-2 family proteins. The pro-apoptotic protein Bax, encoded by a p53 target gene, is one member of this family. We showed in a previous study that nutlin-3 activated the p53 pathway similarly in all BL cell lines.²¹ In this study, we measured the accumulation of Bax in both EBV (–) BL cell lines and EBV (+) latency III BL and LCL treated with nutlin-3 for various periods of time (3, 7 or 24 h). Western blot analysis was carried out to determine the levels of p53 (as a control of its activation) and of Bax. Bax-specific bands were quantified by densitometry and normalized with respect to β -actin levels. In all cell lines, treatment with nutlin-3 induced a gradual increase in Bax levels until 24 h of treatment (Figure 2). These results are consistent with our previous findings and confirm that the accumulation of Bax in response to p53 activation is not correlated with the EBV status of the cells and that the resistance of EBV (+) cells to apoptosis does not result from a defect in Bax accumulation.

Following p53 activation, Bax accumulates in the mitochondria of latency III EBV (+) cells but is not activated. For Bax to be active, it must be translocated from the cytosol (where it is found in healthy cells) to the outer mitochondrial membrane. The redistribution of Bax was assessed by western blot analysis of the mitochondrial and

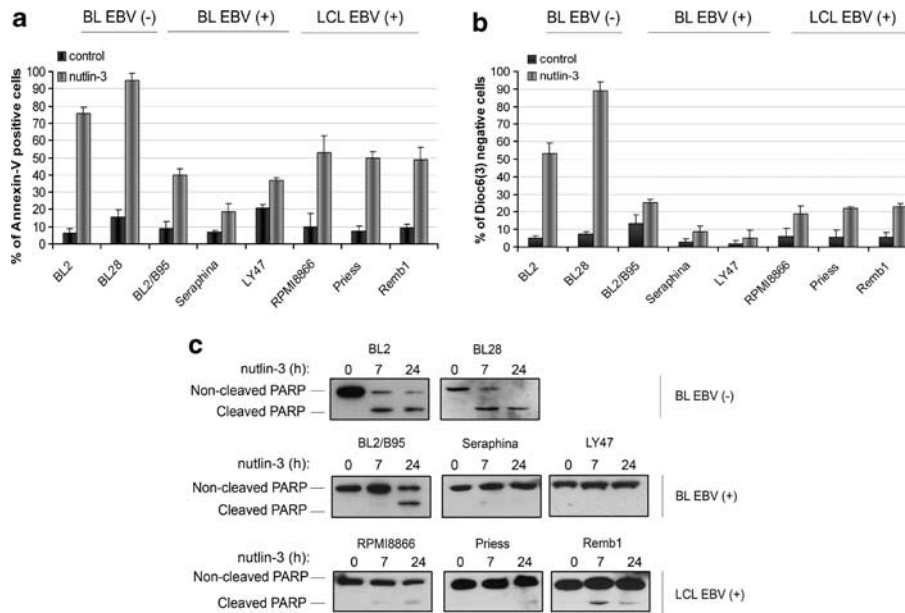


Figure 1 Effect of nutlin-3 treatment on the induction of apoptosis in Epstein-Barr virus (EBV) (–), EBV (+) Burkitt's lymphoma (BL) and lymphoblastoid cell lines (LCLs). (a) Cells were treated with 10 μ M nutlin-3 or with the solvent dimethyl sulfoxide for 24 h. The cells were labeled with annexin V-FITC and PI and analyzed with a FACSCalibur flow cytometer to determine the percentage of cells that were apoptotic. The values presented (means \pm s.d.) are from three independent experiments. (b) Cells were treated with 10 μ M nutlin-3 or the solvent dimethyl sulfoxide for 16 h. The cells were stained with DiOC₆(3) and analyzed on a FACSCalibur flow cytometer. DiOC₆(3) stains mitochondria in a potential-dependent fashion. Cells with a low $\Delta\psi_m$ are therefore only weakly fluorescent, and are described as 'DiOC₆(3) negative'. The values presented (means \pm s.d.) are from three independent experiments. (c) Cells were treated with 10 μ M nutlin-3 for the indicated periods of time. Apoptosis levels were determined by assessing polyadenosine diphosphate-ribose polymerase (PARP) cleavage on western blots. Results are representative of at least three independent experiments

cytosolic fractions of untreated cells and of cells treated with nutlin-3 for 24 h (Figure 3a). Fractionation quality was checked by probing blots with antibodies recognizing proteins known to be localized to mitochondria (Bcl-2) or the cytosol (vinculin). Bax-specific bands were then quantified by densitometry and the results were normalized with respect to Bcl-2 or vinculin levels. In EBV (–) BL cells, nutlin-3 treatment either strongly decreased the amount of Bax in the cytosolic fractions (BL2) or resulted in this protein being undetectable (BL28) in these fractions, whereas Bax levels in the mitochondrial fractions clearly increased. By contrast, in EBV (+) cell lines (BL and LCL), Bax accumulated in both the cytosolic and mitochondrial fractions after treatment with nutlin-3.

We investigated whether the Bax accumulating in the mitochondria was in the activated form by labeling cells with the 6A7 conformation-specific Bax mAb (Figure 3b). This antibody recognizes an N-terminal epitope of Bax that is occluded in the inactive form of the protein but exposed after the activation and insertion of the protein into membranes.²⁶ After treatment with nutlin-3, 52% of EBV (–) BL2 cells were positive for 6A7 labeling (mean fluorescence intensity (MFI): 105 versus 48 for control), whereas latency III EBV (+) cells were only weakly stained (2% (MFI: 38 versus 36), 25% (MFI: 66 versus 39) and 32% (MFI: 68 versus 28) for LY47, BL2/B95 and Remb1 cells, respectively).

To confirm that the activation of Bax is involved in nutlin-3-mediated apoptosis of BL2 cells, we next inhibited the production of this protein with a specific small-interfering RNA, treated the cells with nutlin-3 and then measured

apoptosis levels by assessing PARP cleavage on western blots. In BL2 cells with low levels of Bax, lower levels of PARP cleavage were observed than in controls cells (Supplementary Figure 1). These data show that, in EBV (–) cells treated with nutlin-3, Bax accumulates in mitochondria in its activated form and takes part in the apoptotic process. By contrast, in EBV (+) latency III cells, most of the Bax accumulating in the mitochondria is not in the active conformation.

Bcl-2 is overproduced in latency III EBV (+) cells. At least three EBV-encoded proteins (LMP1, LMP2A and EBNA2) have been shown to induce the upregulation of various anti-apoptotic Bcl-2 family members able to sequester Bax.^{27–29} We therefore carried out western blotting to evaluate the endogenous levels of these anti-apoptotic proteins (Bcl-2, Bcl-xL and Mcl-1) in our cell lines (Figure 4). There was no direct correlation between the EBV status of the various cell lines and basal levels of Bcl-xL or Mcl-1. By contrast, a strong correlation was observed between basal levels of Bcl-2 and EBV status: all latency III EBV (+) cells contained high levels of Bcl-2, whereas EBV (–) cells had low levels of this protein. To confirm that high levels of Bcl-2 were correlated with LMP1 expression,²⁸ we also assessed the level of this viral protein. Large amounts of LMP1 were observed in all EBV (+) cell lines except BL2/B95, which had only low levels of this protein. As LMP1 has also been shown to induce the downregulation of Bax,³⁰ we determined endogenous Bax levels in our various cell lines. No correlation was observed between the EBV status and Bax levels.

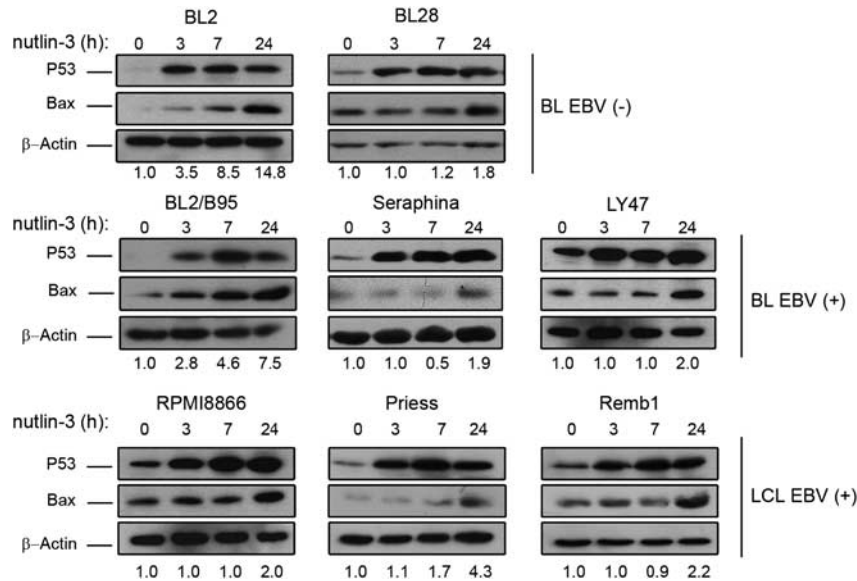


Figure 2 Effect of nutlin-3 treatment on p53 and Bax levels in Burkitt's lymphoma (BL) and lymphoblastoid cell lines (LCLs). Cells were treated with 10 μ M nutlin-3 for the indicated periods of time. The levels of p53 and Bax protein were assessed by western blot analysis. Fold-change values for Bax *versus* the untreated control (0 h), normalized with respect to β -actin levels are shown under the blots

Bcl-2 interacts with Bax in latency III EBV (+) cells, but not in EBV (-) cells. We investigated the role of Bcl-2 in the resistance to apoptosis observed in latency III EBV (+) cells by studying the interactions between Bax and Bcl-2. BL2 EBV (-) cells and BL2/B95 EBV (+) cells (which differ only in terms of their EBV status) were left untreated or treated with nutlin-3 for 7 h. Proteins were then extracted and immunoprecipitation was carried out with an anti-Bax pAb. The immunoprecipitates were then probed for Bax and Bcl-2 (Figure 5). These western blots showed that treatment with nutlin-3 induced a stronger accumulation of Bax in BL2 than in BL2/B95 cells, but that Bcl-2 was coprecipitated with Bax only in BL2/B95 cells, with nutlin-3 treatment having no significant impact on this co-immunoprecipitation. Thus, in EBV (+) latency III cells, Bax and Bcl-2 form stable complexes that may be at least partly responsible for the resistance to apoptosis.

An inhibitor of Bcl-2 disrupts the interactions between Bax and Bcl-2 and allows Bax activation in BL2/B95 EBV (+) cells treated with nutlin-3. We investigated the involvement of interactions between Bax and Bcl-2 in resistance to nutlin-3-induced apoptosis using ABT-737, a BH3 mimetic that antagonizes pro-survival members of the Bcl-2 family, promoting the release of Bax.³¹ We first investigated the effect of this Bcl-2 inhibitor on Bax/Bcl-2 interaction. BL2/B95 EBV (+) cells were treated for 1 h with ABT-737 (10 μ M), incubated with nutlin-3 for 7 h and protein extracts were prepared. We then assessed Bax and Bcl-2 levels by western blotting and interaction between these two molecules by co-immunoprecipitation. Treatment with ABT-737 had no effect on Bcl-2 or Bax levels (Figure 6a), but abolished the co-immunoprecipitation of Bax with Bcl-2, in both untreated cells and cells treated with nutlin-3 (Figure 6b).

We also evaluated the effect of treatment with ABT-737 on the distribution of Bax in untreated cells and cells treated with

nutlin-3. ABT-737 decreased the accumulation of Bax in the cytosol induced by nutlin-3 alone and concomitantly increased the level of Bax in the mitochondria (Figure 6c). We investigated whether the Bax accumulating in the mitochondria was in the activated form by labeling the cells with the 6A7 conformation-specific Bax mAb (Figure 6d). After treatment with ABT-737 alone or nutlin-3 alone, 10% (MFI: 39 *versus* 31) and 23% (MFI: 62) of BL2/B95 cells, respectively, were weakly positive for 6A7 labeling, whereas when the two compounds were used in combination, 41% (MFI: 74) of the cells were strongly labeled with the 6A7 mAb. Thus, a combined treatment with ABT-737 and nutlin-3 induces the release of Bax from the Bax/Bcl-2 complexes and its activation in mitochondria.

An inhibitor of Bcl-2 restores the susceptibility of latency III EBV (+) cells to p53-dependent apoptosis. We then used flow cytometry to determine whether treatment with ABT-737 sensitized the EBV (+) latency III BL and LCL cells to nutlin-3-induced apoptosis (Figure 7a). Consistent with our previous observations, treatment with nutlin-3 for 24 h induced <50% apoptosis in these cells. Treatment with ABT-737 (10 μ M) alone had no cytotoxic effect on BL2/B95 and Seraphina cells but induced $42 \pm 7\%$ apoptosis in LY47 cells. An even stronger effect was observed in LCL cells, in which treatment with 10 μ M ABT-737 for 24 h induced >70% apoptosis (data not shown). In these cell lines, we therefore decreased the concentration of ABT-737 used to reduce its cytotoxic effect. Treatment with 0.25 μ M ABT-737 induced $32 \pm 8\%$, $47 \pm 3\%$ and $39 \pm 2\%$ apoptosis in RPMI8866, Priess and Remb1 cells, respectively. When the two drugs were used in combination, a synergic effect on the induction of apoptosis was observed in BL2/B95 and Seraphina cells ($78 \pm 5\%$ and $81 \pm 3\%$ apoptotic cells, respectively). In the other cell lines, no synergy between ABT-737 and nutlin-3 was observed, these

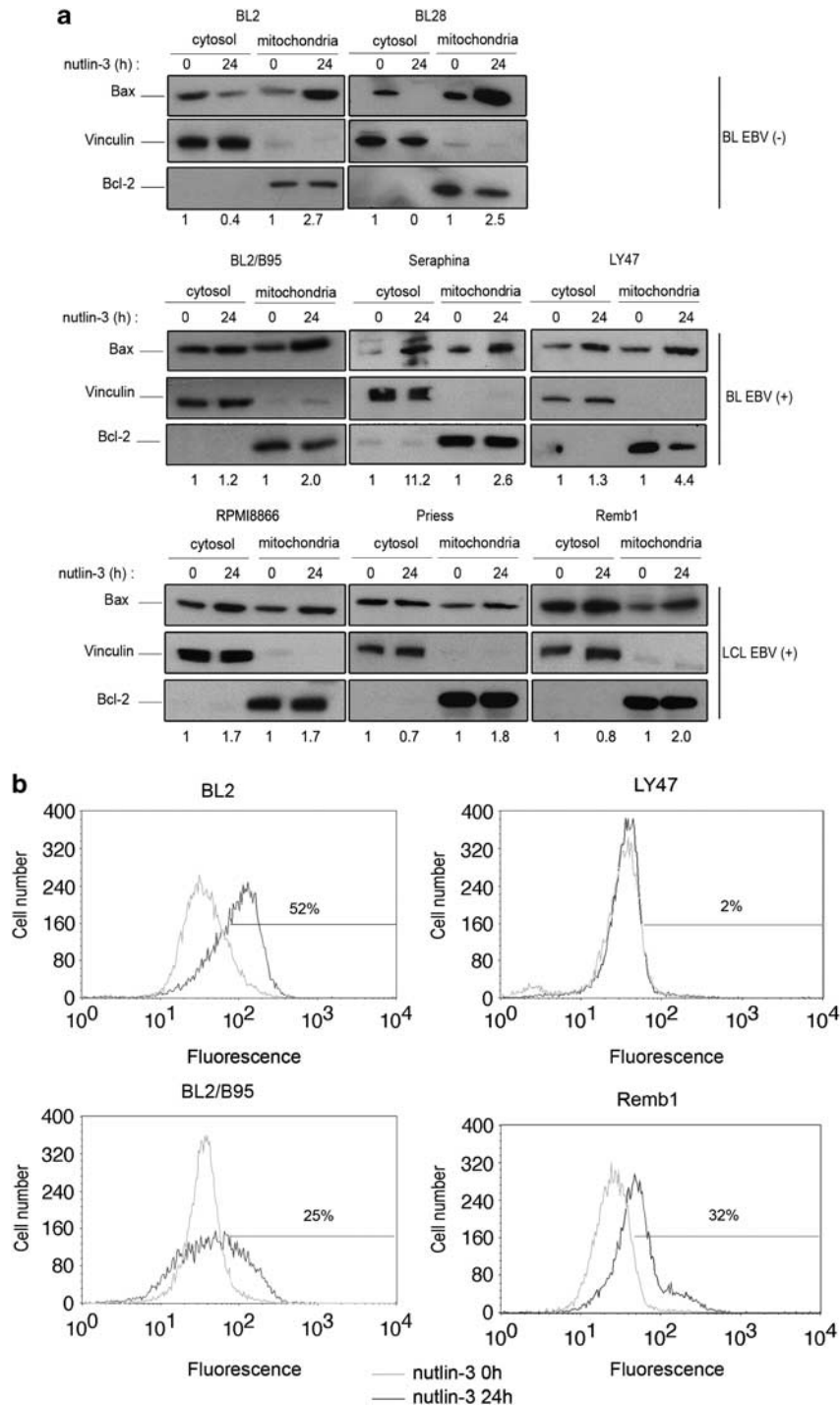


Figure 3 Effect of nutlin-3 treatment on Bax relocalization and activation in EBV (–) and EBV (+) cells. (a) Cells were treated with 10 μ M nutlin-3 or the solvent dimethyl sulfoxide for 24 h. Cytosolic and mitochondrial fractions were analyzed by western blotting with an anti-Bax pAb. Vinculin and Bcl-2 were used as cytosolic and mitochondrial markers, respectively. Fold-change values versus the untreated control (0 h), after normalization with respect to vinculin or Bcl-2 protein levels, are shown under the blots. Results are representative of three independent experiments. (b) BL2 EBV (–) cells and BL2/B95, LY47 and Remb1 EBV (+) cells were treated with 10 μ M nutlin-3 or the solvent dimethyl sulfoxide for 24 h. Cells were fixed in 0.25% paraformaldehyde and labeled with a conformation-specific 6A7 Bax antibody, which recognizes only the active conformation of the protein and Alexa 488-conjugated goat anti-mouse IgG. Cells were then analyzed with a FACSCalibur flow cytometer

compounds instead having an additive effect ($65 \pm 1\%$, $71 \pm 8\%$, $73 \pm 7\%$ and $81 \pm 5\%$ apoptosis in LY47, RPMI8866, Priess and Remb1 cells, respectively). We also evaluated the effect of nutlin-3 and ABT-737 on three cell

lines originating from the same individual: BL40, BL40/B95 and IARC 211. BL2 and BL2/B95 were used as controls. Apoptosis was determined by assessing PARP cleavage on western blots (Figure 7b). No cleavage of PARP was

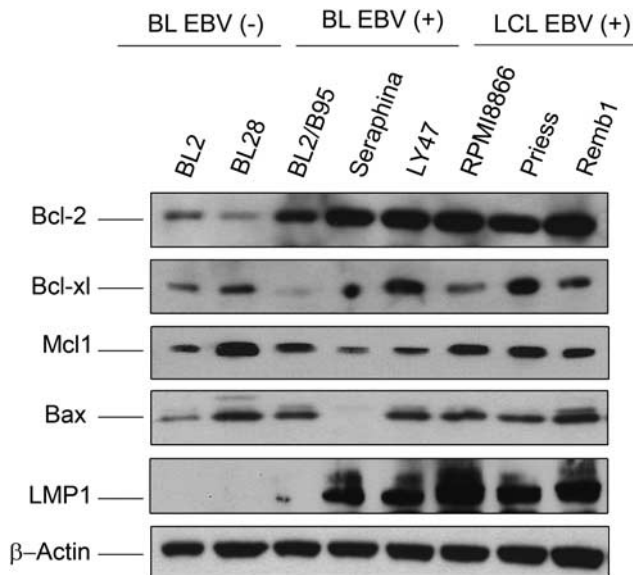


Figure 4 Levels of Bcl-2 family members in EBV (–) and EBV (+) lymphoid cell lines. Levels of the Bax, Bcl-2, Bcl-xl and Mcl-1 proteins as well as those of the viral LMP1 protein were assessed by western blot analysis

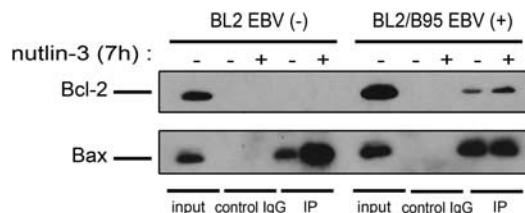


Figure 5 Interaction of Bax with Bcl-2 in BL2 EBV (–) and BL2/B95 EBV (+) cells untreated or treated with nutlin-3. Cells were treated with 10 μ M nutlin-3 or the solvent dimethyl sulfoxide for 7 h. Lysates were subjected to immunoprecipitation with agarose-conjugated anti-Bax pAb (IP) or agarose-conjugated control rabbit IgG (IgG control) and then subjected to western blotting with an anti-Bcl-2 mAb or an anti-Bax pAb. As a control for protein levels before IP, a portion of cell lysate (input) corresponding to 15% of the input for IP was also included in the western blot. All results are representative of three independent experiments

observed after treatment with ABT-737 alone in BL2 and BL2/B95 cells, whereas a strong or partial cleavage was observed in BL40, BL40/B95 and IARC 211 cells. Treatment with nutlin-3 alone induced strong (BL2) or complete (BL40) cleavage of PARP in EBV (–) cell lines, moderate cleavage in BL2/B95 cells and almost no cleavage in BL40/B95 and IARC 211 cells. As expected, treatment with both compounds had an additive effect on cell viability and PARP was totally cleaved in all cell lines.

Finally, as EBV reactivation might also reduce cell viability, we tested the effects of nutlin-3 and ABT-737 on production of the viral BZLF1 protein, a transcriptional activator that mediates the transition between the latent and lytic cycles of EBV. Except in LY47 cells, in which nutlin-3 slightly increased the very low basal levels of BZLF1 protein, nutlin-3, ABT-737 and the combination of these two molecules were unable to induce the production of this protein in any of the EBV (+) cell lines tested, (Supplementary Figure 2). Thus, a combined treatment with ABT-737 and nutlin-3 efficiently induces the

apoptosis of latency III EBV (+) cells producing large amounts of Bcl-2 protein, without affecting the latent stage of the virus.

Discussion

The activation of wt p53, or even the reactivation of mutant p53, alone or in combination with cytotoxic agents (chemotherapy drugs or radiation) is now considered a promising new approach for cancer treatment.^{15,32} Various elements must be considered during the development of such new therapies, including the cellular context, which may greatly modulate the consequences of p53 activation. In this study, we showed that the type III latent EBV infection of lymphoid cells does not prevent the p53 activation induced by an inhibitor of Mdm2, but strongly decreases p53-induced apoptosis. We also found that the activation of p53 induced Bax accumulation similarly in all cell lines and that this pro-apoptotic member of the Bcl-2 family was involved in the apoptotic pathway in EBV (–) cell lines. Bax is primarily a cytosolic protein in healthy living cells. Following the induction of apoptosis, it undergoes a conformational change and is translocated to the mitochondria.³³ After nutlin-3 treatment, Bax underwent conformational changes and relocation in EBV (–) cells, whereas in EBV (+) cells, it accumulated in both the cytosol and the mitochondria but underwent no conformational changes. Thus, the accumulation of Bax in the mitochondria of EBV (+) cells is not sufficient to induce apoptosis.

Several studies have shown that latent infection with EBV protects cells from apoptosis. For example, the viral proteins LMP1 and LMP2A have been shown to upregulate various anti-apoptotic members of the Bcl-2 family by activating the nuclear factor- κ B (NF κ B) pathway,²⁸ or to inhibit the transcription of the pro-apoptotic Bax protein.³⁰ We determined the endogenous levels of Bax, Bcl-2, Bcl-xl and Mcl-1 in our cell lines. Consistent with the results of Rowe *et al.*,²⁸ we observed a strong correlation between basal Bcl-2 level and EBV status: all latency III EBV (+) cells had high levels of Bcl-2, whereas EBV (–) cells had low levels of this protein. By contrast, Bax levels, similar to those of Bcl-xl and Mcl-1, were variable in all cell lines, regardless of EBV status. It has been reported that Bcl-2 inhibits both changes in the conformation of the N-terminus of Bax and the translocation of Bax from the cytosol to the mitochondria.^{34,35} Our results are consistent with these observations, as we observed no specific translocation of Bax to the mitochondria after the treatment with nutlin-3 of cells overproducing Bcl-2. We also showed that Bcl-2 interacted with Bax in EBV (+) cells, but not in EBV (–) cells. This sequestration of Bax may account, at least in part, for the resistance to nutlin-3 observed in EBV (+) latency III cells. Our results are also consistent with those of Forte and Luftig,³⁶ who previously showed that, in LCL, nutlin-3 induced p53 activation and a moderate level of apoptosis (around 40% of annexin-V (+) cells after 48 h of treatment), which was enhanced by previous treatment of the cells with an inhibitor of NF κ B activity. They showed that NF κ B activity controlled steady-state levels of MDM2 protein and concluded that the NF κ B inhibitor sensitized cells to nutlin-3 by decreasing MDM2 levels. However, as the upregulation of

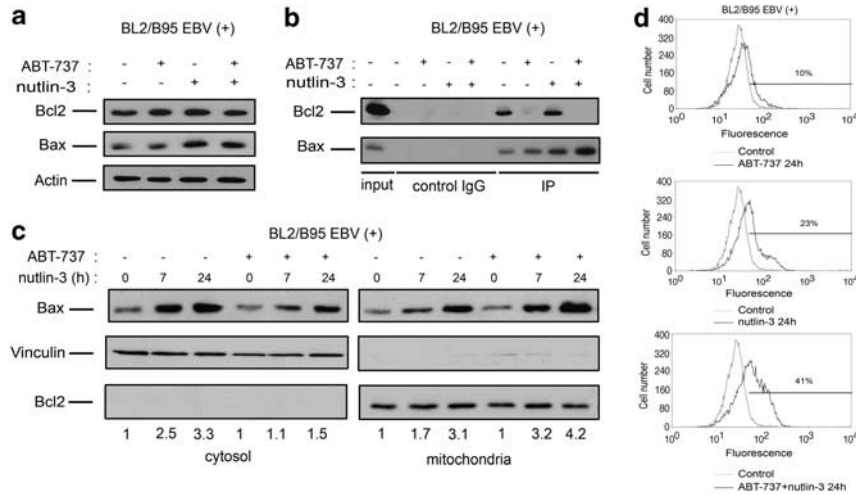


Figure 6 Effect of a combination of nutlin-3 and ABT-737 in the EBV (+) cell lines. BL2/B95 EBV (+) cells were or were not subjected to prior treatment with 10 μ M ABT-737 for 1 h, and were then treated with nutlin-3 for 7 h. (a) The levels of Bcl-2 and Bax proteins were assessed by western blot analysis. (b) Lysates were subjected to immunoprecipitation with agarose-conjugated anti-Bax pAb (IP) or agarose-conjugated control rabbit IgG (IgG control) and then subjected to western blotting with an anti-Bcl-2 mAb or an anti-Bax pAb. As a control for protein levels before IP, a portion of cell lysate (Input) corresponding to 15% of the input for IP was also included in the western blot. All results are representative of three independent experiments. (c) BL2/B95 EBV (+) cells were or were not subjected to prior treatment with 10 μ M ABT-737 for 1 h and were then treated with nutlin-3 for the indicated periods of time. Cytosolic and mitochondrial fractions were analyzed by western blotting with an anti-Bax pAb. Vinculin and Bcl-2 were used as cytosolic and mitochondrial markers, respectively. Fold-change values *versus* the untreated control (0 h), after normalization with respect to the levels of vinculin or Bcl-2 protein, are shown under the blots. The results shown are representative of three independent experiments. (d) BL2/B95 cells were or were not subjected to prior treatment with ABT-737 for 1 h and were then left untreated or treated with nutlin-3 for 24 h. Cells were fixed in 0.25% paraformaldehyde and labeled with a conformation-specific 6A7 Bax antibody, which recognizes only the active conformation of the protein and Alexa 488-conjugated goat anti-mouse IgG. Cells were then analyzed with a FACSCalibur flow cytometer

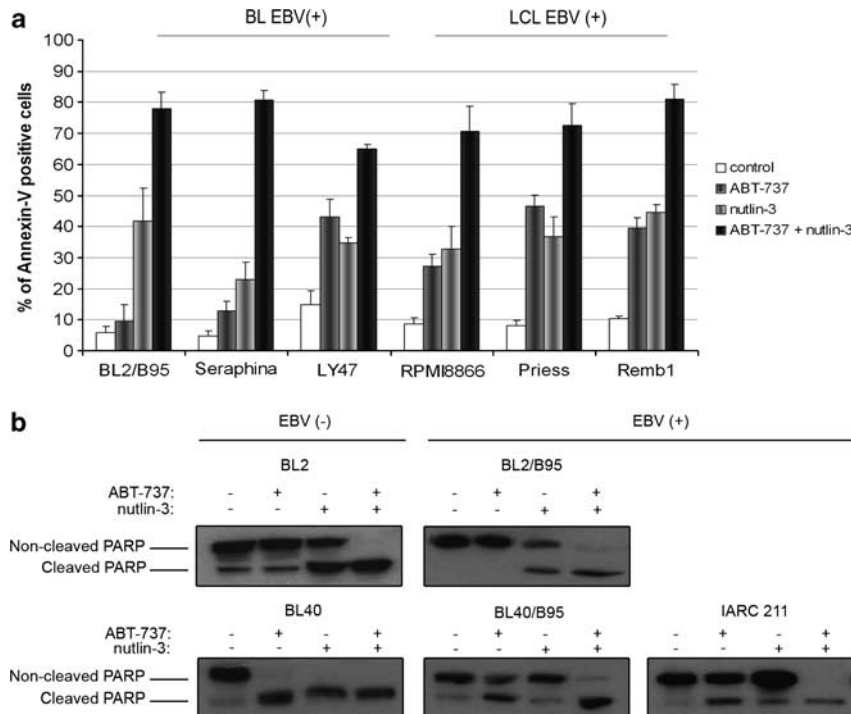


Figure 7 Effect of a combination of nutlin-3 and ABT-737 on the induction of apoptosis in EBV (+) Burkitt's lymphoma (BL) and lymphoblastoid cell lines (LCL). (a) EBV (+) cell lines were or were not subjected to prior treatment with ABT-737 (BL2/B95, Seraphina, LY47: 10 μ M; 8866, Priess, Remb1: 0.25 μ M) for 1 h and were then left untreated or treated with nutlin-3 for 24 h. The cells were labeled with annexin V-FITC and PI and analyzed with a FACSCalibur flow cytometer, to determine the percentage of cells that were apoptotic. The values presented (means \pm s.d.) are from three independent experiments. (b) Cell lines were treated as in (a) and apoptosis levels were determined by assessing polyadenosine diphosphate-ribose polymerase (PARP) cleavage on western blots. Results are representative of at least three independent experiments

Bcl-2 in B cells containing LMP1 is due to NF κ B activation,^{28,37} it could also be deduced from their data that the NF κ B inhibitor sensitizes cells by decreasing Bcl-2 levels.

ABT-737 is a BH3 mimetic that efficiently antagonizes various pro-survival members of Bcl-2 family and promotes the release of Bax and Bak.³¹ In latency III EBV (+) cells, the disruption of Bax/Bcl-2 interaction by treatment with ABT-737 before nutlin-3 treatment promoted the relocalization of Bax to the mitochondria and its activation. Treatment with these two compounds also strongly induced the apoptosis of these cells. The combined inhibition of the MDM2 and Bcl-2 proteins has been reported to strongly induce apoptosis in acute myelogenous leukemia cells overproducing Bcl-2³⁸ and in tumors (including BL) in which Hdmx limits the p53 activation induced by nutlin-3.³⁹ Combined treatment with nutlin-3 and an inhibitor of the anti-apoptotic members of the Bcl-2 family, such as ABT-737, may therefore constitute an attractive new treatment strategy for tumors harboring wild-type p53 but with defects in the p53-induced apoptotic pathway. *In vivo* studies in murine models are now required to evaluate the effects of this combined treatment in terms of side effects and tumor regression and to compare these effects with those of conventional chemotherapy.

Concerning latency III EBV (+) cells, it will also be interesting to use *in vivo* models to assess the efficiency of ABT-737 alone, as we found that, even at very low doses, this compound promoted apoptosis in LCL cells. This treatment may be particularly beneficial in patients with EBV (+) post-transplant lymphoproliferative disorder or nasopharyngeal carcinoma, because both these types of tumor overproduce Bcl-2 or Bcl-xL.⁴⁰

Materials and Methods

Cell lines. All BL cell lines were originally established from endemic or sporadic cases of BL. BL2, BL28, BL40, BL2/B95, BL40/B95 and LY47 were kindly provided by the International Agency for Research on Cancer (IARC, Lyon); Seraphina cells were provided by Professor G Klein (Stockholm); BL2/B95 and BL40/B95 cells were generated by stable infection of the original EBV (–) BL2 and BL40 cells with the B95-8 EBV strain. LCLs were obtained by the *in vitro* immortalization of normal B lymphocytes. IARC 211 cells were established from the normal B lymphocytes of patient BL40 and were obtained from IARC. Priess and Remb1 cells were kindly provided by Dr JG Bodmer (London). These cell lines were cultured in RPMI 1640 medium (PAA) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM glucose, 100 U/ml penicillin and 100 μ g/ml streptomycin and supplemented with 10% heat-inactivated fetal calf serum.

Antibodies and reagents. Nutlin-3 was obtained from Cayman Chemical (SPI-Bio, Montigny-le Bretonneux, France); ABT-737 was kindly provided by Abbott Laboratories (Chicago, IL, USA).

PARP mAb (Ab-2, clone c-2-10) and anti-p53 mAb (Ab-6, OP43) were purchased from Calbiochem (Meudon, France). Anti-Bax pAb (N-20), anti-Bcl-2 mAb (clone 100), anti-Mcl-1 mAb (clone 22), anti-Bcl-xL mAb (7B2.5) and anti-BZLF1 mAb (BZ1) were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Anti-vinculin (clone VIN-11-5) and anti- β actin (clone AC-74) mAbs were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Anti-LMP1 mAb (CS1-4) was obtained from Dako France SAS (Trappes, France). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG and HRP-conjugated donkey anti-rabbit IgG for western blotting were purchased from GE Healthcare (Velizy, France) and Jackson ImmunoResearch Laboratories (Newmarket, UK), respectively.

Induction and quantification of apoptosis. We treated 0.5×10^6 cells for various periods of time, at 37 °C, with nutlin-3 (10 μ M), ABT-737 (10 μ M)

or both these compounds. Cells were washed in PBS, resuspended in annexin buffer (10 mM HEPES/NaOH (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) supplemented with 2.5 μ g/ml FITC-labeled annexin-V (Roche Applied Science, Meylan, France) and incubated at room temperature for 10 min. Cells were then washed, resuspended in annexin buffer supplemented with PI (10 μ g/ml) and analyzed by flow cytometry ($n = 10\,000$; FACSCalibur, Becton-Dickinson, Pont-de-Claix, France). Annexin-V-positive cells (PI negative or PI positive) were considered to be apoptotic.

Determination of mitochondrial membrane potential. After treatment with nutlin-3, ABT-737 or both these compounds, for various periods of time, DiOC₆(3) was added (40 nM) and cells were incubated for an additional 30 min at 37 °C. Cells were washed, resuspended in PBS supplemented with PI and analyzed by flow cytometry ($n = 10\,000$; FACSCalibur, Becton-Dickinson).

Intracellular labeling. Cells (2×10^6), either untreated or treated with nutlin-3 (10 μ M), were fixed by incubation in 0.25% paraformaldehyde for 20 min at room temperature. They were then washed twice and stained with primary antibody (monoclonal anti-Bax antibody, clone 6A7, Sigma-Aldrich) in digitonin (200 μ g/ml) for 30 min at room temperature. The cells were then washed again with PBS and incubated for 30 min with goat anti-mouse IgG conjugated to Alexa 488 (GAM-Alexa-488, Molecular Probes, Leiden, The Netherlands). Samples were then analyzed on a FACSCalibur flow cytometer. Cells labeled with GAM-Alexa-488 only were used as a control.

Preparation of mitochondrial and cytosolic fractions. We resuspended 2×10^6 cells in 100 μ l of ice-cold cell lysis and mitochondria intact buffer (250 mM sucrose, 70 mM KCl, 200 μ g/ml digitonin) and incubated the suspension at 4 °C for 5 min. The samples were then centrifuged at $1000 \times g$ for 5 min at 4 °C. The supernatants (cytosolic fractions) were recovered and stored at –80 °C. The pellets were resuspended in 50 μ l of IP buffer (30 mM Tris HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.2% Triton \times 100, 0.3% NP40, complete protease inhibitor cocktail) and incubated for 10 min at 4 °C. The suspension was then centrifuged at $10\,000 \times g$ for 10 min at 4 °C and the supernatants (mitochondrial fractions) were stored at –80 °C until use.

Immunoprecipitation. We subjected 5×10^6 cells to disruption by sonication (10 s on ice) in 1 ml of CHAPS buffer (1% CHAPS, 1 M Hepes, 5 M NaCl, complete protease inhibitor cocktail). We mixed the resulting lysates with 2 μ g of agarose-conjugated rabbit anti-Bax pAb (clone N-20) or agarose-conjugated control rabbit IgG pAb (Santa Cruz Biotechnology Inc.) and incubated the mixture overnight at 4 °C, with end-over-end rotation. Immune complexes were washed twice with ice-cold CHAPS buffer, twice with PBS and resuspended in loading buffer. The samples were boiled for 5 min and the proteins were separated by electrophoresis in 4–12% Bis-Tris precast gels (Invitrogen, Cergy Pontoise, France) and analyzed by western blotting.

Western-blot analysis. A pellet containing 1×10^6 cells was solubilized by incubation in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 0.1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, complete protease inhibitor cocktail) for 10 min. Sample loading buffer was added and the mixture was boiled for 5 min. Proteins were separated by electrophoresis in 12% polyacrylamide gels or 4–12% Bis-Tris precast gels (Invitrogen) and transferred to PVDF membranes (Millipore, Molsheim, France). Blots were blocked by incubation overnight at 4 °C in 3% non-fat milk powder, 2% glycine in PBS and incubated for 1 h at room temperature with primary antibodies. The blots were then washed and incubated with HRP-conjugated RAM-IgG or HRP-conjugated DAR-IgG. Antibody complexes were detected by enhanced chemiluminescence (Millipore).

Conflict of Interest

The authors declare no conflict of interest.

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