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Tax Protein-induced Expression of Antiapoptotic Bfl-1 Protein Contributes to Survival of Human T-cell Leukemia Virus Type 1 (HTLV-1)-infected T-cells*[S]◆

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Background: Antiapoptotic Bcl-2 proteins are up-regulated in HTLV-1-infected T-cells.
Results: Antiapoptotic Bfl-1 protein is regulated by viral Tax and HBZ proteins and is involved in survival of HTLV-1-transformed T-cells.
Conclusion: Bfl-1, as well as Bcl-x_L, induces resistance to apoptosis in HTLV-1-transformed T-cells.
Significance: We provide evidence for Bfl-1 and Bcl-x_L as potential therapeutic targets in the treatment of leukemia/lymphoma associated with HTLV-1.

Human T lymphotropic virus type 1 (HTLV-1) is the etiologic agent of adult T-cell leukemia/lymphoma (ATLL). ATLL is a severe malignancy with no effective treatment. HTLV-1 regulatory proteins Tax and HTLV-1 basic leucine zipper factor (HBZ) play a major role in ATLL development, by interfering with cellular functions such as CD4$^+$ T-cell survival. In this study, we observed that the expression of Bfl-1, an antiapoptotic protein of the Bcl-2 family, is restricted to HTLV-1-infected T-cell lines and to T-cells expressing both Tax and HBZ proteins. We showed that Tax-induced *bfl-1* transcription through the canonical NF-κB pathway. Moreover, we demonstrated that Tax cooperated with c-Jun or JunD, but not JunB, transcription factors of the AP-1 family to stimulate *bfl-1* gene activation. By contrast, HBZ inhibited c-Jun-induced *bfl-1* gene activation, whereas it increased JunD-induced *bfl-1* gene activation. We identified one NF-κB, targeted by RelA, c-Rel, RelB, p105/p50, and p100/p52, and two AP-1, targeted by both c-Jun and JunD, binding sites in the *bfl-1* promoter of T-cells expressing both Tax and HBZ. Analyzing the potential role of antiapoptotic Bcl-2 proteins in HTLV-1-infected T-cell survival, we demonstrated that these cells are differentially sensitive to silencing of Bfl-1, Bcl-x_L, and Bcl-2. Indeed, both Bfl-1 and Bcl-x_L knock-

downs decreased the survival of HTLV-1-infected T-cell lines, although no cell death was observed after Bcl-2 knockdown. Furthermore, we demonstrated that Bfl-1 knockdown sensitizes HTLV-1-infected T-cells to ABT-737 or etoposide treatment. Our results directly implicate Bfl-1 and Bcl-x_L in HTLV-1-infected T-cell survival and suggest that both Bfl-1 and Bcl-x_L represent potential therapeutic targets for ATLL treatment.

Adult T-cell leukemia/lymphoma (ATLL)[6] is associated with human T-cell leukemia virus type 1 (HTLV-1) infection. It is an aggressive malignancy of mature activated CD4$^+$ T-cells and in rare cases of CD4$^+$CD8$^+$ or CD8$^+$ T-cells. Among the 10–20 million people infected with HTLV-1 worldwide, 2–10% will develop an ATLL after a long period of clinical latency. ATLL is characterized by the persistence and clonal expansion of CD4$^+$-infected T-cells (1, 2), but the molecular mechanisms of ATLL development are complexes and remain incompletely understood. Some of these mechanisms are related to the expression of the regulatory viral Tax protein and of the HTLV-1 basic leucine zipper (bZIP) factor (HBZ) (3). The oncoprotein Tax is highly expressed in early stages of ATLL development, although its expression decreases to become almost undetectable in most ATLL cells (4–6). Strong evidence indicates that Tax plays a central role in T-cell transformation both

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in vitro in human cells and *in vivo* in transgenic animal models. However, Tax-induced immortalization of human primary T-cells is a very rare event (7–13). Tax modulates cellular gene expression and interferes with the control of cell survival, proliferation, and genetic stability of infected cells (14–16). Tax does not directly bind to DNA, but it promotes the recruitment of transcription factors on targeted cellular genes. In particular, Tax activates survival transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) members, which in turn increase the expression of antiapoptotic proteins (16–21). As a consequence, both HTLV-1- and Tax-transformed T-cells show higher resistance to apoptosis than untransformed control cells (18, 22).

Although Tax is not detectable in 60% of ATLL cases, HBZ remains expressed through all stages of the ATLL process (14, 15, 23–27). HBZ controls gene transcription by interacting with Jun members of the AP-1 family through their bZIP domain (28). An increasing number of studies report that HBZ promotes T-cell proliferation and inflammation and suggest that HBZ participates in the maintenance of tumoral phenotype (23, 28–32).

Many viruses responsible for the development of leukemia/lymphoma have evolved to escape immune surveillance. Some of them inhibit apoptosis by encoding viral Bcl-2 (B-cell lymphoma gene-2) analogs, which mimic their cellular antiapoptotic function. Others up-regulate the expression of cellular antiapoptotic Bcl-2 proteins (33–35). Impaired apoptosis associated with an imbalance of the expression of Bcl-2 members in favor of antiapoptotic proteins is a hallmark of human hematopoietic malignancies and is frequently associated with resistance to therapy (36, 37). The NF-κB transcription factors have been shown to directly up-regulate both *bfl-1* (Bcl-2 fetal liver) and *bcl-x$_L$* (Bcl-2-like long) gene expression, and overexpression of both proteins has been associated with increased resistance of tumor cells to apoptotic stimuli or to chemotherapeutic drugs (38–47). Interestingly, several data point out a potential role for NF-κB-mediated *bfl-1* and *bcl-x$_L$* overexpression, by viral proteins, in both B- and T-lymphoma/leukemia. Indeed, the *bfl-1* promoter is activated by EBV latent membrane protein 1 (LMP1), as well as by EBV nuclear antigen 2 (EBNA2) and HTLV-1 Tax proteins (48–50). Similarly, *bcl-x$_L$* gene is activated by EBV LMP2A and HTLV-1 Tax proteins in B- and T-cells, respectively (51–53). Although the regulation of *bcl-x$_L$* by Tax protein has already been documented, the mechanism underlying the regulation of *bfl-1* expression by viral Tax and HBZ proteins and the involvement of Bfl-1 in HTLV-1-infected T-cell survival remain unknown.

Here, we report that Bfl-1 is expressed in HTLV-1-infected T-cell lines but not in uninfected T-cells. We demonstrated that Tax induces Bfl-1 expression through the canonical NF-κB pathway but also synergizes with JunD or c-Jun of the AP-1 family to activate *bfl-1* transcription. By contrast, HBZ modulates Jun-mediated *bfl-1* gene activation. Moreover, both NF-κB and AP-1 bind to different sites of the *bfl-1* promoter in a T-cell line stably expressing *tax*, *hbz*, and *bfl-1* transcripts. Finally, we showed that knockdown of Bfl-1 or Bcl-x$_L$, but not Bcl-2, decreases HTLV-1-infected T-cell survival and that targeting both Bfl-1 and Bcl-x$_L$ restored full cell death. Altogether,

our data strongly suggest that Bfl-1 and Bcl-x$_L$ represent potential therapeutic targets for ATLL treatment.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids—The monoclonal antibodies (mAbs) used were either mouse anti-Bcl-x$_L$ (2H12) (Pharmingen), anti-Bcl-2 (124, Dako SA), anti-Mcl-1 (SC-12756, Tebubio), anti-c-Jun (OP55, Merck), anti-FLAG (F3165, Sigma), anti-c-Myc (Santa Cruz Biotechnology, sc-40), or anti-β-actin (Sigma). We also used rabbit polyclonal antibodies anti-actin (Sigma), anti-GST-Bfl-1 (kindly provided by J. Borst, The Netherlands Cancer Institute), anti-IκBα (44D4, Cell Signaling), and allophycocyanin-conjugated anti-active caspase-3 (Pharmingen). The mouse mAb anti-Tax hybridoma (168A51-42) was a kind gift of AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, National Institutes of Health). Secondary reagents used were ECL anti-rabbit IgG horseradish peroxidase from donkey and anti-mouse IgG horseradish peroxidase from sheep (GE Healthcare).

Plasmids used were as follows: empty-pSG5M, pSG5M-Tax-WT, pSG5M-Tax-M22, and pSG5M-Tax-M47 (54, 55); empty-pJFE and pJFE-Tax-WT (kindly provided by F. Bex, Belgium (56)); empty-pcDNA3, pcDNA-JunB, pcDNA-c-Jun, and pCMV-JunD-FLAG (57, 58); $-1374/+81$-*bfl-1* promoter-driven luciferase gene (Bfl-1-Luc, kindly provided by D. Walls, Dublin, Ireland (50)); pRL-TK-*Renilla* luciferase (pRL-TK-Ruc, Promega); pCMV4-HA-IκBα S32A/S36A (59), and NF-κB and 5′LTR-HTLV-1-driven luciferase gene (NF-κB-Luc and LTR-HTLV-1-Luc (60)). pcDNA-HBZ-Myc encoding sHBZ and the mutated sHBZ forms deleted for activation (HBZΔAD) and for bZIP (HBZΔbZIP) domains were described previously (57, 61).

Cells—HeLa cell line was grown in DMEM (Invitrogen) supplemented with 10% endotoxin-free FCS (Lonza), 2 mM L-glutamine, 10 mM HEPES, and 40 μg/ml gentamicin (Invitrogen). The HTLV-1-infected MT-2, MT-4, C8166-45, SP, and FC36.22 T-cell lines were obtained from AIDS Research and Reference Reagent Program. The HTLV-1-infected T-cell lines C91PL and HuT-102 were provided by E. Wattel (Lyon, France) and R. Mahieux, respectively. Uninfected human leukemia Jurkat (Clone E6–1), Sup-T1, Molt-4 (Clone 8) and CEM, and lymphoma HuT-78 T-cell lines, which served as negative controls, were obtained from AIDS Research and Reference Reagent Program. JPX-9 cells are derivatives of Jurkat, which have a stably integrated *tax* gene under the control of a metallothionein promoter and are kindly provided by M. Nakamura (Tohoku University, Japan (62)). To induce Tax expression, JPX-9 cells were cultured in the presence of 20 μM CdCl$_2$. Stably Tax/HBZ-expressing Jurkat (E12 clone) T-cells were obtained as reported previously (63). The T-cell lines were grown in RPMI 1640 medium (Invitrogen) supplemented as for DMEM medium. FC36.22 and SP cells were cultured in the presence of 4% of homemade IL-2.

We used frozen peripheral blood mononuclear cells (PBMC) from healthy donors (Etablissement de Transfusion Sanguine, Lyon, France) and ATLL patients (Prof. O. Hermine, H. Necker, Paris, France). The clinical status of the ATLL patients at the time of sample collection is summarized in supplemental Table S1. Fresh human PBMC were stimulated with mAbs anti-CD3/

CD28 (Dynabeads Human T-cell activator, Invitrogen) 48 h prior to transduction. Then the Tax-expressing T-cells were obtained by transduction of stimulated PBMC with a lentivirus vector encoding a HTLV-1 Tax-enhanced YFP fusion, as described previously (64). The obtained CD4$^+$ Tax-T-cells were grown in complete RPMI medium supplemented with 1% (v/v) Eagle's minimum essential medium nonessential amino acid, 1 mM sodium pyruvate, and 200 units/ml human IL-2 (PeproTech). Untransduced T-cells from blood were sorted by FACS according to CD4 and CD8 expression using FACSVantage SE option Diva (BD Biosciences). Purity was over 90%.

RNA Extraction and Real Time RT-PCR—0.5–2 × 10^6 T-cells were lysed using TRIzol reagent (Invitrogen), and cDNAs were obtained as described previously (65). 0.1–0.5 × 10^6 transfected HeLa cells were lysed according to the manufacturer's specifications (SV total RNA isolation kit, Promega). The real time RT-PCR was performed with FastStart SYBR Green Master (Roche Applied Science) on a StepOne Plus machine (Applied Biosystems) using 4 μM of each primer (supplemental Table S2). cDNA levels were normalized using the housekeeping *HPRT* and *GusB* genes. Each sample was run in triplicate, and the data were analyzed as described previously (65).

Immunoblotting Analysis—2–5 × 10^6 cells were lysed in 50 μl of Nonidet P-40 lysis buffer (150 mM NaCl, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, and 1% Nonidet P-40) supplemented with protease inhibitor mixture (Roche Applied Science). Cytoplasmic protein extracts (70–100 μg) were separated on a NuPAGE 4–12% BisTris gel (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Protein expression was analyzed by immunoblotting using specific Abs. Protein-Ab complexes were visualized by chemiluminescence (Western Lighting Chemiluminescence Reagent Plus; PerkinElmer Life Sciences).

Transduction Assays—RNA interference sequences are summarized in supplemental Table S3. Corresponding oligonucleotides were cloned into the lentiviral vector pLL3.7 co-expressing GFP as described previously (47). 3 × 10^5 cells were cultured in a 6-well plate for 24 h. The cells were then transduced with lentiviral vector expressing specific shRNA at 16 multiplicities of infection in the presence of Polybrene (Sigma), as performed previously (47). Cells were collected at days 5–7 to evaluate efficiency of transduction (GFP$^+$) by flow cytometry, mRNA, or protein extinction and mortality by flow cytometry after staining with 0.5 μg/ml propidium iodide (PI$^+$).

Transfections and Luciferase Assays—2.5 × 10^5 or 1.25 × 10^5 HeLa cells were plated on 6- or 24-well plates overnight, respectively. Then, cells were transiently transfected with 3 μg/6-well or 1 μg/24-well plasmids using JetPEI according to the manufacturer's protocol (Ozyme). 48 h post-transfection, cells were collected for RNA and protein extractions or for luciferase assays. To compare the amounts of expressing vectors between experiments performed in 6 and 24 wells, amounts are presented in the figures as nanograms or micrograms of interest plasmid/μg of total transfected vectors. Luciferase activities were determined in triplicate using a luciferase assay kit system (Dual-Glo®, Promega) and a luminometer (Infinite M200, Tecan) according to the manufacturer's specifications. Lucifer-

ase activity was calculated using the ratio Luc/Ruc. Normalized luminescence values are presented as relative fold *trans*-activation by comparison with the basal *bfl-1* luciferase activity obtained using empty vector, which was arbitrarily set up to one.

Electrophoretic Mobility Shift Assay (EMSA) and Supershift—For the preparation of nuclear extracts, 10^7 cells were washed with PBS, and the volume of the pellet was determined. Cells were resuspended in a 4-pellet volume of cold lysis buffer (20 mM Tris-HCl (pH 8), 1 mM EDTA, 5 mM DTT) with protease inhibitors (Sigma). After 15 min on ice, cells were lysed by homogenization in a loose-pestle Dounce, and the mixture was centrifuged at 5,000 × g at 4 °C for 10 min in an Eppendorf microcentrifuge. The pellet was resuspended in 4 pellet volumes of nuclear lysis buffer (20 mM Tris-HCl (pH 8), 20% glycerol, 0.42 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM DTT) with protease inhibitors and incubated on ice for 15 min. The mixture was then homogenized in a tight pestle Dounce and centrifuged 10,000 × g at 4 °C for 10 min. The resulting supernatant was used as the nuclear extracts in EMSA.

DNA-protein complexes were formed by a 45-min incubation of the extracts with DNA fragments at 25 °C in a 20-μl final volume reaction (20 mM Tris-HCl (pH 7.9), 10% glycerol, 0.1 mM EDTA, 5 mM MgCl$_2$, 0.05% Nonidet P-40) and with 0.5–1 μg of poly(dI-dC)-poly(dI-dC) (Sigma). Reactions were then complemented with 1 μl of glycerol and loaded either on a 20 × 20-cm nondenaturing 4.5% polyacrylamide gel (TBE 1×, acrylamide/bisacrylamide 60:1 ratio) in the case of [γ-^{32}P]ATP-labeled DNA or a 10 × 10-cm 1% agarose gel in the case of unlabeled DNA. Acrylamide gels were dried, and autoradiographed and agarose gels were "stained" by ethidium bromide. Double-stranded synthetic oligonucleotides corresponding to binding sites from the proximal part of the *bfl-1* promoter were labeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) by using T4 polynucleotide kinase (New England Biolabs) and then purified on a ProbeQuant G-50 Micro-column (GE Healthcare) and used as probes. The sequences of the oligonucleotides used were as follows: κB52-F, 5'-GCAAACAGAAATTCCCCAGCCTC-3' (NF-κB site); AP-104-F, 5'-TTTCATACATGACATGAAA-CACAGCCT-3' (AP-1 site); and AP74-F, 5'-CGCAC-GAAAGTGACTAGGAGGAA-3' (AP-1 site).

To identify transcription factors in the DNA-protein complexes shown in EMSA, we used antibodies specific for c-Jun (sc-74543X, Santa Cruz Biotechnology) and JunD (sc-74X, Santa Cruz Biotechnology) and for various NF-κB pathways, including p105/p50 (Ab7971, Abcam), p100/p52 (ab7972, AbCam), p65/RelA (CS-204359, Millipore), RelB (sc-28689X, Santa Cruz Biotechnology), and c-Rel (sc-71X, Santa-Cruz Biotechnology). These antibodies were incubated with the nuclear extracts from E12 T-cell clone for 15 min at 25 °C before incubation with the DNA fragments to elicit a supershift of DNA-protein complexes. The supershift was detected on agarose gels.

Cell Death Assay—T-cell lines (4 × 10^5/ml) were cultured in the absence or presence of various concentrations of DMSO, ABT-737 (Euromedex, Selleck Chem) (66) or etoposide (Sigma). 48 h after treatment, mortality was determined by flow cytometry after staining with PI or with allophycocyanin-con-

jugated rabbit anti-active caspase-3 antibody after fixation and permeabilization using the BD Cytofix/Cytoperm™ kit.

Statistical Analysis—Statistical analysis of the differences between the groups was performed using *t* test with $p < 0.05$ considered statistically significant.

RESULTS

Antiapoptotic Bfl-1 Protein Is Overexpressed in HTLV-1-infected T-cells—De la Fuente et al. (48) reported an activation of the *bfl-1* promoter in a Tax-transfected T-cell line. To gain a more precise view of the expression of Bfl-1 in the context of HTLV-1 infection, we analyzed mRNA and protein expression of Bfl-1 in HTLV-1-infected and -uninfected T-cells harboring different phenotypes. HTLV-1-transformed T-cell lines were CD4$^+$CD8$^+$ (SP), CD4$^+$ (C91PL, MT-4, MT-2, and HuT-102), and CD8$^+$ (FC36.22), and uninfected T-cells were CD4$^+$CD8$^+$ (Molt-4 and Sup-T1) and CD4$^+$ (CEM, Jurkat and HuT-78) (supplemental Table S4). Interestingly, Bfl-1 mRNA and proteins were detected in all HTLV-1-infected T-cell lines regardless of their phenotype but not in uninfected T-cell lines, except very moderate mRNA levels in HuT-78 (Fig. 1, *A* and *B*). Note that Bfl-1 expression was observed in IL-2-dependent (SP and FC36.22) and -independent (MT-4, MT-2, HuT-102, and C91PL) HTLV-1-infected T-cells (Fig. 1, *A* and *B*). We also compared the expression of Bcl-x$_L$ and Bcl-2 in these T-cell lines. In agreement with previous reports (52, 53), we observed that Bcl-2 and Bcl-x$_L$ messengers and proteins were expressed at different levels in both uninfected and HTLV-1-infected T-cell lines (supplemental Fig. S1, *A* and *B*). The presence of HTLV-1 was confirmed by the expression of Tax and HBZ (Fig. 1, *A* and *B*, and supplemental Fig. S1*A*). Altogether, these data show that, in contrast to Bcl-2 and Bcl-x$_L$, Bfl-1 expression is restricted to HTLV-1-infected CD4$^+$CD8$^+$, CD8$^+$, and CD4$^+$ T-cells.

We then examined the expression of a Bfl-1 protein in PBMC from three healthy donors and four ATLL samples. Although healthy PBMC expressed between 25 and 46% of CD3$^+$CD4$^+$ T-cells, all PBMC from ATLL patients exhibited more than 70% of CD3$^+$CD4$^+$ T-cells (supplemental Table S5). As expected, only the CD4$^+$ ATLL cells showed an activated phenotype. Interestingly, Bfl-1 protein was detected in two acute aggressive ATLL cells but not in two chronic indolent ATLL cells and in healthy control cells (Fig. 1*C* and supplemental Table S1).

Tax Activates bfl-1 Gene Transcription in T-cells—To determine the molecular mechanism of Bfl-1 induction by HTLV-1, we first focused on Tax protein, which is known to be involved in T-cell survival. We used JPX-9 cells, a Jurkat subclone that carries the *tax* gene under the control of the inducible metallothionein promoter. Treatment of JPX-9 cells with CdCl$_2$ induced *tax* mRNA levels within 12 h, and *tax* expression persisted until 48 h (Fig. 2*A*). We observed that *bfl-1* transcript levels were significantly induced only 24 h post-treatment with CdCl$_2$ and further increased with time. These results indicated that *tax* expression preceded that of *bfl-1* and that the presence of *bfl-1* correlated with that of *tax*. Then we measured *bfl-1* mRNA expression in human primary CD4$^+$ T-cells. We observed that CD4$^+$ T-cells expressing *tax* mRNA after transduction expressed higher levels of *bfl-1* mRNA, compared with
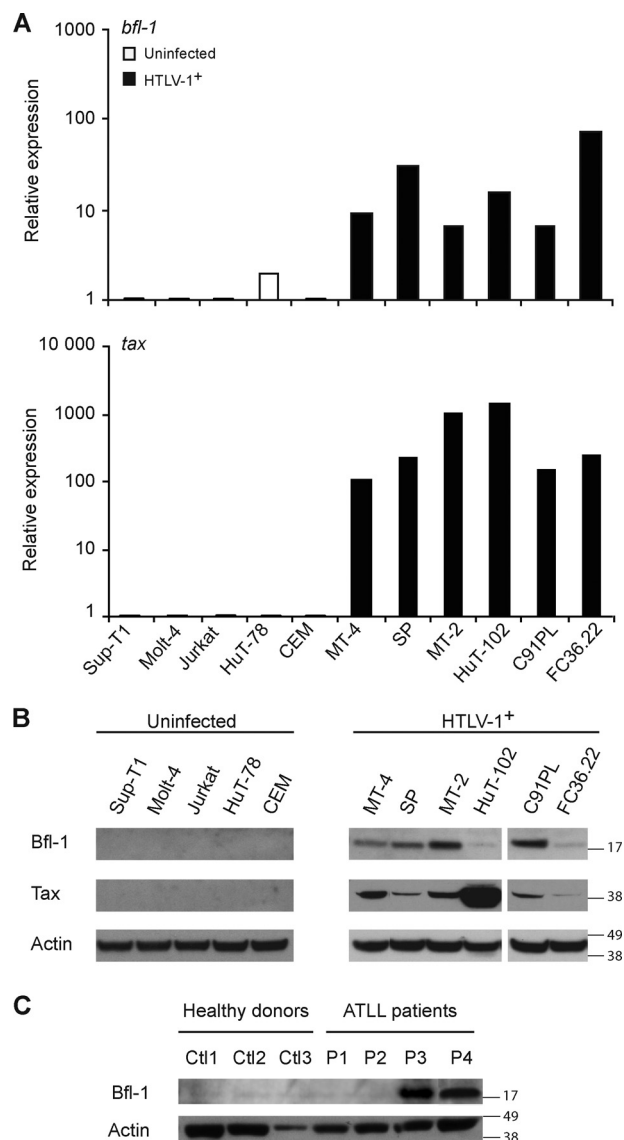
FIGURE 1. Antiapoptotic Bfl-1 transcript and protein are expressed in HTLV-1-infected T-cell lines but not in uninfected leukemic T-cell lines. *A,* relative expression of specific *bfl-1* and *tax* mRNA was normalized using housekeeping genes after real time RT-PCR of uninfected (Sup-T1, Molt-4, Jurkat, HuT-78, and CEM) and HTLV-1-infected (MT-4, SP, MT-2, HuT-102, C91PL, and FC36.22) T-cell lines. *B,* expression of Bfl-1 and Tax proteins in uninfected and HTLV-1-infected T-cell lines was determined by Western blotting. The loading control was the housekeeping actin protein. Results are from one representative experiment of two to three. *C,* expression of Bfl-1 protein in human PBMC from healthy controls (*Ctl 1–3*), chronic (*P1–2*), and acute (*P3–4*) ATLL patients were determined by Western blotting. The loading control was the housekeeping actin protein.

untransduced T-cells (Fig. 2*B*). Note that untransduced T-cells and Tax-transduced CD4$^+$ T-cells expressed similar levels of *bfl-1* mRNA to uninfected HuT-78 and HTLV-1-infected MT-2 and C91PL CD4$^+$ T-cells, respectively (Figs. 1*A* and 2*B*).

To further demonstrate that Tax activates *bfl-1* gene expression, we knocked down the expression of Tax using the lentivirus-based vector pLL3.7 expressing Tax-specific shRNA in stably Tax-expressing E12 T-cell clone. Tax shRNA induced more than 80 and 60% of inhibition of *tax* and *bfl-1* mRNA expression, respectively, compared with Ctl shRNA (Fig. 2*C*). Thus, these data indicate that Tax induces *bfl-1* gene expression in T-cells.
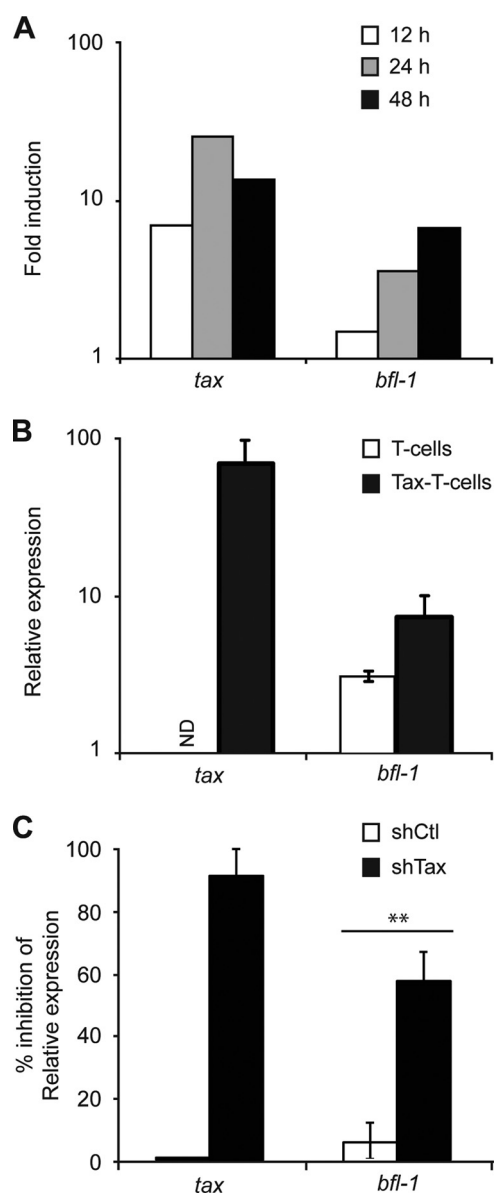
FIGURE 2. ***bfl-1* gene is transcriptionally activated by Tax in human T-cells.** *A,* expression of *tax* and *bfl-1* messengers in JPX-9 cells at different times post-treatment with 20 μM CdCl$_2$. The relative expression of specific *tax* and *bfl-1* mRNA was normalized using housekeeping genes after real time RT-PCR. Data are presented as fold induction by comparison with untreated cells, where relative expression was arbitrarily set up to one. Results are from one representative experiment of two. *B,* expression of *bfl-1* transcript in primary T-cells and Tax-expressing T-cells derived from transduced PBMC by real time RT-PCR. The relative expression of *tax* and *bfl-1* mRNA was normalized using housekeeping genes. Results are expressed as means ± S.D. of three independent experiments. *ND,* not determined. *C,* expression of *tax* and *bfl-1* messengers after knockdown of Tax in E12 T-cell clone. Cells were transduced with lentivirus expressing Luc-Ctl (*ShCtl*) or Tax (*ShTax*) shRNA. After 5 days, the relative expression of specific *tax* and *bfl-1* mRNA was normalized using housekeeping genes after real time RT-PCR. The results are presented as percent of inhibition of *tax* and *bfl-1* expression compared with untransduced T-cell clone. The *bars* represent the means ± S.D. of three to four independent experiments. *t* test has been performed between Ctl and Tax shRNA-transduced T-cells (0.001 < *p* value < 0.01).

Tax trans-Activates the Transcription of bfl-1 Gene via the Canonical NF-κB Pathway—As Tax *trans*-activates the expression of numerous genes via the NF-κB pathway (20), and as *bfl-1* promoter contains NF-κB-binding sites (38, 50, 67), we then investigated the role of the NF-κB pathway in Tax-medi-

ated *bfl-1* transcription (Fig. 3). HeLa cells were transfected with expressing vectors encoding for either WT Tax (Tax-WT), Tax-M47 (ΔCREB/ATF), or Tax-M22 (ΔNF-κB) mutants (Fig. 3A). The *tax* and *bfl-1* mRNA levels were then measured by real time RT-PCR (Fig. 3B). Although mRNA levels of Tax-WT and Tax mutants were quite similar (Fig. 3B, *left panel*), only Tax-WT and Tax-M47 mutant efficiently induced *bfl-1* mRNA expression (Fig. 3B, *right panel*). By contrast, Tax-M22 mutant induced only a weak *bfl-1* mRNA expression, suggesting that NF-κB pathway is involved.

To further confirm the involvement of the NF-κB pathway in Tax-mediated *bfl-1* transcription, we used the dominant-negative IκBα S32A/S36A (IκBαDN) mutant construct that prevents the phosphorylation and thus the degradation of IκBα, an inhibitor of the canonical pathway. HeLa cells were co-transfected with empty- or Tax-expressing vectors, *bfl-1*-luciferase promoter plasmid (Bfl-1-Luc), and increasing amounts of IκBαDN-expressing vectors. As shown in Fig. 3C, overexpression of IκBαDN resulted in a dose-dependent reduction of the Tax-mediated *bfl-1* promoter activation (Fig. 3C, *upper panel*). Expression of IκBα and Tax proteins was confirmed by immunoblotting (Fig. 3C, *lower panel*). We observed that IκBαDN was unable to totally inhibit the Tax-mediated *bfl-1* promoter activation even when over 100 ng of IκBαDN-expressing vector were transfected (Fig. 3C, *upper panel,* and data not shown). By contrast, IκBαDN completely inhibited Tax-mediated NF-κB-responsive promoter when used at 25 ng (supplemental Fig. S2, *left panel*). As expected, IκBαDN had no effect on HTLV-1-LTR *trans*-activation by Tax (supplemental Fig. S2, *right panel*), showing that this inhibitor was specific to the NF-κB pathway. Finally, we analyzed the effect of IκBαDN on *bfl-1* messenger expression mediated by Tax. HeLa cells were co-transfected with Tax-WT- and IκBαDN-expressing vectors, and *bfl-1* transcript levels were measured by real time RT-PCR. Overexpression of IκBαDN resulted in a 70% inhibition of the Tax-mediated endogenous *bfl-1* mRNA expression (Fig. 3D).

Altogether, these findings demonstrate that Tax *trans*-activates the *bfl-1* gene through the NF-κB pathway, but it also suggests that other transcription factors might be involved in this regulation. Reinforcing such an hypothesis, we observed that the NF-κB inhibitor Bay11-7082 reduced *bfl-1* mRNA level in HTLV-1-infected C91PL and MT-4 T-cell lines (data not shown).

c-Jun and JunD Cooperate with Tax to Activate bfl-1 Gene Transcription—Previous reports showed that the Tax-M22 mutant, deleted for its NF-κB binding ability, was also unable to activate the AP-1-responsive promoter (68). Furthermore, the *bfl-1* promoter contains AP-1-binding sites (50, 67). Therefore, we tested whether AP-1 may also be involved in *trans*-activation of the *bfl-1* promoter by Tax. We first investigated the role of AP-1 transcription factors on the activation of *bfl-1* gene expression by performing luciferase reporter assays. Co-transfection of HeLa cells with either JunB, c-Jun, or JunD, and *bfl-1*-luciferase promoter-expressing vectors showed that both c-Jun and JunD activated the *bfl-1* promoter in a dose-dependent manner, although JunB did not (supplemental Fig. S3). We next analyzed the potential cooperation between either Tax and c-Jun or Tax and JunD using suboptimal amounts of AP-1-
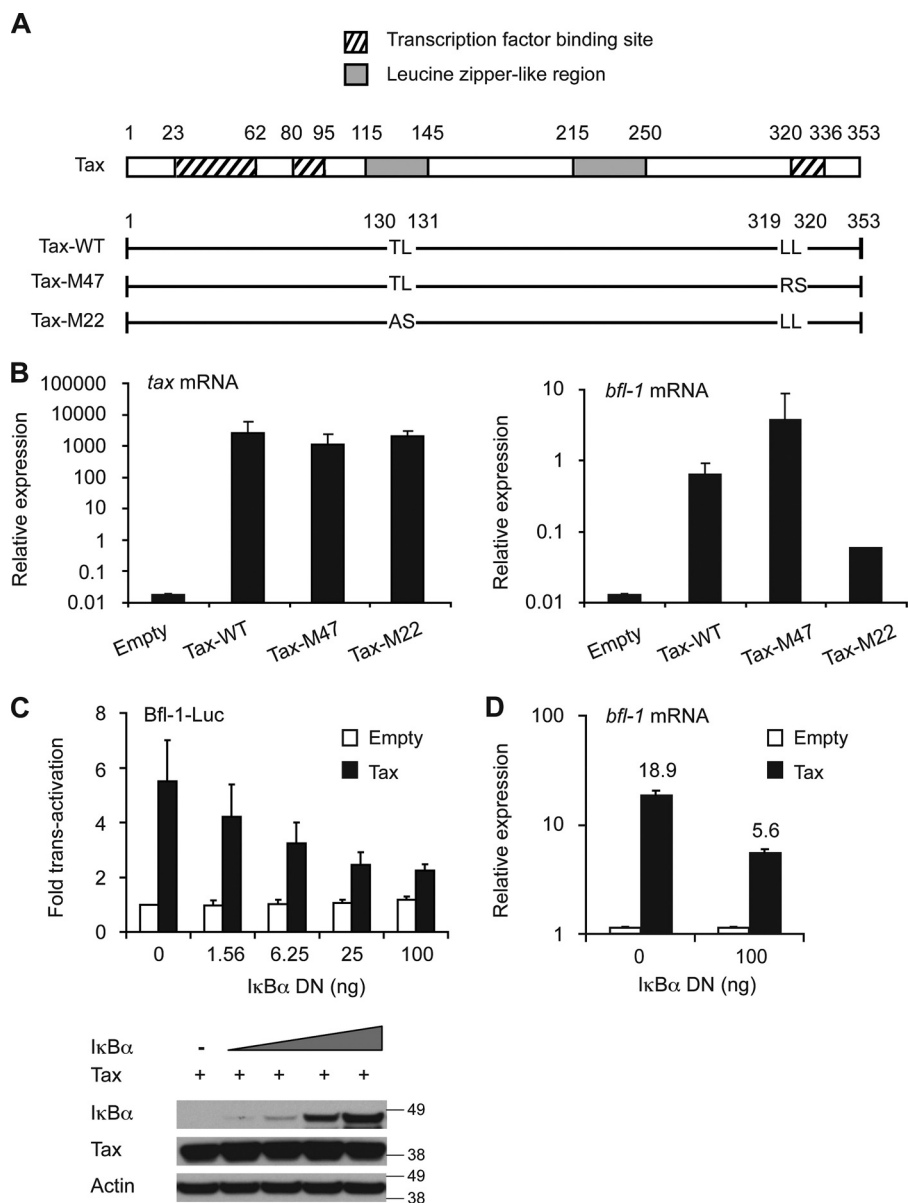
FIGURE 3. **Tax *trans*-activates *bfl-1* gene via the canonical NF-κB-pathway.** *A,* schematic diagram of wild type Tax of HTLV-1 and its mutants (Tax-M47 and Tax-M22) used in this study. Specific mutations interrupting the transcriptional pathways are indicated, Tax-M47 (L319R/L320S, ΔCREB) and Tax-M22 (T130A/L131S, ΔNF-κB). Tax is composed of transcription factor binding sites and leucine zipper-like regions. The numbers shown indicate amino acid positions. *B,* effect of Tax-WT, Tax-M47, and Tax-M22 on the expression of endogenous *bfl-1* transcripts. Empty vector, Tax-WT, Tax-M22, or Tax-M47 expression vectors (0.3 μg) were transfected into HeLa cells. After 2 days, the relative expression of *tax* (*left panel*) and *bfl-1* (*right panel*) mRNAs was normalized using housekeeping gene by real time RT-PCR. Results are expressed as means ± S.D. of three independent experiments. *C,* effect of IκBαDN on Tax-mediated activation of the *bfl-1* promoter. *Upper panel,* HeLa cells were co-transfected with Bfl-1-Luc promoter (0.3 μg), with pRL-TK-Ruc (0.1 μg) plasmids, with increasing amounts of IκBαDN (1.56–100 ng) and with empty or Tax-WT (0.3 μg) expression vectors. After 2 days, luciferase activity was normalized to TK-Ruc. Normalized luciferase values were expressed as *trans*-activation (*n*-fold) over control empty vector. The *bars* represent the means ± S.D. of three independent experiments. *Lower panel,* protein levels for IκBα and Tax in lysates of HeLa samples were determined by Western blotting. β-Actin was used as a loading control. *D,* effect of IκBαDN on Tax-mediated endogenous *bfl-1* mRNA expression. HeLa cells were co-transfected with IκBαDN (100 ng) and empty or Tax-WT (0.3 μg) expression vectors. After 2 days, the relative expression of *bfl-1* mRNA was normalized using housekeeping gene by real time RT-PCR. Results are expressed as means ± S.D. of triplicates.

expressing vectors. Interestingly, overexpression of Tax with c-Jun or JunD resulted in an increased activity of the *bfl-1* promoter activity in a dose-dependent manner, compared with Tax, c-Jun, or JunD alone (Fig. 4, *A* and *B, upper panels*). Immunoblotting experiments confirmed the expression of Tax, c-Jun, or JunD proteins in transfected HeLa cells (Fig. 4, *A* and *B, lower panels*). To corroborate these results in T-cells, the Jurkat T-cell line was nucleofected with the *bfl-1* promoter expressing vector and empty-, Tax-, and c-Jun- or JunD-expressing vec-

tors. As expected, we observed that Tax cooperated with c-Jun or JunD in Jurkat T-cells to activate the *bfl-1* promoter (data not shown). We next examined the cooperation of Tax with either c-Jun or JunD on endogenous *bfl-1* gene expression. For that purpose, HeLa cells were co-transfected either with Tax- and c-Jun-expressing or Tax- and JunD-expressing vectors, and *bfl-1* mRNA expression was evaluated by real time RT-PCR. We observed that *bfl-1* gene transcription was highly increased in cells co-expressing Tax and c-Jun or Tax and JunD as com-
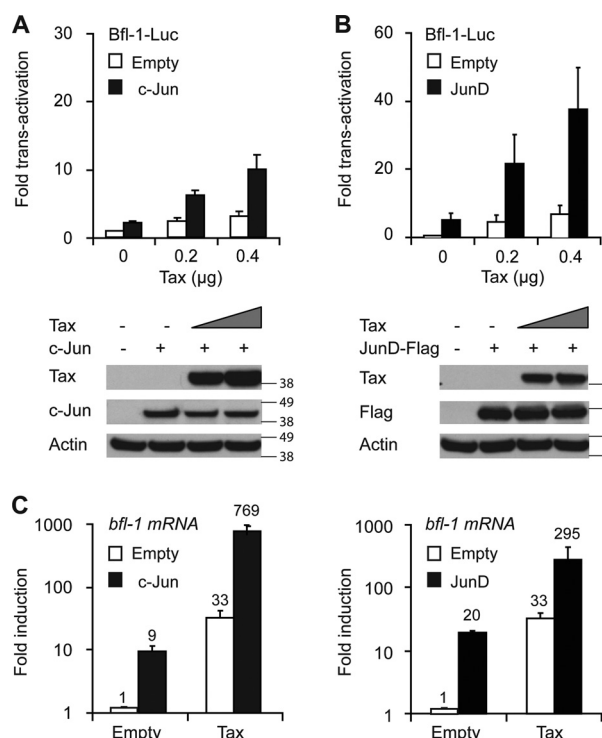
FIGURE 4. Tax synergizes with either c-Jun or JunD to *trans*-activate the *bfl-1* gene. *A,* effect of Tax on c-Jun-mediated activation of the *bfl-1* promoter. *Upper panel,* HeLa cells were co-transfected with Bfl-1-Luc promoter (0.3 μg) and pRL-TK (0.1 μg) plasmids and either with c-Jun expression vectors (6.25 ng) or increasing amounts of Tax-WT expression vectors (0.2 and 0.4 μg). Luciferase activity was normalized and presented as in Fig. 3. Results are expressed as means ± S.D. of three independent experiments. *Lower panel,* protein levels for Tax and c-Jun in lysates of HeLa samples were determined by Western blotting. β-Actin was used as a loading control. Results are from one representative experiment of two. *B,* effect of Tax on JunD-mediated activation of the *bfl-1* promoter. *Upper panel,* HeLa cells were co-transfected with Bfl-1-Luc promoter (0.3 μg) and pRL-TK (0.1 μg) plasmids and either with different amounts of Tax-WT expression vectors (0.2 and 0.4 μg) or JunD expression vectors (6.25 ng). Luciferase activity was normalized and presented as in Fig. 3. Results are expressed as means ± S.D. of three independent experiments. *Lower panel,* protein levels for Tax and JunD in lysates of HeLa samples were determined by Western blotting. β-Actin was used as a loading control. Results are from one representative experiment of two. *C,* effect of Tax on Jun-mediated endogenous *bfl-1* mRNA expression. HeLa cells were transfected with Tax-WT (0.3 μg), c-Jun (6.25 ng), or JunD (6.25 ng) expressing vectors alone or co-transfected with Tax-WT plus c-Jun or Tax-WT plus JunD expressing vectors. After 2 days, the relative expression of *bfl-1* mRNA was normalized using housekeeping gene by real time RT-PCR. Results are expressed as means ± S.D. of triplicates.

pared with cells expressing only Tax, c-Jun, or JunD (Fig. 4*C*). Thus, these results show for the first time that Tax acts synergistically either with c-Jun or JunD to *trans*-activate the *bfl-1* gene.

HBZ Regulates Jun-induced bfl-1 Promoter Activation via Both Activation and Basic Leucine Zipper Domains—As HBZ modulates gene expression via c-Jun and JunD (29, 57, 58, 69), we assessed on the ability of HBZ viral protein to regulate *bfl-1* gene activation. We first analyzed the role of HBZ on the activation of *bfl-1* gene expression by performing luciferase reporter assays. Co-transfection of HeLa cells with HBZ-expressing vector (Fig. 5*A*) and *bfl-1*-luciferase promoter plasmid showed that HBZ was unable to activate the *bfl-1* promoter, whatever the amount of HBZ expressing vector used (Fig. 5, *B* and *C, upper panels*). We next analyzed the potential effect of HBZ on c-Jun- or JunD-mediated *bfl-1* promoter activation
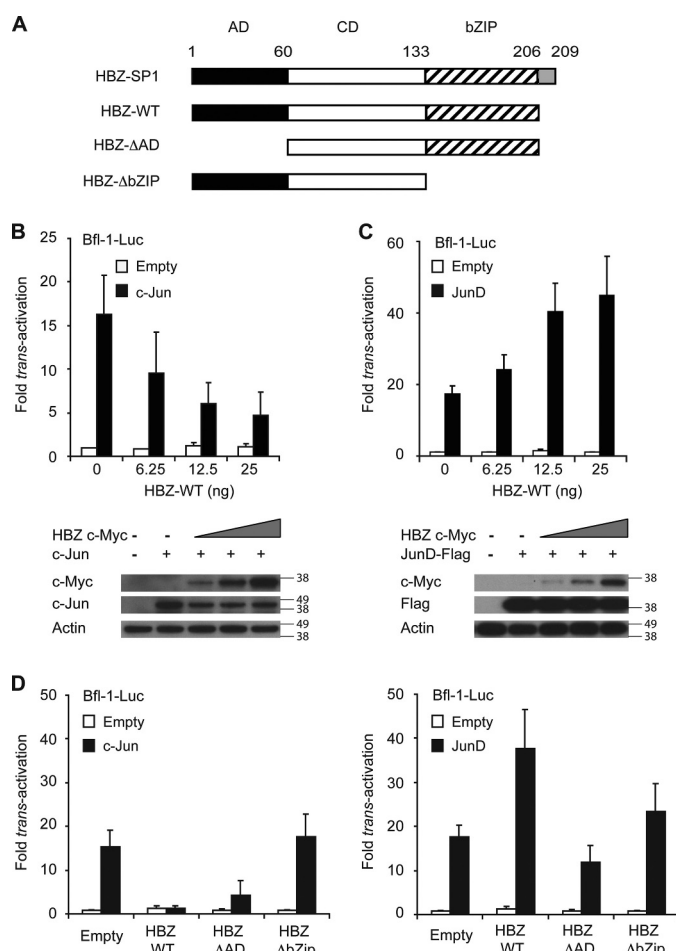

FIGURE 5. HBZ modulates Jun-mediated *bfl-1* promoter activation via both activation and leucine zipper domains. *A,* schematic diagram of full-length HBZ (*HBZ-WT*) and its mutants (*HBZ-ΔAD* and *HBZ-ΔbZIP*) used in this study. HBZ is composed of an N-terminal activation domain (*AD*), a central domain (*CD*), and a C-terminal bZIP. The *numbers* shown indicate amino acid positions. Δ, deletion. *B,* HBZ inhibits c-Jun-mediated activation of the *bfl-1* promoter. *Upper panel,* HeLa cells were co-transfected with Bfl-1-Luc promoter (0.3 μg) and pRL-TK (0.1 μg) plasmids and either with increasing amounts of WT HBZ (6.25–25 ng) or c-Jun (25 ng). Luciferase activity was normalized and presented as in Fig. 3. Results are expressed as means ± S.D. of three independent experiments. *Lower panel,* protein levels for WT HBZ and c-Jun in lysates of HeLa samples were determined by Western blotting. β-Actin was used as a loading control. Results are from one representative experiment of two. *C,* HBZ synergizes with JunD to activate the *bfl-1* promoter. *Upper panel,* HeLa cells were co-transfected with Bfl-1-Luc promoter (0.3 μg) and pRL-TK (0.1 μg) plasmids and either with increasing amounts of WT HBZ (6.25–25 ng) or JunD (25 ng) expression vectors. Luciferase activity was normalized and presented as in Fig. 3. Results are expressed as means ± S.D. of three independent experiments. *Lower panel,* protein levels for WT HBZ and JunD in lysates of HeLa samples were determined by Western blotting. β-Actin was used as a loading control. Results are from one representative experiment of two. *D,* both activation and leucine zipper are required for full HBZ activities. HeLa cells were co-transfected with Bfl-1-Luc promoter (0.3 μg) and pRL-TK (0.1 μg) plasmids and either with HBZ (WT, ΔAD, ΔbZIP, 25 ng), or c-Jun (25 ng, *left panel*) or JunD (25 ng, *right panel*) expressing vectors alone or with HBZ (WT, ΔAD, ΔbZIP) plus c-Jun or HBZ (WT, ΔAD, ΔbZIP) plus JunD expressing vectors. After 2 days, luciferase activity was normalized and presented as in Fig. 3. Results are expressed as means ± S.D. of three independent experiments.

using optimal amounts (25 ng) of Jun-expressing vectors. Interestingly, overexpression of HBZ inhibited c-Jun-mediated activation of the *bfl-1* promoter in a dose-dependent manner (Fig. 5*B, upper panel*). By contrast, overexpression of HBZ with JunD resulted in the amplification of JunD-mediated *bfl-1* promoter

activation in a dose-dependent manner (Fig. 5C, *upper panel*). Immunoblotting experiments confirmed the expression of HBZ, c-Jun, or JunD in transfected HeLa cells (Fig. 5, *B* and *C, lower panels*).

We further examined the role of activation domains and bZIP domains in HBZ modulating Jun-induced *bfl-1* promoter activation, using the well characterized HBZ mutants (HBZ-ΔAD and -ΔbZIP) (Fig. 5A). Vectors expressing HBZ-WT or its deleted forms and the *bfl-1* promoter were transiently co-transfected into HeLa cells with or without c-Jun- or JunD-expressing vectors. As shown in Fig. 5D, HBZΔAD partially repressed c-Jun-mediated *bfl-1* promoter activation, whereas HBZΔbZIP was unable to do so (*left panel*). By contrast, HBZΔAD was unable to enhance JunD-mediated *bfl-1* promoter activation, whereas HBZΔbZIP had no significant effect on JunD-mediated *bfl-1* promoter *trans*-activation (Fig. 5D, *right panel*).

Altogether these results demonstrate the following: (i) HBZ suppresses c-Jun-mediated *bfl-1* promoter activation; (ii) HBZ acts synergistically with JunD to increase the activation of the *bfl-1* promoter; and (iii) both the activation and basic leucine zipper domains of HBZ are required to modulate c-Jun- or JunD-induced *bfl-1* promoter activation.

NF-κB, c-Jun, and JunD Binding Activity to the bfl-1 Promoter in T-cells—We have shown that Tax induced *bfl-1* gene activation via both NF-κB and AP-1 pathways and that HBZ modulates Jun-mediated *bfl-1* promoter activity. To determine the resulting activity of both Tax and HBZ on the endogenous expression of *bfl-1* transcripts, we measured *bfl-1* mRNA levels by real time RT-PCR in the E12 cells, a clone derived from Jurkat T-cells that stably express Tax and HBZ, and in C8166-45 T-cells carrying but not expressing the HTLV-1 viral proteins, except Tax and HBZ. As shown in Fig. 6A, both E12 and C8166-45 T-cells expressed a high level of *tax* and a low level of *hbz* transcripts. More interestingly, *bfl-1* is highly expressed in these T-cells (Fig. 6A), suggesting that Tax may have a dominant-positive effect when its expression is higher than that of HBZ.

We next determined whether Tax and/or HBZ are responsible for NF-κB and/or AP-1 binding to respective consensus elements in the proximal part of the *bfl-1* promoter. EMSA was performed with double-stranded oligonucleotides representing the NF-κB at position −52 and AP-1 elements at positions −104 or −74. Consistent with the luciferase assays, protein complexes bound to both NF-κB (Fig. 6B, *lane 2*) and AP-1 (Fig. 6B, *lanes 6* and *10* for *AP-104* and *AP74*, respectively) sites were detected with nuclear extracts from E12 T-cell clone. The specificity of DNA-protein complexes in extracts was determined by competition studies with unlabeled competitors. As expected, cold κB52, AP-104, and AP74 double-stranded oligonucleotides efficiently competed with their homologously labeled probes and decreased binding of nuclear extracts in a dose-dependent manner (Fig. 6B, *lanes 3* and *4* for *κB52*, *lanes 7* and *8* for AP-104, and *lanes 11* and *12* for AP74). Then, the exact composition of the transcription factor DNA-protein complexes in E12 T-cell clone was ascertained using antibody supershifting EMSA. Supershift analyses were performed on agarose gels to achieve a better resolution of the antibody-protein-DNA complexes. Antibodies against p65/RelA, c-Rel,

RelB, p100/p52, and p105/p50 all induced a supershift of the DNA-protein complexes using the κB52 site of the *bfl-1* gene as probe (Fig. 6C, *lanes 2–6*, respectively) with a variable degree of shift depending on the antibody used. Antibodies against c-Jun and JunD induced a supershift of complexes formed with either an AP-104 or AP-74 DNA-binding site (Fig. 6C, *lanes 8* and *9* and *11* and *12*, respectively). Note that isotype controls were unable to induce supershift, demonstrating the specificity of interactions between NF-κB and AP-1 with the *bfl-1* promoter (data not shown). Taken together, our data indicate that NF-κB, c-Jun, and JunD proteins bind to the proximal part of the κB and AP-1 elements of the *bfl-1* promoter in E12 T-cells expressing Tax, HBZ, and Bfl-1. Moreover, these results suggest that both canonical and alternative NF-κB pathways are involved in the κB binding activity of the *bfl-1* promoter.

Bfl-1 Is Involved in HTLV-1-infected T-cell Survival—We finally asked whether Bfl-1 participates in the survival of HTLV-1-infected T-cell lines. As the impact of the antiapoptotic Bcl-2 proteins in HTLV-1-infected T-cell survival has never been studied, we also compared the involvement of Bfl-1 to that of the antiapoptotic Bcl-2 or Bcl-x$_L$ proteins. We thus knocked down the expression of these proteins using a lentivirus-based vector pLL3.7 that co-expressed specific shRNA and GFP as reporter genes, thus permitting transduced cells to be tracked by flow cytometry. After 5–7 days of infection, the transduction efficiency of uninfected (Jurkat) and HTLV-1-infected (C91PL and MT-4) T-cell lines ranged from 87 to 98% (data not shown). Bcl-2, Bcl-x$_L$, and Bfl-1 silencing by the specific shRNAs was confirmed by immunoblotting (Fig. 7A). We observed that Bcl-x$_L$ and Bfl-1 knockdown induced a moderate death of C91PL T-cells (Fig. 7B, *middle panel*) with only 20 and 40% of cell death detected in C91PL cells transduced with Bcl-x$_L$ and Bfl-1 shRNA, respectively, suggesting that Bfl-1 expression may offset the Bcl-x$_L$ knockdown and conversely. In contrast, we observed that MT4 T-cells were highly sensitive to Bfl-1 silencing, whereas cell death resulting from Bcl-x$_L$ knockdown never exceeded 25% after 7 days in these cells (Fig. 7B, *right panel*). Finally, Ctl or Bcl-2 shRNA did not induce significant death in transduced C91PL and MT-4 T-cells, and the viability of uninfected Jurkat T-cells was not altered by any shRNA tested (Fig. 7B). Altogether, these data directly involve Bfl-1, but also Bcl-x$_L$, in the survival of HTLV-1-transformed T-cell lines.

HTLV-1-infected T-cell Survival Is Fully Compromised by Combination of Bfl-1 Knockdown and ABT-737 Treatment—As Bcl-x$_L$ and Bfl-1 knockdown induced, respectively, only 20 and 40% of cell death in C91PL (Fig. 7B), we assessed whether targeting both Bfl-1 and Bcl-x$_L$ proteins might compromise C91PL cell survival. Among the small compounds that target Bcl-2 antiapoptotic proteins, ABT-737 has been described to specifically block Bcl-2, Bcl-x$_L$, and Bcl-w activity, but not Bfl-1 and Mcl-1 activity (66). We first explored survival of the C91PL T-cell line after ABT-737 exposure. ABT-737 caused death of C91PL T-cells in a concentration-dependent manner (Fig. 8A). As a control and in agreement with a previous report (70), Jurkat T-cells were poorly sensitive to ABT-737 (data not shown). We then combined treatment with ABT-737 and Bfl-1 shRNA. Interestingly, Bfl-1 knockdown resulted in
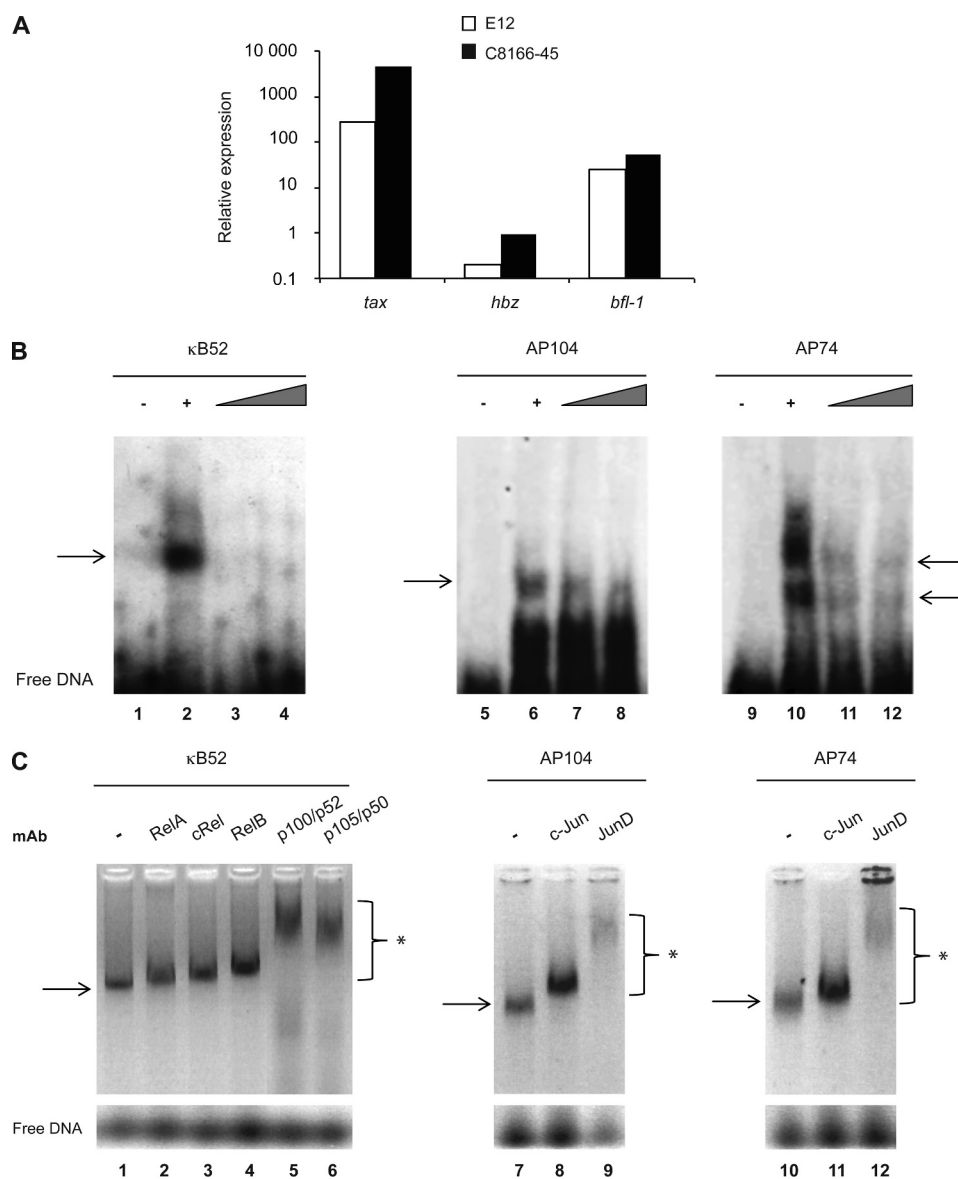
FIGURE 6. **Constitutive activation of NF-κB, c-Jun, and JunD in E12 T-cell clone expressing both Tax and HBZ.** *A,* expression of *bfl-1* transcript in stably Tax/HBZ-expressing Jurkat cells (E12) and in HTLV-1-infected C8166–45 T-cell line expressing only Tax and HBZ by real time RT-PCR. The relative expression of specific *tax*, *hbz*, and *bfl-1* mRNA was normalized using housekeeping genes. Results are from one representative experiment of three. *B,* increase of NF-κB and AP-1 binding activities in nuclear extracts from E12 T-cell clone. Labeled *bfl-1* promoter containing the NF-κB- (κB52 probe, *left panel*) and AP-1 (AP-104 and AP74 probes, *middle and right panels*)-binding sites were incubated alone (*lanes 1, 5,* and *9,* respectively) or with 100 ng of nuclear extract (*lanes 2–4, 6–8,* and *10–12*) in EMSA reactions. Specificity of the DNA-protein complex formation at the NF-κB and AP-1 sites in competition EMSA; 100 and 200 ng of cold κB52 (*lanes 3* and *4*), AP-104 (*lanes 7* and *8*), and AP74 (*lanes 11* and *12*) oligonucleotides were added as competitors. Free probes are indicated at the *bottom* of the gels. Specific protein-DNA complexes are indicated by *arrows*. *C,* NF-κB- and AP-1-binding complexes were recognized by specific mAbs. Nuclear extracts from E12 T-cell clone were preincubated without (indicated as −) or with the mAbs indicated on *top* of each lane, before adding the κB52 (*left panel*), AP-104 (*middle panel*), and AP74 (*right panel*) probes to the binding mixture. Free DNA is indicated at the *bottom* of the gels. Supershifted bands are indicated by *brace* plus *. Results are from one representative experiment of three.

complete sensitization of C91PL T-cells to ABT-737 treatment (Fig. 8*B*). Such cell death was not observed in C91PL cells transduced with Bfl-1 shRNA plus DMSO or with Ctl shRNA plus ABT-737. As a negative control, Bfl-1 knockdown did not significantly induce cell death of ABT-737-treated Jurkat T-cell line (data not shown).

Thus, our data demonstrate for the first time that Bfl-1 expression confers resistance of the HTLV-1-infected C91PL T-cell line to ABT-737 treatment, and the data suggest that the concurrent up-regulation of both Bfl-1 and Bcl-x$_L$ can synergize to induce survival of HTLV-1-infected T-cell lines.

HTLV-1-infected T-cell Survival Is Compromised by Combination of Bfl-1 Knockdown and Etoposide Treatment—As acute aggressive ATLL cells were resistant to chemotherapeutic agents (71), we further determined whether expression of Bfl-1 could be associated with resistance to drugs such as etoposide. We first explored viability of the C91PL T-cell line after exposure to etoposide for 48 h. Etoposide caused death of C91PL T-cells in a concentration-dependent manner (Fig. 8*C*), with a maximum of 60% of dead cells, even for concentrations higher than 1.5 μM (data not shown). We next assessed whether Bfl-1 silencing affected etoposide-induced apoptosis of HTLV-1-
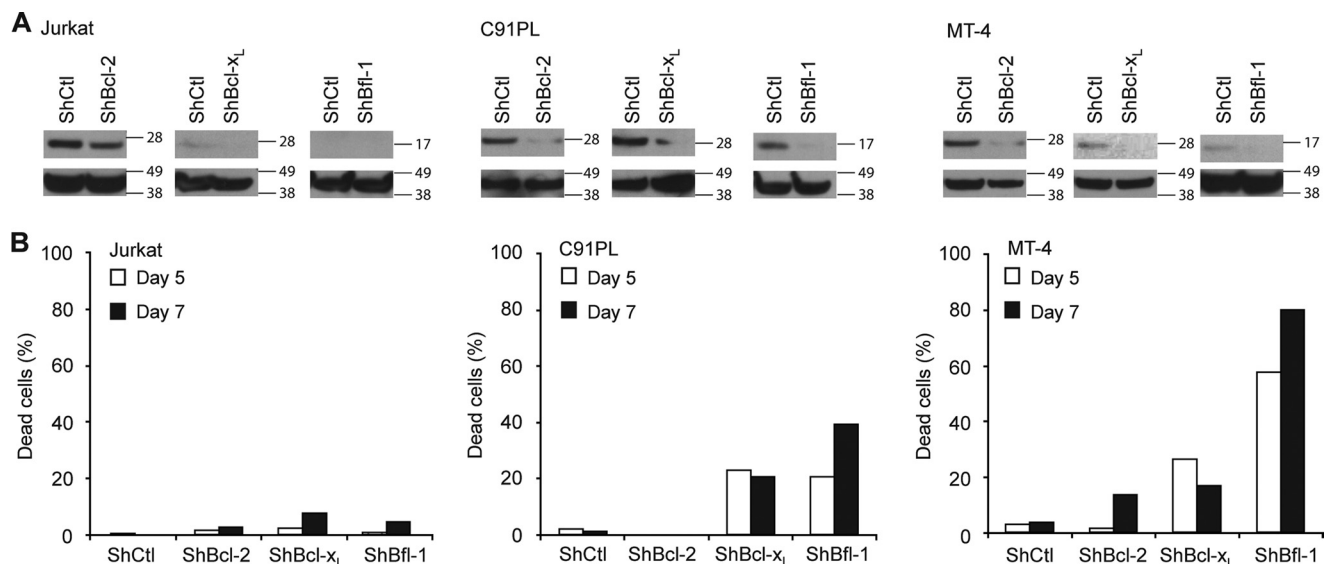
FIGURE 7. **Down-regulation of Bfl-1 expression induced spontaneous HTLV-1-infected T-cell death.** Cells were transduced with lentivirus expressing Luc-Ctl, Bcl-2, Bcl-x$_L$, or Bfl-1 shRNA. *A,* after 5 days, the knockdown of Bcl-2, Bcl-x$_L$, or Bfl-1 by the specific shRNA in transduced Jurkat, C91PL, and MT-4 T-cell lines was controlled by Western blotting using specific mAbs against Bcl-2, Bcl-x$_L$, or Bfl-1, respectively (*upper panels*). Comparable protein loading was confirmed using a mAb specific for the housekeeping β-actin protein (*lower panels*). *B,* percent of dead cells (PI$^+$) was measured by flow cytometry 5–7 days post-transduction. Results are from one representative experiment of three to five.
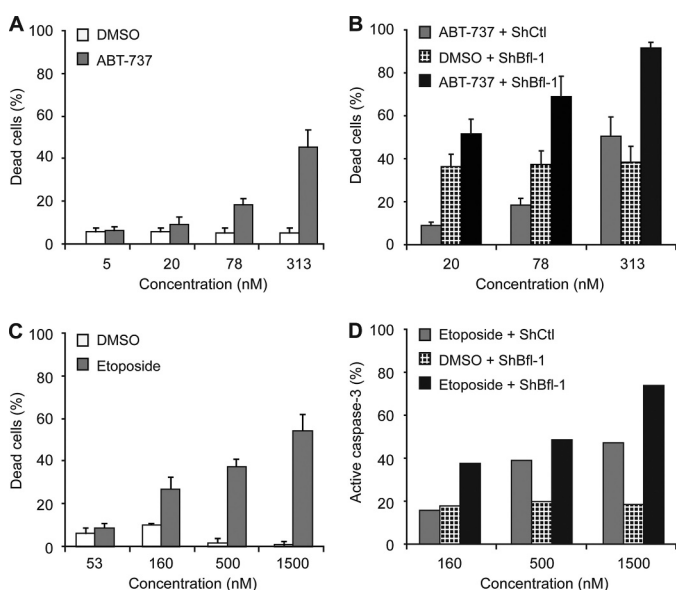

FIGURE 8. **Knockdown of Bfl-1 by shRNA sensitizes HTLV-1-infected C91PL T-cells to ABT-737- or etoposide-induced apoptosis.** *A,* effect of ABT-737 on HTLV-1-infected C91PL T-cell death. Cells were treated with different concentrations of vehicle (DMSO) or ABT-737. After 2 days, percentages of cell death (PI$^+$) were measured by flow cytometry. Results are expressed as means ± S.D. of three independent experiments. *B,* C91PL cells were transduced with Bfl-1 or scramble Bfl-1 Ctl shRNA and 5 days later exposed to different concentrations of DMSO or ABT-737 for 2 additional days. Cell death was assessed by PI staining and measured by flow cytometry. Results are expressed as means ± S.D. of three independent experiments. *C,* effect of etoposide on HTLV-1-infected C91PL T-cell death. Cells were treated with different concentrations of vehicle (DMSO) or etoposide. After 2 days, the percentages of cell death were measured as in *A*. Results are expressed as mean ± S.D. of three independent experiments. *D,* C91PL cells were transduced with Bfl-1 or scramble Bfl-1 Ctl shRNA and 5 days later were exposed to different concentrations of etoposide for 2 additional days. Apoptosis was assessed by anti-active caspase-3 Ab staining and measured by flow cytometry. Results are from one representative experiment of two.

infected C91PL T-cells. C91PL cells were first transduced with Ctl or Bfl-1 shRNA. After 5 days, cells were treated with various concentrations of etoposide for 48 h, and the percentage of apoptotic cells, determined with anti-active caspase 3 Ab, was measured by flow cytometry. As shown in Fig. 8*D*, Bfl-1 shRNA, but not Ctl shRNA, efficiently increased sensitivity to etoposide. Such an effect is not observed in the presence of DMSO (Fig. 8*D*). Altogether, these data demonstrate a role for Bfl-1 in resistance to etoposide-induced apoptosis of HTLV-1-infected T-cells.

DISCUSSION

The role of the antiapoptotic Bfl-1 and Bcl-x$_L$ proteins in tumor survival and chemoresistance to current drugs has been documented (72). ATLL development is a multistep oncogenic process, which affects among others cellular functions, proliferation, and cell survival. The outcome of aggressive ATLL remains very poor because HTLV-1-infected cells are resistant to most conventional chemotherapy. Understanding the impact of antiapoptotic proteins of the Bcl-2 family on HTLV-1-infected T-cell survival may provide clues for the development of new therapeutic strategies. Although the expression of Bcl-2 and Bcl-x$_L$ antiapoptotic proteins has been extensively documented in HTLV-1-infected T-cell lines and in ATLL cells, the role of Bfl-1 has never been described. Here, we showed the following: (i) HTLV-1-infected T-cells express Bfl-1 protein, whereas uninfected leukemia/lymphoma T-cells do not; (ii) the regulatory viral Tax protein induces Bfl-1 expression through both NF-κB and AP-1 pathways, whereas the HBZ protein modulates AP-1-induced Bfl-1 expression; and (iii) Bfl-1 and Bcl-x$_L$, but not Bcl-2, are involved in the survival of HTLV-1-infected T-cells.

The first link between HTLV-1 infection and the deregulation of *bfl-1* expression was revealed by gene expression profile experiments indicating that *bfl-1* gene is significantly up-regu-

lated in PHA/IL-2-stimulated HTLV-1-infected human peripheral blood T-cells, compared with stimulated uninfected T-cells (73). Reinforcing this observation, our results, based on the analysis of six HTLV-1-infected and five uninfected T-cell lines, indicate that Bfl-1 expression is restricted to HTLV-1-infected T-cell lines. Interestingly, we also observed Bfl-1 protein expression in two acute ATLL cells, but not in two chronic ATLL cells. Such results suggest that Bfl-1 expression might be related to the aggressive status of acute ATLL associated with a poor prognosis and multidrug resistance, although the chronic ATLL is considered as an indolent subtype (71). However, more samples need to be studied to definitively demonstrate overexpression of Bfl-1 in aggressive ATLL cells.

More and more studies point out the growing complexity of the mechanisms regulating viral Tax and HBZ protein expression and their impact in leukemogenesis (see reviews in Refs. 14, 21, 28). According to our first observation in primary thymocytes (74), we found here that Tax alone up-regulates endogenous *bfl-1* in primary mature T-cells, indicating that Bfl-1 expression in HTLV-1-infected T-cells is at least Tax-dependent. Our data show unequivocally that Tax-mediated Bfl-1 expression occurs in part through the NF-κB pathway, involving both the canonical and alternative pathways. Supporting the involvement of NF-κB in HTLV-1-mediated T-cell survival, we and others observed that Bay11-7082, an inhibitor of the canonical NF-κB pathway, partly down-regulates Bcl-x$_L$ (75) and Bfl-1 (our work), and partially induced cell death in HTLV-1-infected T-cell lines (data not shown). These observations support the hypothesis that signaling pathways, other than the NF-κB pathway, are involved in overexpression of the antiapoptotic Bfl-1 protein. We showed that JunD and c-Jun, but not JunB, potently activate the *bfl-1* promoter and stimulate endogenous expression of *bfl-1*. Jun members are responsible for the expression of genes involved in cell proliferation, survival, and T-cell transformation (76, 77). Although Tax is able to activate the expression and to increase the activity of JunD, c-Jun, c-Fos, and Fra-1/2 *in vitro* (20, 78–80), only JunD seems to be constitutively activated in ATLL cells (27, 78). We report here that JunD or c-Jun synergized with Tax to activate *bfl-1* transcription. We observed both c-Jun and JunD binding activities in two sites of the proximal part of the *bfl-1* promoter in E12 T-cell clone highly expressing Tax. Altogether, these data demonstrate for the first time that NF-κB and AP-1 members mediate Tax-induced Bfl-1 expression. Thus, we propose that both NF-κB and AP-1, especially JunD, transcription factors, are involved in Tax-mediated antiapoptotic activity through the up-regulation of the *bfl-1* protein. In keeping with such an hypothesis, we observed that knockdown of Tax down-regulates *bfl-1* expression and induces death of the E12 T-cell clone expressing both Tax and HBZ (data not shown). These findings are in agreement with previous observations showing that the mouse CTLL-2 T-cells expressing the Tax mutant defective for its ability to activate both NF-κB and AP-1 (Tax-M22) were unable to survive in the absence of IL-2, in contrast to cells expressing Tax-WT (79, 81).

Although Tax plays an important role in the initiation to leukemogenesis, HBZ seems to rather maintain the tumoral phenotype of ATLL cells. Numerous studies showed that HBZ modulates NF-κB and AP-1 pathways to enhance proliferation of HTLV-1-infected T-cells in the late stages of ATLL (for review see Ref. 28). The present data demonstrate that HBZ is unable to *trans*-activate the *bfl-1* gene in T-cells and that it inhibits Tax-mediated *bfl-1 trans*-activation. Supporting our observation, Zhi *et al.* (82) reported that HBZ decreases Tax-mediated NF-κB activation. Zhao et al. (83) indicated a selective suppression of the canonical NF-κB pathway by HBZ, which interacts with the p65 subunit of NF-κB, thus preventing its DNA binding activity and inducing its degradation through a proteasome-dependent pathway. However, HBZ-mediated repression of the canonical NF-κB pathway is not efficient when Tax is overexpressed (19, 83, 84). HBZ has also been described to modulate gene expression, especially hTERT, depending on its Jun partner (29). Here, we report that ectopic expression of HBZ inhibits c-Jun-mediated *bfl-1* gene activation and enhances *bfl-1* promoter activity induced by JunD in transfected cells. We also showed that Bfl-1 is highly expressed in T-cell lines, which constitutively express a low level of HBZ and a high level of Tax, supporting the notion that Tax seems to be the major inducer of Bfl-1, compared with HBZ, when Tax is highly expressed. However, we cannot exclude that other viral proteins may regulate Bfl-1 expression. For example, accessory p30[II] protein of HTLV-1 is known to modulate apoptotic pathways, by regulating the transcriptional activity of NF-κB and AP-1 (85). In agreement with this hypothesis, we found no correlation between the expression of Tax and/or HBZ and that of Bfl-1 in HTLV-1-infected T-cell lines.

Finally, our results directly implicate both Bfl-1 and Bcl-x$_L$ in the survival of HTLV-1-infected T-cell lines and support the hypothesis that these two antiapoptotic proteins may be crucial for the extended survival of (pre-)leukemic T-cells. Similarly to what was previously observed by us and others in B lymphomas (47, 86), silencing of the Bfl-1 antiapoptotic protein induces significant cell death of HTLV-1-infected T-cells in a caspase-dependent manner. In agreement with the model proposed by Certo *et al.* (87), our observation suggests that inhibition of Bfl-1 antiapoptotic protein by itself, either by down-regulating its expression or through interaction with BH-3-only proteins or BH3 mimetics, may be sufficient to provoke cell apoptosis.

Drugs used in ATLL treatment, such as the NF-κB inhibitor Bay11-7082, suppress Bfl-1 and Bcl-x$_L$ expression and induce apoptosis of HTLV-1-infected T-cell lines (data not shown). Other drugs, such as arsenic trioxide, have also been shown to induce down-regulation of Bcl-x$_L$, but not that of Bcl-2, and apoptosis in HTLV-1-infected T-cell lines and in *ex vivo* ATLL cells (88). Apoptosis of tumor cells can be triggered by small compounds that mimic BH3-only proteins and target antiapoptotic Bcl-2 proteins (70). However, ABT-737, which targets Bcl-2, Bcl-x$_L$ and Bcl-w (66), does not induce an efficient *in vitro* apoptosis of ATLL cells expressing at least one member of the Bcl-2 family, such as Bcl-2, Bcl-x$_L$, Bcl-w, and Mcl-1 (89). The low sensitivity of ATLL cells to ABT-737-induced apoptosis may indeed be explained by overexpression of Mcl-1, but also Bfl-1, because both proteins are known to be involved in ABT-737 resistance (90–93). In agreement with such an hypothesis, we demonstrate that down-regulation of Bfl-1 expression by shRNA strategy sensitizes the HTLV-1-infected C91PL T-cell

line to ABT-737 treatment. Taken together, our results implicate Bfl-1 and Bcl-x$_L$ as crucial regulators of the extended survival of leukemic T-cells in the context of HTLV-1 infection. A previous report indicates that anti-apoptotic activity of Bcl-x$_L$ contributes to chemotherapeutic drug resistance of acute ATLL cells (89). To date, the role of Bfl-1 in resistance to conventional chemotherapeutic agents has only been demonstrated in B-cell malignancies (47, 86, 94). In this study, we show for the first time that Bfl-1 silencing increases etoposide-induced caspase-dependent apoptosis in HTLV-1-infected T-cells. But, etoposide treatment in combination with ABT-737 (89) or with Bfl-1 silencing (our work) shows no synergistic effect, suggesting that both Bfl-1 and Bcl-x$_L$ may be simultaneously targeted to restore strong chemosensitivity of HTLV-1-infected T-cells. Taken together, these data indicate that these two antiapoptotic proteins may be considered as potential therapeutic targets in T-cell malignancy associated with HTLV-1.

REFERENCES

1. Cavrois, M., Wain-Hobson, S., Gessain, A., Plumelle, Y., and Wattel, E. (1996) Adult T-cell leukemia/lymphoma on a background of clonally expanding human T-cell leukemia virus type-1-positive cells. *Blood* **88,** 4646–4650

2. Etoh, K., Tamiya, S., Yamaguchi, K., Okayama, A., Tsubouchi, H., Ideta, T., Mueller, N., Takatsuki, K., and Matsuoka, M. (1997) Persistent clonal proliferation of human T-lymphotropic virus type I-infected cells *in vivo*. *Cancer Res.* **57,** 4862–4867

3. Li, M., and Green, P. L. (2007) Detection and quantitation of HTLV-1 and HTLV-2 mRNA species by real time RT-PCR. *J. Virol. Methods* **142,** 159–168

4. Franchini, G., Wong-Staal, F., and Gallo, R. C. (1984) Human T-cell leukemia virus (HTLV-I) transcripts in fresh and cultured cells of patients with adult T-cell leukemia. *Proc. Natl. Acad. Sci. U.S.A.* **81,** 6207–6211

5. Sugamura, K., Fujii, M., Kannagi, M., Sakitani, M., Takeuchi, M., and Hinuma, Y. (1984) Cell surface phenotypes and expression of viral antigens of various human cell lines carrying human T-cell leukemia virus. *Int. J. Cancer* **34,** 221–228

6. Furukawa, Y., Osame, M., Kubota, R., Tara, M., and Yoshida, M. (1995) Human T-cell leukemia virus type-1 (HTLV-1) Tax is expressed at the same level in infected cells of HTLV-1-associated myelopathy or tropical spastic paraparesis patients as in asymptomatic carriers but at a lower level in adult T-cell leukemia cells. *Blood* **85,** 1865–1870

7. Grassmann, R., Berchtold, S., Radant, I., Alt, M., Fleckenstein, B., Sodroski, J. G., Haseltine, W. A., and Ramstedt, U. (1992) Role of human T-cell leukemia virus type 1 X region proteins in immortalization of primary human lymphocytes in culture. *J. Virol.* **66,** 4570–4575

8. Grossman, W. J., Kimata, J. T., Wong, F. H., Zutter, M., Ley, T. J., and Ratner, L. (1995) Development of leukemia in mice transgenic for the *tax* gene of human T-cell leukemia virus type I. *Proc. Natl. Acad. Sci. U.S.A.* **92,** 1057–1061

9. Bellon, M., Baydoun, H. H., Yao, Y., and Nicot, C. (2010) HTLV-I Tax-de-

10. Robek, M. D., and Ratner, L. (1999) Immortalization of CD4$^+$ and CD8$^+$ T lymphocytes by human T-cell leukemia virus type 1 Tax mutants expressed in a functional molecular clone. *J. Virol.* **73,** 4856–4865

11. Pozzatti, R., Vogel, J., and Jay, G. (1990) The human T-lymphotropic virus type I *tax* gene can cooperate with the *ras* oncogene to induce neoplastic transformation of cells. *Mol. Cell. Biol.* **10,** 413–417

12. Nerenberg, M., Hinrichs, S. H., Reynolds, R. K., Khoury, G., and Jay, G. (1987) The *tat* gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice. *Science* **237,** 1324–1329

13. Hasegawa, H., Sawa, H., Lewis, M. J., Orba, Y., Sheehy, N., Yamamoto, Y., Ichinohe, T., Tsunetsugu-Yokota, Y., Katano, H., Takahashi, H., Matsuda, J., Sata, T., Kurata, T., Nagashima, K., and Hall, W. W. (2006) Thymus-derived leukemia-lymphoma in mice transgenic for the *Tax* gene of human T-lymphotropic virus type I. *Nat. Med.* **12,** 466–472

14. Journo, C., Douceron, E., and Mahieux, R. (2009) HTLV gene regulation. Because size matters, transcription is not enough. *Future Microbiol.* **4,** 425–440

15. Ishikawa, C., Nakachi, S., Senba, M., Sugai, M., and Mori, N. (2011) Activation of AID by human T-cell leukemia virus Tax oncoprotein and the possible role of its constitutive expression in ATL genesis. *Carcinogenesis* **32,** 110–119

16. Grassmann, R., Aboud, M., and Jeang, K. T. (2005) Molecular mechanisms of cellular transformation by HTLV-1 Tax. *Oncogene* **24,** 5976–5985

17. Zane, L., Sibon, D., Legras, C., Lachuer, J., Wierinckx, A., Mehlen, P., Delfau-Larue, M. H., Gessain, A., Gout, O., Pinatel, C., Lançon, A., Mortreux, F., and Wattel, E. (2010) Clonal expansion of HTLV-1 positive CD8$^+$ cells relies on cIAP-2 but not on c-FLIP expression. *Virology* **407,** 341–351

18. Taylor, J. M., and Nicot, C. (2008) HTLV-1 and apoptosis. Role in cellular transformation and recent advances in therapeutic approaches. *Apoptosis* **13,** 733–747

19. Silbermann, K., and Grassmann, R. (2007) Human T cell leukemia virus type 1 Tax-induced signals in cell survival, proliferation, and transformation. *Signal Transduction* **7,** 34–52

20. Hall, W. W., and Fujii, M. (2005) Deregulation of cell-signaling pathways in HTLV-1 infection. *Oncogene* **24,** 5965–5975

21. Chlichlia, K., and Khazaie, K. (2010) HTLV-1 Tax. Linking transformation, DNA damage, and apoptotic T-cell death. *Chem. Biol. Interact.* **188,** 359–365

22. Fujita, M., and Shiku, H. (1995) Differences in sensitivity to induction of apoptosis among rat fibroblast cells transformed by HTLV-I *tax* gene or cellular nuclear oncogenes. *Oncogene* **11,** 15–20

23. Satou, Y., Yasunaga, J., Yoshida, M., and Matsuoka, M. (2006) HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T-cell leukemia cells. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 720–725

24. Takeda, S., Maeda, M., Morikawa, S., Taniguchi, Y., Yasunaga, J., Nosaka, K., Tanaka, Y., and Matsuoka, M. (2004) Genetic and epigenetic inactivation of *tax* gene in adult T-cell leukemia cells. *Int. J. Cancer* **109,** 559–567

25. Usui, T., Yanagihara, K., Tsukasaki, K., Murata, K., Hasegawa, H., Yamada, Y., and Kamihira, S. (2008) Characteristic expression of HTLV-1 basic zipper factor (HBZ) transcripts in HTLV-1 provirus-positive cells. *Retrovirology* **5,** 34

26. Nagakubo, D., Jin, Z., Hieshima, K., Nakayama, T., Shirakawa, A. K., Tanaka, Y., Hasegawa, H., Hayashi, T., Tsukasaki, K., Yamada, Y., and Yoshie, O. (2007) Expression of CCR9 in HTLV-1$^+$ T-cells and ATL cells expressing Tax. *Int. J. Cancer* **120,** 1591–1597

27. Nakayama, T., Hieshima, K., Arao, T., Jin, Z., Nagakubo, D., Shirakawa, A. K., Yamada, Y., Fujii, M., Oiso, N., Kawada, A., Nishio, K., and Yoshie, O. (2008) Aberrant expression of Fra-2 promotes CCR4 expression and cell proliferation in adult T-cell leukemia. *Oncogene* **27,** 3221–3232

28. Matsuoka, M. (2010) HTLV-1 bZIP factor gene. Its roles in HTLV-1 pathogenesis. *Mol. Aspects Med.* **31,** 359–366

29. Kuhlmann, A. S., Villaudy, J., Gazzolo, L., Castellazzi, M., Mesnard, J. M., and Duc Dodon, M. (2007) HTLV-1 HBZ cooperates with JunD to enhance transcription of the human telomerase reverse transcriptase gene (hTERT). *Retrovirology* **4,** 92

30. Satou, Y., Yasunaga, J., Zhao, T., Yoshida, M., Miyazato, P., Takai, K., Shimizu, K., Ohshima, K., Green, P. L., Ohkura, N., Yamaguchi, T., Ono, M., Sakaguchi, S., and Matsuoka, M. (2011) HTLV-1 bZIP factor induces T-cell lymphoma and systemic inflammation in vivo. *PLoS Pathog.* **7,** e1001274

31. Hagiya, K., Yasunaga, J., Satou, Y., Ohshima, K., and Matsuoka, M. (2011) ATF3, an HTLV-1 bZIP factor-binding protein, promotes proliferation of adult T-cell leukemia cells. *Retrovirology* **8,** 19

32. Arnold, J., Zimmerman, B., Li, M., Lairmore, M. D., and Green, P. L. (2008) Human T-cell leukemia virus type-1 antisense-encoded gene, *Hbz*, promotes T-lymphocyte proliferation. *Blood* **112,** 3788–3797

33. White, E. (2006) Mechanisms of apoptosis regulation by viral oncogenes in infection and tumorigenesis. *Cell Death Differ.* **13,** 1371–1377

34. Postigo, A., and Ferrer, P. E. (2009) Viral inhibitors reveal overlapping themes in regulation of cell death and innate immunity. *Microbes Infect.* **11,** 1071–1078

35. Polster, B. M., Pevsner, J., and Hardwick, J. M. (2004) Viral Bcl-2 homologs and their role in virus replication and associated diseases. *Biochim. Biophys. Acta* **1644,** 211–227

36. Adams, J. M., and Cory, S. (2007) The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* **26,** 1324–1337

37. Kirkin, V., Joos, S., and Zörnig, M. (2004) The role of Bcl-2 family members in tumorigenesis. *Biochim. Biophys. Acta* **1644,** 229–249

38. Zong, W. X., Edelstein, L. C., Chen, C., Bash, J., and Gélinas, C. (1999) The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-κB that blocks TNFα-induced apoptosis. *Genes Dev.* **13,** 382–387

39. Wang, C. Y., Guttridge, D. C., Mayo, M. W., and Baldwin, A. S., Jr. (1999) NF-κB induces expression of the Bcl-2 homologue A1/Bfl-1 to preferentially suppress chemotherapy-induced apoptosis. *Mol. Cell. Biol.* **19,** 5923–5929

40. Simonian, P. L., Grillot, D. A., and Nuñez, G. (1997) Bcl-2 and Bcl-XL can differentially block chemotherapy-induced cell death. *Blood* **90,** 1208–1216

41. Schneider, T. J., Grillot, D., Foote, L. C., Núñez, G. E., and Rothstein, T. L. (1997) Bcl-x protects primary B-cells against Fas-mediated apoptosis. *J. Immunol.* **159,** 4834–4839

42. Olsson, A., Norberg, M., Okvist, A., Derkow, K., Choudhury, A., Tobin, G., Celsing, F., Osterborg, A., Rosenquist, R., Jondal, M., and Osorio, L. M. (2007) Up-regulation of *bfl-1* is a potential mechanism of chemoresistance in B-cell chronic lymphocytic leukemia. *Br. J. Cancer* **97,** 769–777

43. Lee, H. H., Dadgostar, H., Cheng, Q., Shu, J., and Cheng, G. (1999) NF-κB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes. *Proc. Natl. Acad. Sci. U.S.A.* **96,** 9136–9141

44. Lam, L. T., Davis, R. E., Pierce, J., Hepperle, M., Xu, Y., Hottelet, M., Nong, Y., Wen, D., Adams, J., Dang, L., and Staudt, L. M. (2005) Small molecule inhibitors of IκB kinase are selectively toxic for subgroups of diffuse large B-cell lymphoma defined by gene expression profiling. *Clin. Cancer Res.* **11,** 28–40

45. Grumont, R. J., Rourke, I. J., and Gerondakis, S. (1999) Rel-dependent induction of A1 transcription is required to protect B-cells from antigen receptor ligation-induced apoptosis. *Genes Dev.* **13,** 400–411

46. Chen, C., Edelstein, L. C., and Gélinas, C. (2000) The Rel/NF-κB family directly activates expression of the apoptosis inhibitor Bcl-x$_L$. *Mol. Cell. Biol.* **20,** 2687–2695

47. Brien, G., Trescol-Biemont, M. C., and Bonnefoy-Bérard, N. (2007) Down-regulation of Bfl-1 protein expression sensitizes malignant B-cells to apoptosis. *Oncogene* **26,** 5828–5832

48. de la Fuente, C., Wang, L., Wang, D., Deng, L., Wu, K., Li, H., Stein, L. D., Denny, T., Coffman, F., Kehn, K., Baylor, S., Maddukuri, A., Pumfery, A., and Kashanchi, F. (2003) Paradoxical effects of a stress signal on pro- and anti-apoptotic machinery in HTLV-1 Tax-expressing cells. *Mol. Cell Biochem.* **245,** 99–113

49. D'Souza, B., Rowe, M., and Walls, D. (2000) The *bfl-1* gene is transcriptionally up-regulated by the Epstein-Barr virus LMP1, and its expression promotes the survival of a Burkitt's lymphoma cell line. *J. Virol.* **74,** 6652–6658

50. D'Souza, B. N., Edelstein, L. C., Pegman, P. M., Smith, S. M., Loughran, S. T., Clarke, A., Mehl, A., Rowe, M., Gélinas, C., and Walls, D. (2004) Nuclear factor κB-dependent activation of the antiapoptotic *bfl-1* gene by the Epstein-Barr virus latent membrane protein 1 and activated CD40 receptor. *J. Virol.* **78,** 1800–1816

51. Portis, T., and Longnecker, R. (2004) Epstein-Barr virus (EBV) LMP2A mediates B-lymphocyte survival through constitutive activation of the Ras/PI3K/Akt pathway. *Oncogene* **23,** 8619–8628

52. Nicot, C., Mahieux, R., Takemoto, S., and Franchini, G. (2000) Bcl-X$_L$ is up-regulated by HTLV-I and HTLV-II *in vitro* and in *ex vivo* ATLL samples. *Blood* **96,** 275–281

53. Mori, N., Fujii, M., Cheng, G., Ikeda, S., Yamasaki, Y., Yamada, Y., Tomonaga, M., and Yamamoto, N. (2001) Human T-cell leukemia virus type I tax protein induces the expression of anti-apoptotic gene *Bcl-xL* in human T-cells through nuclear factor-κB and c-AMP-responsive element-binding protein pathways. *Virus Genes* **22,** 279–287

54. Smith, M. R., and Greene, W. C. (1990) Identification of HTLV-I *tax* trans-activator mutants exhibiting novel transcriptional phenotypes. *Genes Dev.* **4,** 1875–1885

55. Meertens, L., Chevalier, S., Weil, R., Gessain, A., and Mahieux, R. (2004) A 10-amino acid domain within human T-cell leukemia virus type 1 and type 2 Tax protein sequences is responsible for their divergent subcellular distribution. *J. Biol. Chem.* **279,** 43307–43320

56. Lamsoul, I., Lodewick, J., Lebrun, S., Brasseur, R., Burny, A., Gaynor, R. B., and Bex, F. (2005) Exclusive ubiquitination and sumoylation on overlapping lysine residues mediate NF-κB activation by the human T-cell leukemia virus Tax oncoprotein. *Mol. Cell. Biol.* **25,** 10391–10406

57. Thébault, S., Basbous, J., Hivin, P., Devaux, C., and Mesnard, J. M. (2004) HBZ interacts with JunD and stimulates its transcriptional activity. *FEBS Lett.* **562,** 165–170

58. Basbous, J., Arpin, C., Gaudray, G., Piechaczyk, M., Devaux, C., and Mesnard, J. M. (2003) The HBZ factor of human T-cell leukemia virus type I dimerizes with transcription factors JunB and c-Jun and modulates their transcriptional activity. *J. Biol. Chem.* **278,** 43620–43627

59. Sun, S., Elwood, J., and Greene, W. C. (1996) Both amino- and carboxyl-terminal sequences within IκBα regulate its inducible degradation. *Mol. Cell. Biol.* **16,** 1058–1065

60. Meertens, L., Pise-Masison, C., Quere, N., Brady, J., Gessain, A., and Mahieux, R. (2004) Utilization of the CBP but not the p300 co-activator by human T-lymphotropic virus type-2 Tax for p53 inhibition. *Oncogene* **23,** 5447–5458

61. Hivin, P., Frédéric, M., Arpin-André, C., Basbous, J., Gay, B., Thébault, S., and Mesnard, J. M. (2005) Nuclear localization of HTLV-I bZIP factor (HBZ) is mediated by three distinct motifs. *J. Cell Sci.* **118,** 1355–1362

62. Nagata, K., Ohtani, K., Nakamura, M., and Sugamura, K. (1989) Activation of endogenous c-*fos* proto-oncogene expression by human T-cell leukemia virus type I-encoded p40tax protein in the human T-cell line, Jurkat. *J. Virol.* **63,** 3220–3226

63. Lemasson, I., Robert-Hebmann, V., Hamaia, S., Duc Dodon, M., Gazzolo, L., and Devaux, C. (1997) Transrepression of *lck* gene expression by human T-cell leukemia virus type 1-encoded p40tax. *J. Virol.* **71,** 1975–1983

64. Chung, H. K., Young, H. A., Goon, P. K., Heidecker, G., Princler, G. L., Shimozato, O., Taylor, G. P., Bangham, C. R., and Derse, D. (2003) Activation of interleukin-13 expression in T-cells from HTLV-1-infected individuals and in chronically infected cell lines. *Blood* **102,** 4130–4136

65. Cottalorda, A., Verschelde, C., Marçais, A., Tomkowiak, M., Musette, P., Uematsu, S., Akira, S., Marvel, J., and Bonnefoy-Berard, N. (2006) TLR2 engagement on CD8 T-cells lowers the threshold for optimal antigen-induced T-cell activation. *Eur. J. Immunol.* **36,** 1684–1693

66. Oltersdorf, T., Elmore, S. W., Shoemaker, A. R., Armstrong, R. C., Augeri, D. J., Belli, B. A., Bruncko, M., Deckwerth, T. L., Dinges, J., Hajduk, P. J., Joseph, M. K., Kitada, S., Korsmeyer, S. J., Kunzer, A. R., Letai, A., Li, C., Mitten, M. J., Nettesheim, D. G., Ng, S., Nimmer, P. M., O'Connor, J. M., Oleksijew, A., Petros, A. M., Reed, J. C., Shen, W., Tahir, S. K., Thompson, C. B., Tomaselli, K. J., Wang, B., Wendt, M. D., Zhang, H., Fesik, S. W., and Rosenberg, S. H. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumors. *Nature* **435,** 677–681

67. Edelstein, L. C., Lagos, L., Simmons, M., Tirumalai, H., and Gélinas, C. (2003) NF-κB-dependent assembly of an enhanceosome-like complex on

the promoter region of apoptosis inhibitor Bfl-1/A1. *Mol. Cell. Biol.* **23,** 2749–2761

68. Peloponese, J. M., Jr., and Jeang, K. T. (2006) Role for Akt/protein kinase B and activator protein-1 in cellular proliferation induced by the human T-cell leukemia virus type 1 tax oncoprotein. *J. Biol. Chem.* **281,** 8927–8938

69. Matsumoto, J., Ohshima, T., Isono, O., and Shimotohno, K. (2005) HTLV-1 HBZ suppresses AP-1 activity by impairing both the DNA-binding ability and the stability of c-Jun protein. *Oncogene* **24,** 1001–1010

70. Vogler, M., Dinsdale, D., Dyer, M. J., and Cohen, G. M. (2009) Bcl-2 inhibitors. Small molecules with a big impact on cancer therapy. *Cell Death Differ.* **16,** 360–367

71. Tsukasaki, K., Hermine, O., Bazarbachi, A., Ratner, L., Ramos, J. C., Harrington, W., Jr., O'Mahony, D., Janik, J. E., Bittencourt, A. L., Taylor, G. P., Yamaguchi, K., Utsunomiya, A., Tobinai, K., and Watanabe, T. (2009) *J. Clin. Oncol.* **27,** 453–459

72. Vogler, M. (2012) BCL2A1. The underdog in the BCL2 family. *Cell Death Differ.* **19,** 67–74

73. Harhaj, E. W., Good, L., Xiao, G., and Sun, S. C. (1999) Gene expression profiles in HTLV-I-immortalized T-cells: deregulated expression of genes involved in apoptosis regulation. *Oncogene* **18,** 1341–1349

74. Villaudy, J., Wencker, M., Gadot, N., Gillet, N. A., Scoazec, J. Y., Gazzolo, L., Manz, M. G., Bangham, C. R., and Dodon, M. D. (2011) HTLV-1 propels thymic human T-cell development in "human immune system" Rag2$^{-/-}$ γ c$^{-/-}$ mice. *PLoS Pathog.* **7,** e1002231

75. Mori, N., Yamada, Y., Ikeda, S., Yamasaki, Y., Tsukasaki, K., Tanaka, Y., Tomonaga, M., Yamamoto, N., and Fujii, M. (2002) Bay 11-7082 inhibits transcription factor NF-κB and induces apoptosis of HTLV-I-infected T-cell lines and primary adult T-cell leukemia cells. *Blood* **100,** 1828–1834

76. Shaulian, E. (2010) AP-1-The Jun proteins. Oncogenes or tumor suppressors in disguise? *Cell. Signal.* **22,** 894–899

77. Eferl, R., and Wagner, E. F. (2003) AP-1. A double-edged sword in tumorigenesis. *Nat. Rev. Cancer* **3,** 859–868

78. Mori, N., Fujii, M., Iwai, K., Ikeda, S., Yamasaki, Y., Hata, T., Yamada, Y., Tanaka, Y., Tomonaga, M., and Yamamoto, N. (2000) Constitutive activation of transcription factor AP-1 in primary adult T-cell leukemia cells. *Blood* **95,** 3915–3921

79. Iwai, K., Mori, N., Oie, M., Yamamoto, N., and Fujii, M. (2001) Human T-cell leukemia virus type 1 Tax protein activates transcription through AP-1 site by inducing DNA binding activity in T-cells. *Virology* **279,** 38–46

80. Fujii, M., Iwai, K., Oie, M., Fukushi, M., Yamamoto, N., Kannagi, M., and Mori, N. (2000) Activation of oncogenic transcription factor AP-1 in T-cells infected with human T-cell leukemia virus type 1. *AIDS Res. Hum. Retroviruses* **16,** 1603–1606

81. Iwanaga, Y., Tsukahara, T., Ohashi, T., Tanaka, Y., Arai, M., Nakamura, M., Ohtani, K., Koya, Y., Kannagi, M., Yamamoto, N., and Fujii, M. (1999) Human T-cell leukemia virus type 1 Tax protein abrogates interleukin-2 dependence in a mouse T-cell line. *J. Virol.* **73,** 1271–1277

82. Zhi, H., Yang, L., Kuo, Y. L., Ho, Y. K., Shih, H. M., and Giam, C. Z. (2011) NF-κB hyper-activation by HTLV-1 tax induces cellular senescence but can be alleviated by the viral anti-sense protein HBZ. *PLoS Pathog.* **7,** e1002025

83. Zhao, T., Yasunaga, J., Satou, Y., Nakao, M., Takahashi, M., Fujii, M., and Matsuoka, M. (2009) Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-κB. *Blood* **113,** 2755–2764

84. Matsuoka, M., and Jeang, K. T. (2011) Human T-cell leukemia virus type 1 (HTLV-1) and leukemic transformation. Viral infectivity, Tax, HBZ, and therapy. *Oncogene* **30,** 1379–1389

85. Bindhu, M., Nair, A., and Lairmore, M. D. (2004) Role of accessory proteins of HTLV-1 in viral replication, T-cell activation, and cellular gene expression. *Front. Biosci.* **9,** 2556–2576

86. Morales, A. A., Olsson, A., Celsing, F., Osterborg, A., Jondal, M., and Osorio, L. M. (2005) High expression of *bfl-1* contributes to the apoptosis resistant phenotype in B-cell chronic lymphocytic leukemia. *Int. J. Cancer* **113,** 730–737

87. Certo, M., Del Gaizo Moore, V., Nishino, M., Wei, G., Korsmeyer, S., Armstrong, S. A., and Letai, A. (2006) Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* **9,** 351–365

88. Mahieux, R., Pise-Masison, C., Gessain, A., Brady, J. N., Olivier, R., Perret, E., Misteli, T., and Nicot, C. (2001) Arsenic trioxide induces apoptosis in human T-cell leukemia virus type 1- and type 2-infected cells by a caspase-3-dependent mechanism involving Bcl-2 cleavage. *Blood* **98,** 3762–3769

89. Ishitsuka, K., Kunami, N., Katsuya, H., Nogami, R., Ishikawa, C., Yotsumoto, F., Tanji, H., Mori, N., Takeshita, M., Miyamoto, S., and Tamura, K. (2011) Targeting Bcl-2 family proteins in adult T-cell leukemia/lymphoma. *In vitro* and *in vivo* effects of the novel Bcl-2 family inhibitor ABT-737. *Cancer Lett.* **317,** 218–225

90. Chen, S., Dai, Y., Harada, H., Dent, P., and Grant, S. (2007) Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. *Cancer Res.* **67,** 782–791

91. van Delft, M. F., Wei, A. H., Mason, K. D., Vandenberg, C. J., Chen, L., Czabotar, P. E., Willis, S. N., Scott, C. L., Day, C. L., Cory, S., Adams, J. M., Roberts, A. W., and Huang, D. C. (2006) The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* **10,** 389–399

92. Yecies, D., Carlson, N. E., Deng, J., and Letai, A. (2010) Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood* **115,** 3304–3313

93. Vogler, M., Butterworth, M., Majid, A., Walewska, R. J., Sun, X. M., Dyer, M. J., and Cohen, G. M. (2009) Concurrent up-regulation of BCL-XL and BCL2A1 induces approximately 1000-fold resistance to ABT-737 in chronic lymphocytic leukemia. *Blood* **113,** 4403–4413

94. Cheng, Q., Lee, H. H., Li, Y., Parks, T. P., and Cheng, G. (2000) Up-regulation of Bcl-x and Bfl-1 as a potential mechanism of chemoresistance, which can be overcome by NF-κB inhibition. *Oncogene* **19,** 4936–4940